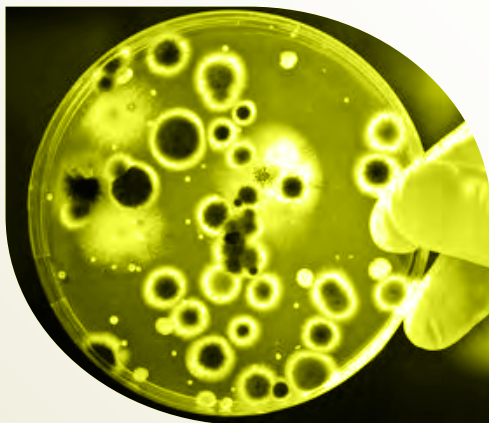
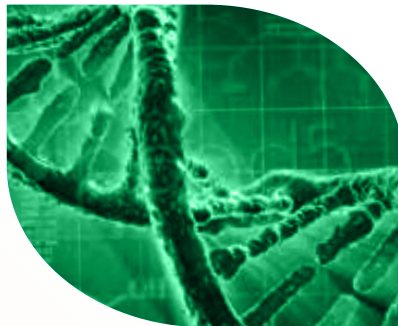
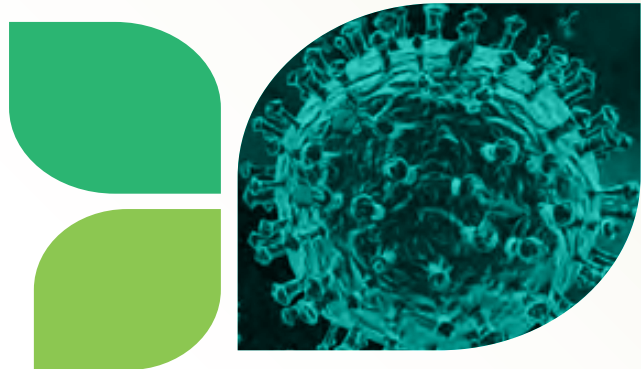




Department of Biotechnology
Ministry of Science & Technology
Government of India



2018

LIFE SCIENCES PROTOCOL MANUAL

DBT Star College Scheme

Compiled by

Dr. P. Hemalatha Reddy, Principal, SVC
Dr. Suman Govil, Senior Advisor, DBT

LIFE SCIENCES PROTOCOL MANUAL

January, 2018

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Ministry of Science & Technology

Government of India

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www.dbtindia.nic.in

Graphic Design

Institute of Communication Design Pvt. Ltd. (www.iocd.in)

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Cover Photo Credits (In order of appearance, from top to bottom)

Picture 1: <https://cdn-images-1.medium.com> (Antibody- virus interaction)

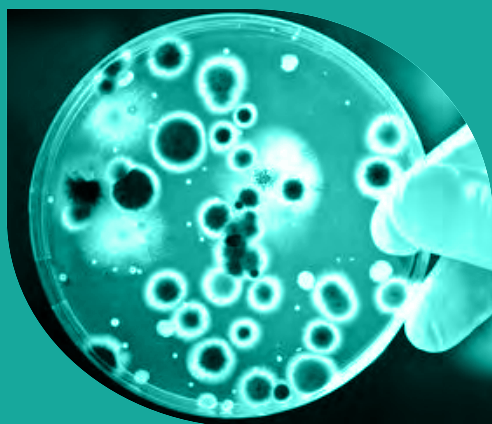
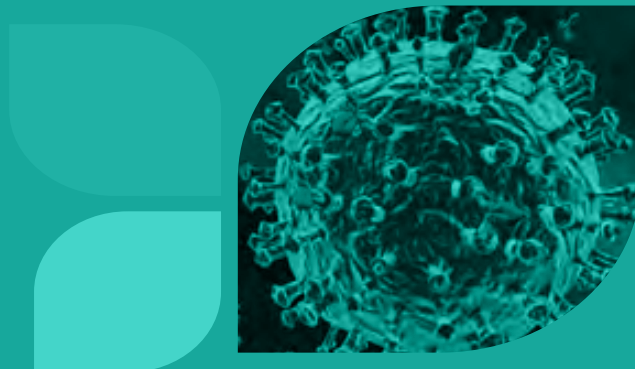
Picture 2: Dr. Sunita Shailajan, Ramnarain Ruia college, Mumbai (*Saraca asoca*)

Picture 3: <http://www.rtc.rs> (Double helix structure of DNA)

Picture 4: <https://www.thestreet.com> (Mould growth on petri plate)



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Dr. P. Hemalatha Reddy, Principal, SVC
Dr. Suman Govil, Senior Advisor, DBT



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Chair DBT Task Force for Star Project, 2014-17

Preface

All learning depends upon curiosity, especially more so in Science. Any new knowledge acquired by any individual spontaneously generates more questions. Most of these questions are “what if ...?”. This, in turn stimulates one to experiment. Since learning of science and advancements in science are based on the continuing enquiry and, therefore, experiments, students of science need to be trained, not only in the basics of principles in different disciplines but also in methods of scientific studies.

This involves learning the basic experimental methodology in laboratory to get trained to conduct experiments and interpret the results. Learning the laboratory methods and techniques thus is an integral part of training of students of science at every level. Unfortunately, this basic component of learning and practicing science in the country has become progressively weaker in recent times, partly because the facilities and resources in academic institutions did not keep pace with the increasing number of students and partly because the teachers and administrators also opted for easier alternatives. Obviously the graduates coming out of such training remain ill-prepared for more advanced scientific studies and research.

In this background, the “Star College Scheme” started by the Department of Biotechnology has been a significant development for undergraduate students across the country. A remarkable feature of this programme is that it aims to support all science departments in an undergraduate college that demonstrate some promise and are competent and willing to improve their teaching programmes across different disciplines so that students get better opportunities for integrative and trans-disciplinary learning. This programme also provides good support for developing the under-graduate laboratories in all the participating departments so that more and more students get an opportunity to perform hands-on laboratory work. My association with this scheme made me realize that the conditions of learning and teaching in a large number of colleges across the country are in a dismal state. Moreover, while the class-room laboratory facilities improved in the colleges participating in the Star College Scheme, their utilization often remained sub-optimal because the teachers in many colleges were not adequately conversant with the laboratory work.

In view of this unfortunate prevailing condition, the present effort was initiated, as a part of the Star College Scheme of DBT, to collate standardized protocols from different colleges and teachers/researchers for laboratory exercises that can be used in under-graduate (or even post-graduate) Biology courses. This indeed is a very welcome initiative. I am sure the resulting Life Sciences Protocol Manual would go a long way in helping improve the learning experience of a very large number of Biology students across the country.

I congratulate the Department of Biotechnology to have conceptualized the Star College Scheme and Dr. Suman Govil and her team in DBT for the successful steering of this programme since its inception with a strong commitment to its cause. I greatly appreciate the sincere and hard work put in by Professor P. Hemalatha Reddy and her team (Drs. Sunita Shailajan, Sasikumar Menon, Shivani Patel, Shalini Sehgal and Professor Suman Kundu) that has resulted in this very useful compilation. I also record my appreciation for all those colleges and teachers who contributed the protocols used by them for this manual.

I am sure this manual would be widely used to further catalyze the spirit of enquiry and experimentation in learners and teachers.

LIFE SCIENCES PROTOCOL MANUAL

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Message By Hon'ble Union Minister

Dr. Harsh Vardhan

Minister of Science & Technology and Earth Sciences
Government of India, New Delhi



सत्यमेव जयते

मंत्री
विज्ञान और प्रौद्योगिकी एवं पृथ्वी विज्ञान
भारत सरकार
नई दिल्ली - 110001

MINISTER
SCIENCE & TECHNOLOGY AND EARTH SCIENCES
GOVERNMENT OF INDIA
NEW DELHI - 110001

MESSAGE

Department of Biotechnology has initiated Star College Scheme in 2008 to build and nurture excellence in undergraduate science education. So far, around 200 colleges have been supported. Emphasis of this programme is on practical training and minor research exposure to undergraduate students. As a part of this programme, colleges have conducted workshops and training courses and have brought out a number of laboratory manuals. These cover innovative practicals and some routine practicals as per curriculum prescribed by respective universities. I have learnt that Department has sanctioned 2 projects to Sri Venkateswara College, Delhi and Ramnarain Ruia College, Mumbai to examine protocols and SOPs submitted by various colleges in life sciences and physical and chemical sciences respectively. In life sciences, 450 protocols have been received from various Star colleges, which have gone through 2 to 3 levels of scrutiny by expert members in last 6 months. In the first phase, 60 protocols have been selected and have been compiled in a uniform format for benefit of academic and student community. I am very glad that these protocols will be available to all undergraduate science colleges in the country. I am confident that teaching faculty and students will benefit immensely from this exercise. I compliment DBT Star College team, Principal of Sri Venkateswara College and all faculty members for taking this initiative in compiling unique, innovative practicals in a uniform format for benefit of undergraduate teachers and students.

(Dr. Harsh Vardhan)





Message By Hon'ble Minister of State

Y. S. Chowdary

Minister of State for Science & Technology and Earth Sciences
Government of India, New Delhi

वाई एस चौदरी
Y S CHOWDARY



राज्य मंत्री
विज्ञान और प्रौद्योगिकी एवं पृथ्वी विज्ञान
भारत सरकार
नई दिल्ली - 110003

MINISTER OF STATE FOR
SCIENCE & TECHNOLOGY AND EARTH SCIENCES
GOVERNMENT OF INDIA
NEW DELHI - 110003


MESSAGE

Star College Scheme, a flagship programme of Department of Biotechnology (DBT), lays emphasis on practical training to undergraduate students and to those doing research on topics of day to day relevance. I am glad that the focus of this programme is on inter-disciplinary interactions amongst different science departments, lectures by eminent scientists and visits to nearby research institutions & universities. I am happy to note that colleges have come up with unique concepts and are organizing interesting competitions based on principles of science such as dance performance, cross word, mime act, skits etc so that students can learn while having fun.

I am told that a large number of workshops and training courses were organized for teachers and students of colleges and schools. As a result of this gigantic exercise, number of laboratory manuals have been generated by the participating colleges. DBT has assigned a project to two colleges viz., Ramnarain Rui College, Mumbai and Sri Venkateswara College, Delhi to examine the protocols for unique, innovativeness, correctness of data, checking plagiarism, referencing etc. Out of 450 protocols in life sciences, after 3 levels of scrutiny, 60 protocols have been shortlisted and compiled in a uniform format for benefit of all undergraduate science colleges in the country.

I congratulate DBT and all faculty members for taking this initiative and methodically compiling, screening and refining these protocols. I am confident that this exercise will be highly beneficial to all UG teachers and students.

New Delhi
24.07.2017


[Y S Chowdary]





Message By Secretary, DBT

K. VijayRaghavan

Secretary, Department of Biotechnology, Ministry of Science & Technology
Government of India, New Delhi



सत्यमेव जयते

के. विजयराघवन

K. VijayRaghavan

सचिव
भारत सरकार
विज्ञान और प्रौद्योगिकी मंत्रालय
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Message

Star College Scheme is a unique, innovative initiative of Department of Biotechnology to promote excellence in undergraduate science education. This programme has provided a forum to all participating science departments in colleges supported to come together and has resulted in melting of departmental boundaries. Colleges are very enthusiastic and have benefitted immensely by interactions in expert committees, visits of mentors and learning from peers from other colleges. I understand from my colleague, Dr. Suman Govil, Adviser, DBT that all participating colleges are showing increase in number of applications as compared to sanctioned seats, increase in cut off percentage at the time of admission, decrease in dropout rates and better performance in results. Thus, we are able to attract students to pursue science at undergraduate level, engage them and retain them which is evident from admissions in post graduate science courses. A few students are also able to publish outcome of their projects or present papers in seminars. Number of colleges have also instituted "Star Student Innovation Awards" to recognise the contributions made by outstanding students. This present initiative to compile the SOPs and practicals conducted by participating colleges is a welcome step as it will benefit not only participating colleges but all undergraduate colleges in the country. I compliment my colleagues, Dr. Suman Govil, Adviser, DBT, Dr. Garima Gupta, Sc.E, DBT for conceiving this idea and Dr. Hemalatha Reddy, Principal, Sri Venkateswara College for taking it forward. I am confident that all faculty and students would benefit immensely from this effort.


(K. VijayRaghavan)

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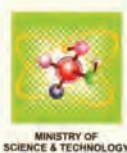


Message By Senior Advisor, DBT



Dr. Suman Govil

Senior Advisor, Department of Biotechnology, Ministry of Science & Technology
Government of India, New Delhi

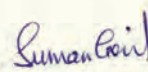


भारत सरकार
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MESSAGE

Star College Scheme was initiated by the Department of Biotechnology to attract students to pursue undergraduate science education and to engage them by providing hands-on experience, so that they learn by doing. Students are encouraged to do minor research projects, which are relevant to them in their day to day life such as safety of drinking water, flora and fauna of campus, measuring haemoglobin, blood sugar, blood group etc. The Department has taken due care to provide multiple copies of routine equipment required for practicals. Opportunities for interaction with leaders by visits to nearby laboratories and institutes as well as lectures by eminent guest faculty have broadened the vision of students and faculty. Colleges are also benefiting by interaction with their peers through participation in DBT Expert Committees, mentors and advisory committee members. So far, 433 proposals have been received and 154 colleges have been supported under strengthening component of the scheme. Based on review of progress on completion of 3 years, 26 colleges have been accorded Star status, which is highly selective. All the participating colleges have shown enhanced interest in science courses, which is evident from number of applicant vs. seats, increase in cut off percentage at the time of admission, decrease in dropout rate, better undergraduate results and higher admissions in post graduate courses. Number of workshops for laboratory staff, students and faculty from schools and colleges have been conducted, resulting in compilation of laboratory manuals. To ensure uniformity, correctness, to detect plagiarism and to improve quality, the Department sanctioned two projects to Ramnarain Ruia College, Mumbai and Sri Venkateswara College, Delhi to examine laboratory manuals in physical sciences and life sciences respectively. The present compilation is first of its kind and is result of several rounds of expert committee meetings to ensure correctness of data and to complete referencing for selected unique, innovative practicals conducted by colleges supported under the scheme.

I thank Dr. Hemalatha Reddy, Principal, Sri Venkateswara College, Delhi, members of Expert Committee, all contributors for undertaking this mammoth exercise for the benefit of student and teacher community.


(Dr. Suman Govil)
Senior Advisor, DBT





Professor L. S. Shashidhara

Dean, Research & Development and Chair of Biology, IISER Pune
Vice-President, Indian National Science Academy (INSA), New Delhi
Co-Chair, DBT Task Force for Star Project, 2014-17

Importance Of Laboratory Experience For Undergraduate Students Of Biology

Scientific pursuit of knowledge is necessarily based on evidence and reasoning. A body of knowledge cannot be assumed right unless it is validated. As validation is an endless process, science progresses more by falsification of ideas, hypotheses and theories. Teaching/learning methods of science would not only help refining the knowledge further as more and more people get involved in this process, it also helps learners to develop/adopt rationality, critical thinking and analytical methods in their professional and personal life. True science education, thus, requires research-based or inquiry-based methods, wherein laboratory courses play very important role.

Amongst all disciplines of science, Biology is far more complex than even an expert biologist could fathom. Its understanding requires interdisciplinary approaches and experimental learning. Therefore, laboratory courses that highlight the basic principles of life processes and their connection to chemical, physical and mathematical sciences are essential components of biology education. A well-designed laboratory program therefore would try to integrate different disciplines and provide comprehensive outlook on the biological complexity.

With this realization, Department of Biotechnology, Govt. of India, initiated star-college scheme to help implement laboratory courses and project-based learning experiences to undergraduate students of science across India. It involves financial support to set up and run laboratories and mentorship in pedagogy and teaching/learning assessment. Large number of colleges have benefited from this, although it is still a very small proportion in the fast-growing higher education system of India, a country of 1.2 billion people. In this context, it is apt and heartening to see that Star College Principals and teachers took on themselves to develop a comprehensive laboratory manual that can be used, like a ready-reckoner, by all teachers across tens of thousands of colleges in India. The whole hearted involvement of Dr. Suman Govil, Senior Advisor, DBT is highly commendable.

Quality of the content of each laboratory course in this compendium is excellent; language and illustrations are self-explanatory, crisp and allows teachers using this to further innovate based on local needs and demands. This self-less endeavor is also an indication of values and ethics that our education system upholds.

I congratulate the co-ordinator, Dr. P. Hemalatha Reddy and the reviewers, Dr. Sunita Shailajan, Dr. Shivani Patel, Dr. Shalini Sehgal, Dr. Sasikumar Menon and Professor Suman Kundu and all the authors of this manual and thank on behalf of the country for the enormous efforts they put in.

I sincerely hope, the efforts put in by the coordinators and the authors of this manual, would motivate teachers and students to further improvise and customize the content of this manual to develop curiosity-driven project-based learning methods that would enhance both the learning experience for students and teaching experience for faculty, and as a bonus, all colleges would pursue high-quality research.

The Star College Scheme- DBT Star Colleges transform undergraduate science education by branding and nurturing excellence.

Dr. Suman Govil, Senior Advisor, DBT

Department of Biotechnology conceived Star College Scheme in 2007 to attract best brains towards basic sciences and to nurture their talent by providing opportunities for interaction with leaders and giving emphasis on practical training. Star college scheme is the brainchild of Dr. M.K. Bhan, ex-Secretary, DBT, who wanted to have colleges of the calibre of “St. Stephens College” in every district of the country, so that students do not have to migrate and can get best education in their native place. Realising the importance of inter-departmental and inter-disciplinary interactions, the Department has taken unconventional and bold step by providing support to all science departments and not restricting it to only life sciences. The scheme addresses all important dimensions of undergraduate education by laying emphasis on hands-on exposure in practical classes, strengthening infrastructure in laboratory by providing multiple copies of routine equipment required for class room practicals, opportunities for interaction with guest faculty and visits to laboratories, universities and industries. All undergraduate students from participating science departments are expected to take up minor research projects, preferably inter-departmental, on subjects of day to day relevance. Opportunities for training of laboratory staff and faculty improvement programmes are an integral component. The scheme has been credited by participating colleges to have blurred the boundaries of participating departments, leading to interaction amongst faculty across traditional disciplinary silos.

The existing schemes of University Grants Commission such as colleges with potential for excellence, colleges of excellence, autonomous colleges and FIST (Fund for Improvement of S&T infrastructure in universities and higher educational institutions) by Department of Science & Technology were thoroughly studied and Star College Scheme was introduced to bring complementarity by giving emphasis on recurring grant for consumables to ensure practical training.

The Department envisaged support to undergraduate colleges under two categories: strengthening component and star college status. Criteria for selection are outlined below:

Two categories of support under star college scheme

Criteria for selection

- Strengthening of support for UG in life sciences Rural /Tribal / Urban / Girls
- Existing infrastructure and per student expenditure in last 3 years by different science departments
- No. of years in existence
- No. of ongoing science courses
- No. of regular/contractual faculty per department (qualification relax)
- Recognition by UGC / AICTE/NAAC/ DST

Star college status (highly selective)

- No. of years — minimum 10 years
- No. of faculty with Ph. D.
- Minimum no. of ongoing 4-5 life science courses & 1-2 applied courses
- Existing infrastructure 8 per student expenditure in last 3 years
- Recognition by UGC/ AICTE/ NAAC/DST
- Cut off percentage for admission, drop out rate Si percentage of result

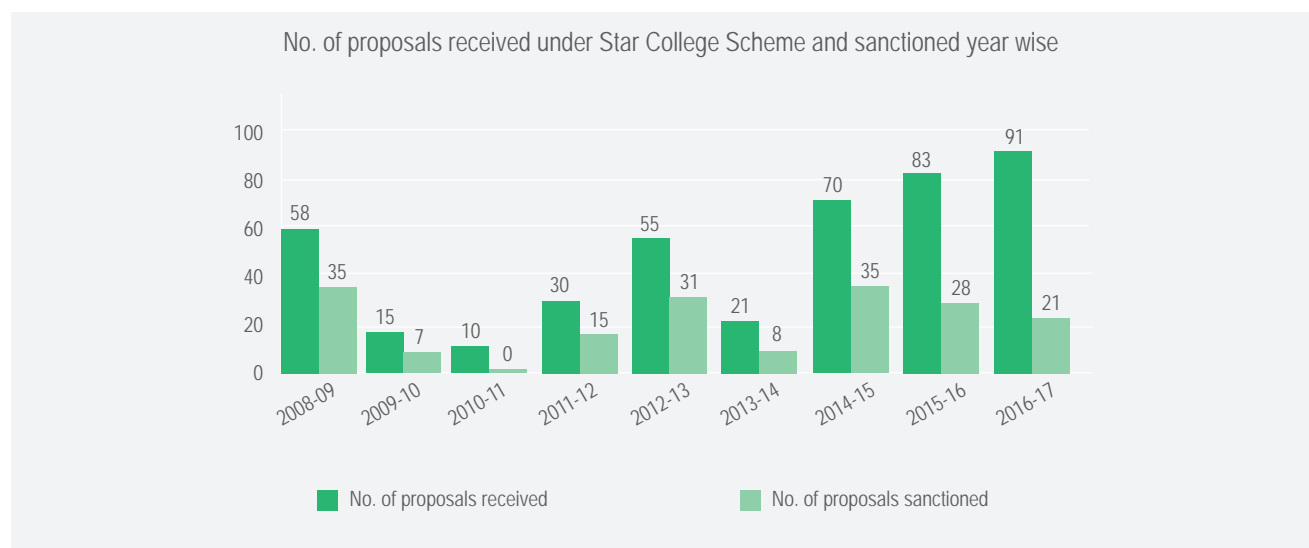
Format for submission of application, guidelines can be accessed at www.dbtindia.nic.in.

After 1st round of call for proposals under the scheme, the Expert Committee felt that it was difficult to award star status to any college in the beginning and star status should be awarded based on evaluation of performance. Initially all selected colleges should be supported under strengthening component only for a period of 3 years. Criteria and guidelines for according Star status after performance review on completion of 3 years or continuation of support under strengthening component for another tenure or discontinuation of support have been formulated based on feedback from Expert Committee and Coordinators from participating colleges.

The advertisement clearly brings out that the scheme has provision of support to different science departments such as physics, mathematics, chemistry, statistics, bioinformatics, computer science in addition to life science departments such as botany, zoology, biochemistry, microbiology, food science etc. However, initially, colleges apply mainly for life science departments and then, based on interaction in Expert Committee meeting, they apply for addition of other departments. Of late, it has been decided to include departments like mass media, journalism, management, economics, commerce, if they clearly bring out how science teaching will be improved by collaborating with them.

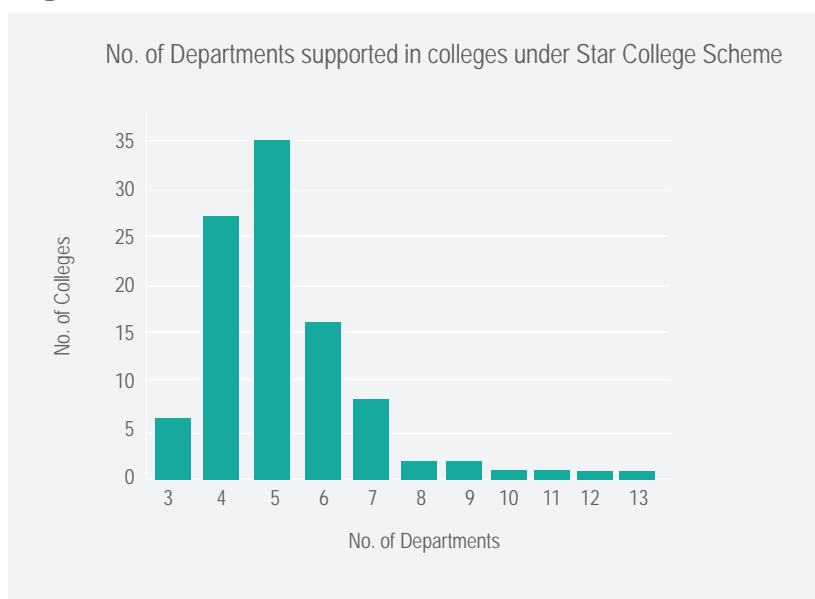
So far, 518 colleges have applied and 180 colleges have been supported under strengthening component. Year wise number of proposals received and sanctioned is given in Fig. 1.

Fig.1



12 colleges have been accorded Star status on completion of one tenure and 14 colleges have been given Star status on completion of two tenures. Several colleges have been continued under strengthening component for second tenure. 67 colleges have been discontinued support due to lack of innovative, inter-disciplinary component. Number of departments supported under Star College scheme vary from 3 to 13. Fig.2 depicts number of departments supported in number of colleges. Maximum colleges have 5-6 departments supported.

Fig. 2



The Scheme has pan India presence and location of colleges supported under the scheme is shown in the Map of India (Fig.3). Star college scheme has limited presence in Madhya Pradesh, Rajasthan, Uttar Pradesh, Chattisgarh, Jharkhand. In spite of specific advertisements seeking proposals from these regions and proactive steps taken by the department, outreach remains limited.

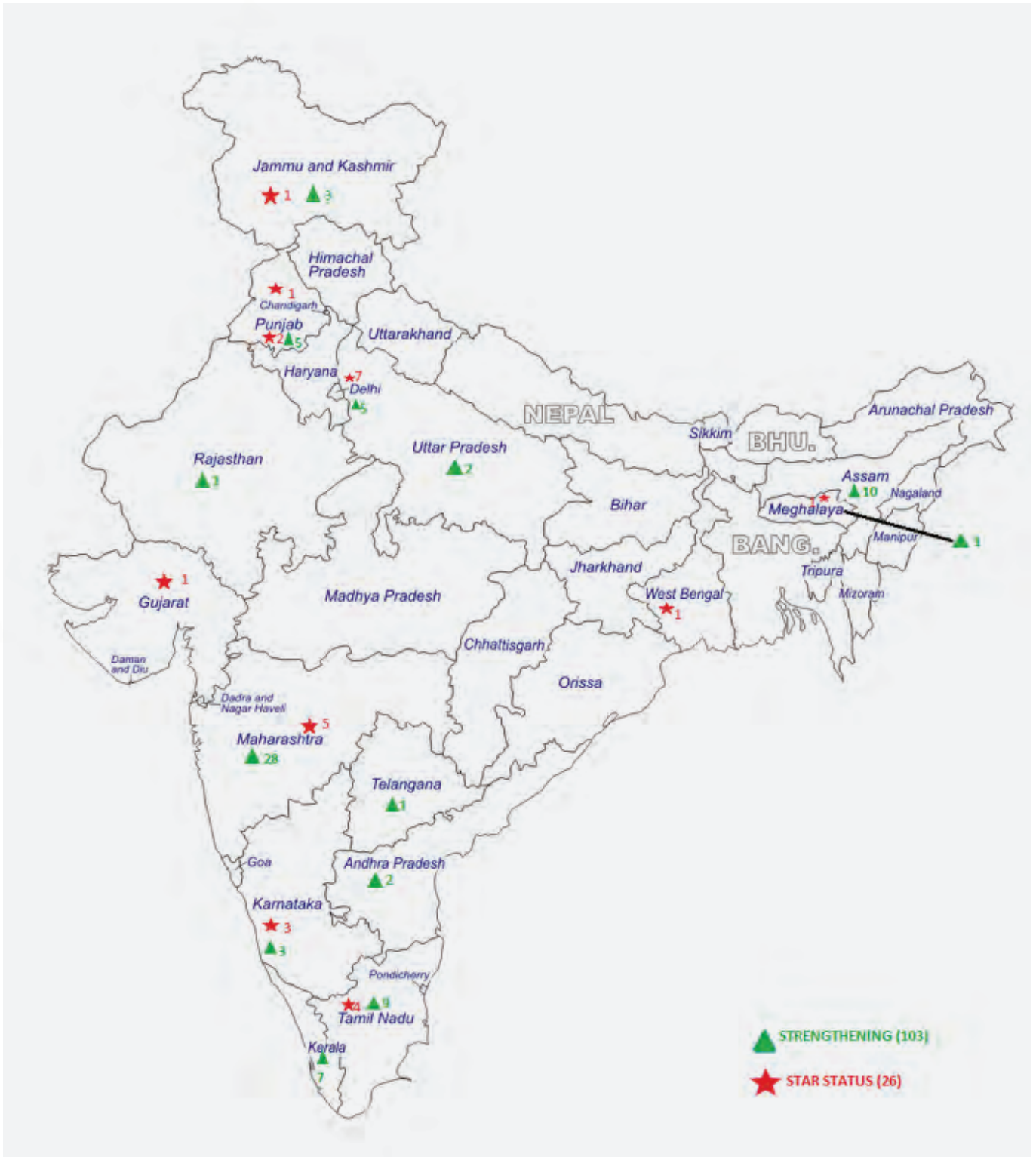


Fig.3 Number of colleges supported under Star College Scheme

Impact of the Scheme

Participating colleges are learning from their own peers in different colleges, experts and mentors in advisory committee meetings, DBT Task Force meeting and meeting of course coordinators. Inclusion of unique, innovative practicals and interdisciplinary projects, interaction opportunities and feedback analysis from all stakeholders have led to a slow and steady improvement in quality of science education as well as their NAAC rankings, extramural funding etc. This small investment made by the department has paid rich dividends by transforming undergraduate science education landscape.

Qualitative Parameters

The scheme expects colleges to conduct practicals prescribed as per the approved curriculum of affiliating universities. Some of the practicals which could not be conducted due to lack of availability of multiple copies of equipment or costly consumables are now being done, number of students per team for practicals has considerably reduced and several demonstrations have been substituted by experiments. Young students are also getting much needed opportunities for interaction with leaders in the field through guest lectures, training workshops and visits to nearby research institutions. Faculty are also able to attend workshops, training courses, present papers in conferences. Collaborative teacher's training workshops jointly supported by DBT and British Council are being organised by IISER, Pune to train teachers in pedagogical tools, who are then expected to conduct regional workshops to train teachers.

Similarly, workshops for laboratory staff on collection and handling of biological material, handling and disposal of hazardous chemicals and wastes, preparation of solutions are also routinely organised. Several colleges are also conducting outreach activities by organising open day, exhibition, popular lectures, practicals for students and teachers from neighbouring schools and colleges.

The philosophy of the programme is to provide equal opportunities to all students from participating departments in selected colleges and not to restrict the projects to interested, better students only. Interesting, interdisciplinary projects of day to day relevance to students such as blood group testing, testing of portability of drinking water, safety of milk, qualitative estimation of baby foods, natural dye isolation from flowers etc., are being undertaken by students. Minor research project is an enriching experience for students as they learn how to identify a research problem, conduct literature survey, design SOPs and protocols and interpret their own research results. This research exposure at undergraduate level helps students to make correct career decision and interest fuelled leads to natural progression to science courses at post graduate level. These unique, innovative initiatives are successful in fostering creativity by activity based learning through relevant projects so as to connect knowledge with real life situations.

Several fun based learning activities like quizzes, debates, science principles based relay race have enabled the colleges to fuel imagination and improve creativity quotient of teachers as well as students. A large number of laboratory manuals have been prepared by the colleges which have been evaluated by Expert Committees to detect plagiarism, to avoid overlapping and to bring uniformity. After several rounds of deliberations, first laboratory manual covering around 60 practicals in life sciences is expected shortly. Similar exercise is on for physical and chemical sciences also.

These laboratory manuals would be accessible on DBT website for all undergraduate sciences colleges.

Quantitative parameters

Most of participating colleges have witnessed gradual increase in number of students admitted to different undergraduate science courses (Fig.4),

Fig.4

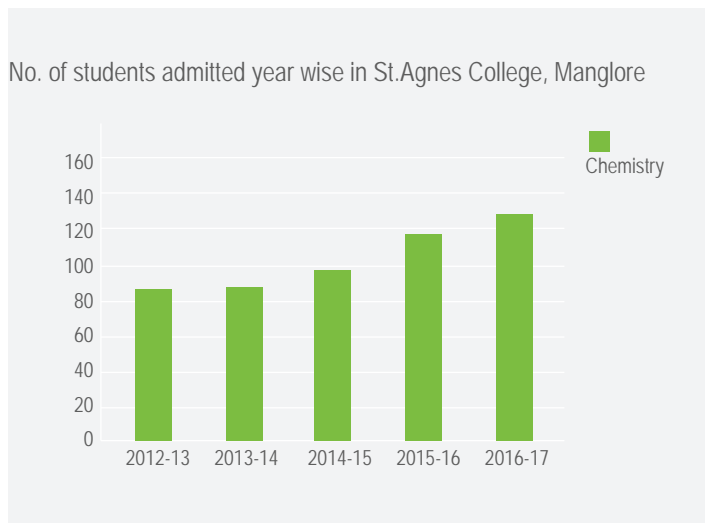


Fig.5

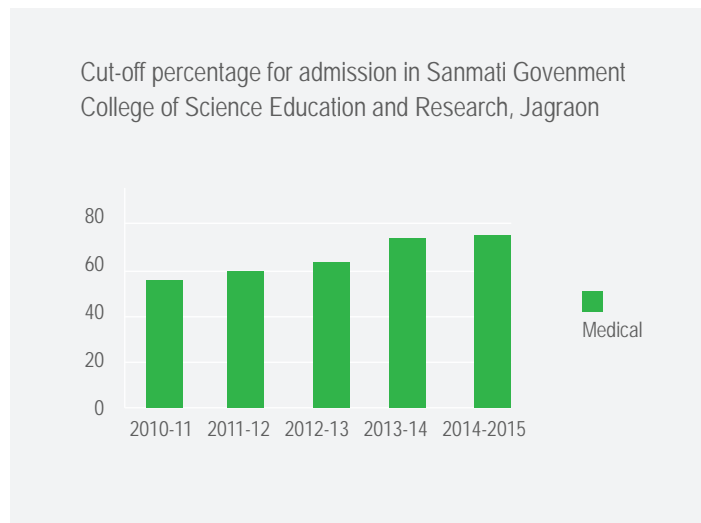


Fig.6

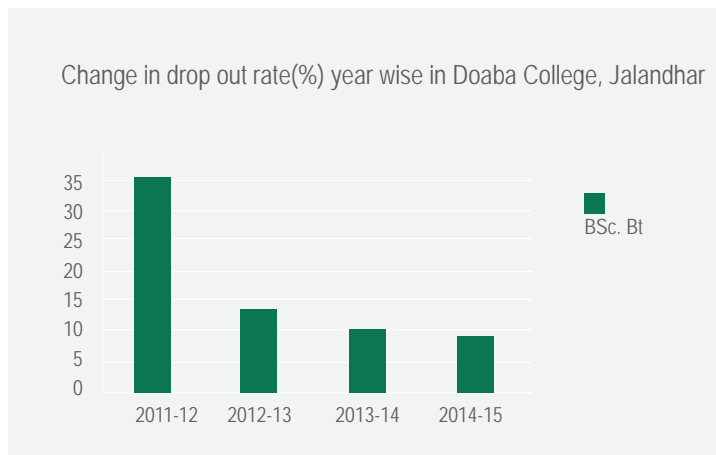


Fig.7

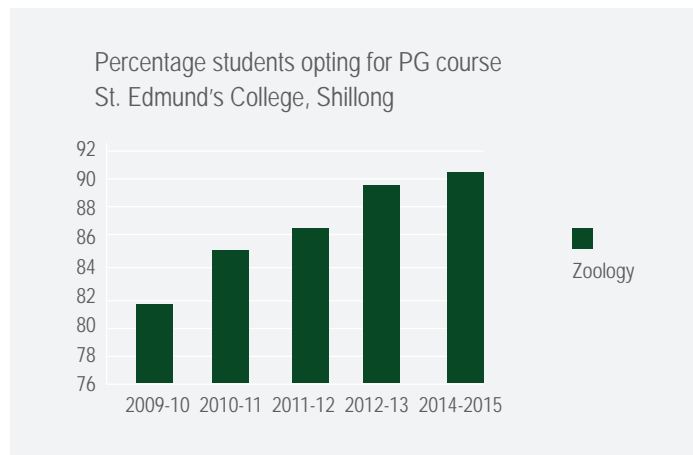


Fig.8



Thus, this programme has managed to attract young students to pursue science, engage in interesting activities and retain students to pursue higher education in science. Data presented in this article is illustrative and representative and has been randomly selected.

Star College scheme is a dynamic programme and is continuously evolving based on feedback from expert committee members, participating colleges, teachers and students. In last few years, enthusiasm of faculty and students is contagious and it is highly satisfying when students from colleges in remote areas like Mangaldai college, Assam and Handique Girls college, Guwahati, share their excitement while presenting their project results and claim that they joined the college because they read in the prospectus that college has been supported under Star College scheme which will give them opportunities to conduct practicals and research projects. Of course, Star College scheme is like a drop in the ocean as this is limited to around 200 colleges out of over 25,000 colleges in the country. Needless to mention, this programme is like developing a model system which can then be replicated on large scale.

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Editorial Note

It gives me immense pleasure to write editorial note on behalf of our team.

We believe that without good practical training at the bench, sound understanding of the fundamentals may not be possible. This conceptual clarity at undergraduate level will go a long way while metamorphosing young students into good scientists.

With an objective to strengthen undergraduate practical training, DBT Star College Scheme has equipped the laboratories in colleges in all corners of the country. The learning of science at bench has been made more meaningful and enjoyable. The off shoot of this programme is to develop E-manual with Standard Operating Procedures and protocols to various life sciences students. It is indeed, a mammoth task, to collect and curate the protocols submitted by many teachers across the nation. The genesis of this entire exercise is during various expert committee meetings with the Senior Advisor Dr. Suman Govil, DBT, the Chairman Professor Lakhotia and Professor Shashidhara for bringing uniformity in the instrumentation in science labs across the country. They felt that the uniformity should also extend to the procedures being followed.

The uniqueness of the manual is that the protocols have been contributed by the faculty members of different science colleges under the Star College Scheme, from the various parts of India. These protocols have been performed, standardized, tested and the results especially the photographs have been taken during the experiment by the faculty members, making this manual a one of its kind. The DBT made an initial effort in the collection of around 450 protocols from the reports and support documents such as manuals submitted under the Star College Scheme. The protocols went through an exhaustive scrutiny by the initial Screening Committee. A Core committee of selected experts was then constituted for the reviewing, harmonizing and development of the manual as there was a lack of uniformity in the presentation of protocols received. Then, the reverted protocols, received within the stipulated time frame, were reviewed and modified by the Core Committee, maintaining the essence of the protocol of the contributor. Plagiarism, language, content and quality of pictures have been taken into consideration while selecting the protocols. Those protocols which are not featured in this manual have to be rewritten and revised as per the guidelines formulated by the Core Committee.

This compilation is the collective wisdom of many experts many editors and many reviewers. The entire committee worked hard irrespective of time and space. Without the driving force of Dr. Suman Govil it would not have taken this shape. The passionate teachers with whom we have worked give us the confidence that the science will flourish in this country in future.

While compiling we have learnt a lot and hope while performing the practical you will get the benefit
.....

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Acknowledgments

The Star College Scheme for strengthening of life sciences and biotechnology education and training has made a tremendous impact on undergraduate science teaching across the country. Developing resources and Standard Operating Protocols (SOPs) is one of the initiatives under this scheme apart from strengthening the infrastructure. This manual is the final outcome of this initiative which has been supported by many through their motivation, contribution and hard work.

We wish to express our heartfelt thanks to Dr. Harsh Vardhan, Minister for Science & Technology and Earth Sciences for his understanding of the need for standardization of techniques in undergraduate science teaching and Dr. Y. S. Chowdary, Minister of State for Science & Technology and Earth Sciences for his motivation. We wish to put on records the sincere appreciation for Dr. K. Vijay Raghavan, Secretary, DBT for encouraging the idea of developing uniform protocols and SOPs at undergraduate level. We thank Dr. Suman Govil, Senior Advisor, DBT for her constant support, involvement and critique to bring out this compilation. The support extended by Dr. Garima Gupta, Scientist E who is part of the Star College Scheme Administration, DBT is commendable. The role of DBT finance and administration deserves a mention as it helped in smooth execution of this project.

We are immensely grateful to Professor S. C. Lakhota (Chair, DBT Task Force for the year 2014-17) for emphasizing the role of strong fundamentals in strengthening undergraduate science teaching. We also thank Professor L. S. Shasidhara (Co- chair DBT Task Force for the year 2014-17) for having faith and belief in the potential of undergraduate science faculty across the country and his unconditional support.

The committee will also like to thank all the contributors across the various Star Scheme Colleges who submitted the protocols and also the reviewers who streamlined the contents. The editorial insights, helped in creating this document. We also thank the Principal, Ramnarain Ruia College and Principal, V. G. Vaze College, Mumbai for helping us by providing logistics during our meetings at their institutes. We also thank Dr. N. Latha, Coordinator and research scholars of Bioinformatics facility of Sri Venkateswara College for making the facility available for this project on day to day basis

The efforts of Dr. Meeta Bhardwaj and Ms. Jyoti JRF, DBT Project in this initiative are highly acknowledged. Mr. MLN Murthy also deserves a mention for managing the project finances. Last but not the least; we appreciate Ms. Manisha Pritilaya and Ms. Avneet Kaur for the creative design of the manual .

We, the members of Core Committee thank each other for the mutual trust and confidence in this long journey.

LIFE SCIENCES PROTOCOL MANUAL

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BOTANY

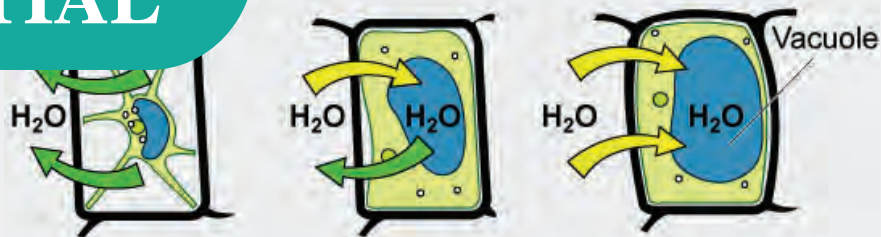


Separator Photo Credit

Mimosa pudica

Contributed by Dr. Sunita Shailajan, Ramnarain Ruia College.

DETERMINATION OF WATER POTENTIAL



1. Aim

To determine the water potential (ψ_w) by:

- A. plasmolytic method
- B. weight method
- C. density method

2. Introduction

According to the thermodynamic laws, every component of a system possesses free energy capable of doing work under constant temperature condition. Osmotic movements of water involve certain work done. In fact the main driving force behind this movement is the difference between the free energy of water on two sides of the semi permeable membrane.

For non-electrolytes, free energy/mole is known as chemical potential (ψ). With reference to water, this is known as water potential (ψ_w).

Osmotic pressure (OP) in a solution results due to the presence of solute and the latter lowers the water potential. Therefore, osmotic pressure in solution is a quantitative index of the lowering of water potential in a solution and using thermodynamic terminology is called Osmotic potential (ψ_s). OP and ψ_s are numerically equal but ψ_s carries a negative sign (-). The hydrostatic pressure developed is known as pressure potential (ψ_p)

$$\text{So, } \psi_w = \psi_s + \psi_p$$

If we neglect ψ_p , Then $\psi_w = \psi_s$

We know that, OP and ψ_s values are numerically similar but the signs are different, so, $\psi_s = \text{OP}$

Therefore, ψ_w can be determined from the volume of osmotic pressure by plasmolytic method as follows

OP=CRT where,

OP=Osmotic pressure.

C = Concentration of cell sap for which osmotic pressure is to be maintained.

R= Gas constant.

T = Temperature (in degree kelvin)

In this experiment, the solutions are of different molar concentrations. There is presence of cell sap of a certain molar concentration and the semi permeable membrane is the cell membrane. The concentration of the cell sap can be found out by counting the number of plasmolysed cells when exposed to solutions of different concentrations.

Plasmolysis is the shrinkage of protoplasm from the cell wall under action of certain solutions, where concentration is greater than that of cell sap. When the plant tissue is placed in hypertonic solution, it is seen that most of the plant cells are plasmolysed. However, at the isotonic point, when the concentrations of both solutions are equal, incipient plasmolysis takes place where the concentration or withdrawal of protoplasm from the cell wall has just commenced. The cell wall is said to be undergoing incipient plasmolysis.

At this point, since the concentration of cell sap is equivalent to that of the external solution and as the latter is known, the concentration of the sap can be found out and consequently, osmotic pressure can be determined.

$$\Psi_w = \Psi_s + \Psi_p$$

$$\text{DPD} = \text{DP} - \text{TP (WP)}$$

So, Ψ_w is equivalent to DPD.

The DPD value can be indirectly measured from the OP value at that major concentration where there are neither absorption nor desorption.

$$\text{So, } \text{DPD} = \text{OP} = \text{CRT}$$

Where C = Molar concentration

R = Universal gas constant

T = Absolute temperature

A. By Plasmolytic Method

3. Materials Required

3.1. Biological Materials: Leaves of *Rhoeo discolor* (syn. *Tradescantia spathacea*).

3.2. Chemicals/Reagents: Sucrose.

3.3. Equipment: Chemical balance, weight box, compound microscope.

3.4. Glassware/Plastic ware: 12 pairs of petri dish, beakers, measuring cylinder, slides.

3.5. Miscellaneous: Forceps, needle, blade, cover slip, distilled water.

4. Preparation of Reagents

4.1. Preparation of Molar solution:

A molar solution is a solution of 1 mole (=molecular weight) of substance dissolved in 1 litre (1000 cc) of distilled water.

In current experiment 1 M sucrose is being used as stock solution

Sucrose is the carbohydrate which has molecular weight of 342.3 g. Hence, we have to take 342.3 g of sucrose to dissolve in 1000 cc of distilled water to make 1 molar solution.

4.2. Preparation of Different Concentrations of Molar solution:

Molar solutions of various concentrations are required for this purpose. The molar solutions of different concentrations are prepared from stock solution already made (Table 1).

1M stock solution(cc)	Distilled water (cc)	Total volume (cc)	Working strength (M)
1	9	10	0.1
2	8	10	0.2
3	7	10	0.3
4	6	10	0.4
5	5	10	0.5
6	4	10	0.6
7	3	10	0.7
8	2	10	0.8
9	1	10	0.9
10	0	10	1.0

Table 1: Preparation of molar solutions of different concentrations

5. Procedure

- i. Peel off a small segment from lower surface of leaf by tearing the leaf obliquely with single jerk or by scrapping it with safety blade.
- ii. Mount the peel in a drop of water on slide and then place a coverslip and observe under microscope.
- iii. Make different molar concentrations of sugar solution such as 0.1-1 M.
- iv. Take another peel and similarly mount the peel of *Tradescantia* in a drop of sugar solution of different concentrations on different slides.
- v. After 30 minutes observe each preparation under microscope.

6. Observations

Tradescantia leaf

Sr. No.	Molar concentration (M)	No. of cells considered	No of cells Plasmolysed	No of cells Non-Plasmolysed	Percentage of Plasmolysed Cells (%)	Percentage of Non- Plasmolysed Cells (%)

Table 2: Percentage of plasmolysed and non-plasmolysed cells with increase in molar concentration.

From the above results, plot a graph between the percentage of plasmolysed cells against the sucrose concentrations. From the graph, the concentration at which 50% cells are plasmolysed is noted down (the stage of incipient plasmolysis).

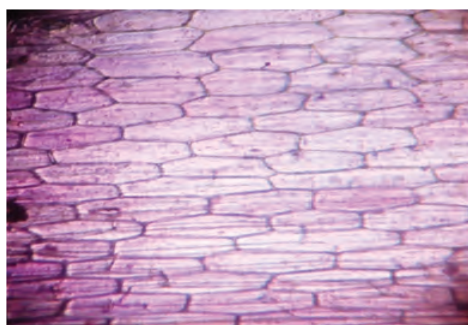


Fig. 1: Turgid cells

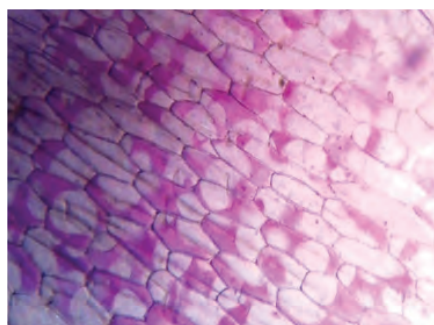


Fig. 2: Plasmolysed cells

7. Result

From the graph, the isotonic point is calculated. At this concentration, 50% of the cells are plasmolysed. Therefore, from the relation,

$OP = CRT$, we have,

C = Concentration at which isotonic point is found.

$R = 0.082 \text{ L atm mol}^{-1} \text{ K}^{-1}$

$T = 273 + (\text{Room temperature in } ^\circ\text{C})$

8. Precautions

- Distilled water should be used for preparation of any solutions.
- Petri plates used need to be kept covered during experimentation.
- Equal exposure time for cells for each concentration is to be strictly maintained.

B. Weight method

3. Materials Required

- 3.1. **Biological Material:** Fresh potato tuber.
- 3.2. **Chemicals/Reagents:** Sucrose ($C_{12}H_{22}O_{11}$).
- 3.3. **Equipment:** Weighing balance.
- 3.4. **Glassware/Plastic ware:** Measuring cylinder, test tube, beaker, petri dishes.
- 3.5. **Miscellaneous:** Distilled water, cork borer, blotting paper.

4. Procedure

- i. A few uniformly bored pieces of potato tuber are made with the help of a borer.
- ii. Each piece is weighed accurately (initial weight) and put into petri dishes containing sucrose solution of known concentration.
- iii. The set up is kept undisturbed for an hour and then tuber pieces were taken out and blotted dry. The pieces are weighed again (final weight).
- iv. The same is repeated with all the pieces of potato tuber noticing the initial and final weight of each bore.

5. Observations

It is seen that the pieces of tuber gained weight with a gradual increase in concentration of molar solution up to a certain point beyond which a corresponding decrease in weight is observed. The results are plotted on a graph by taking molar concentration as the abscissa and the percentage of weight as co-ordinate.

S. No.	Molar concentration (M)	Initial weight (W1) (mg)	Final weight (W2) (mg)	Difference in weight (W2 -W1) (mg)	Percentage of difference in weight	Remark

Table 3: Percentage weight of potato tuber with the increase in molar concentration.

6. Result

Therefore, as previous method,

$$\text{DPD} = \text{OP} - \text{TP}$$

Where, DPD = Diffusion pressure deficit

OP = Osmotic pressure

TP = Turgor pressure

Again, we know that,

OP = CRT, where C = Concentration of molar solution (where there is no change in tuber weight)

$$R = 0.082 \text{ L atm mol}^{-1} \text{ K}^{-1}$$

$$T = (273\text{K} + \text{Room Temperature } ^\circ\text{C})$$

Again, DPD of a cell is equivalent to water potential but differ in sign,

Therefore, Water potential (ψ_w) = DPD

Thus, the value of DPD as well as water potential can be calculated directly from the graph determining the molar concentration at which there is no turgor pressure.

The relationship used is:

$$\text{DPD} = \text{OP} - \text{TP}$$

Where, TP = 0

Therefore DPD = OP

7. Precautions

- i. Distilled water is to be used for all solutions.
- ii. Drying with blotting paper is necessary before weighing the samples.

C. By density method

3. Materials Required

3.1. Biological Material: Peeled potato tuber.

3.2. Chemicals/Reagents: Sucrose solution of different molar concentrations (0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M), methylene blue.

3.3. Equipment: Weighing balance.

3.4. Glassware/Plastic ware: Test tubes, pipette, dropper.

3.5. Miscellaneous: Forceps, cork borer, test tube stand.

4. Procedure

- i. Weigh 34.2 gm of sugar and dissolve in 100 cc of distilled water to make 1 M of stock solution.
- ii. Take 10 test tubes and make different molar concentrations of sucrose (0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M) solution in them in duplicate (set A and set B).
- iii. Cut small cylindrical pieces of potato of about 2 cm in size. Dry these potato pieces between folds of filter paper.
- iv. Among two sets of test tubes with molar concentrations, put small pieces of potato tuber in set A.

- v. Add some drops of methylene blue in the test tubes of set B till the solution becomes sufficiently blue.
- vi. Leave these test tubes undisturbed for about 20 minutes.
- vii. Put a drop of colored solution from set B into the test tube of corresponding molar concentration of set A.
- viii. Now observe the movement of drop in sugar solution whether it moves up and down or simply diffuses.

5. Observations

- i. If a drop rises, the test tube solution has decrease in density over its initial concentration.
- ii. If a drop falls down, it means density of test tube solution has increased.
- iii. If a drop diffuses out, it means the test tube solution has no change in its density & record your reading.

6. Result

Water potential is calculated by the movement of drop of colored solution using the formula

$$OP = CRT,$$

Where C = Concentration of molar solution (where the drop disperses)

$$R = 0.082 \text{ L atm mol}^{-1} \text{ K}^{-1}$$

$$T = (273 + \text{Room Temperature } ^\circ\text{C}) \text{ K}$$

7. Precautions

- i. Sugar solution must be accurately prepared.
- ii. Fresh potato tuber should be used.

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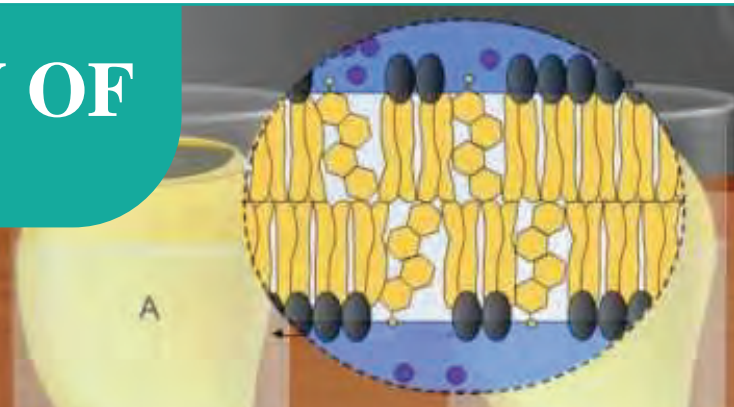
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DEMONSTRATION OF OSMOSIS



1. Aim

To demonstrate osmosis by potato osmoscope

2. Introduction

Osmosis is the phenomenon of movement of solvent molecules from their higher concentration to lower concentration or from a solution of low concentration to high concentration through semi-permeable membrane. A semi-permeable membrane is a membrane which allows only solvent molecules to pass through it but not the solute molecules. In a living cell, the cell membrane, tonoplast and the membrane surrounding the chloroplast, mitochondria, and nucleus are called selectively permeable membrane.

Osmosis is of two types:

- i. Exosmosis: It is the movement of solvent molecules from a plant cell, when it is placed in a hypertonic solution.
- ii. Endosmosis: It is the movement of solvent molecules into a plant cell, when it is placed in a hypotonic solution.

In isotonic solution, the cell wall neither loses nor absorbs any water; the net volume remains the same. The absorption of water by the root hairs from the soil and the movement of water from one living cell to another within the plant is due to osmosis.

3. Materials Required

3.1. Biological Material: Potato tuber.

3.2. Chemicals/Reagents: Sugar solution.

3.3. Glassware/Plasticware: Beakers, petri plates.

3.4. Miscellaneous: Filter paper, razor, scalpel, common pins, cork borer, water.

4. Procedure

- i. Take a large potato tuber and peel off its outer skin with the help of a scalpel.
- ii. Cut its one end flat and make a cavity in the potato tuber almost up to the bottom with the help of a cork borer leaving a flat base at the bottom.
- iii. Put some sugar solution up till half of the cavity of the potato tuber and mark the level with the help of a common pin.
- iv. Put the potato osmometer-1 in a Petri plate containing water and keep it undisturbed for some time.
- v. Similarly prepare another potato osmometer-2 and pour water up till half of its cavity and mark the level with the help of common pin. Make sure the size of potatoes and the level of water filled in the cavity should be same.
- vi. Place this osmometer in a Petri plate with sugar solution and keep it as such for some time.

5. Observations

In the first potato osmometer, the level of sugar solution will rise up, where as in the second potato osmometer, the level of water will fall down.

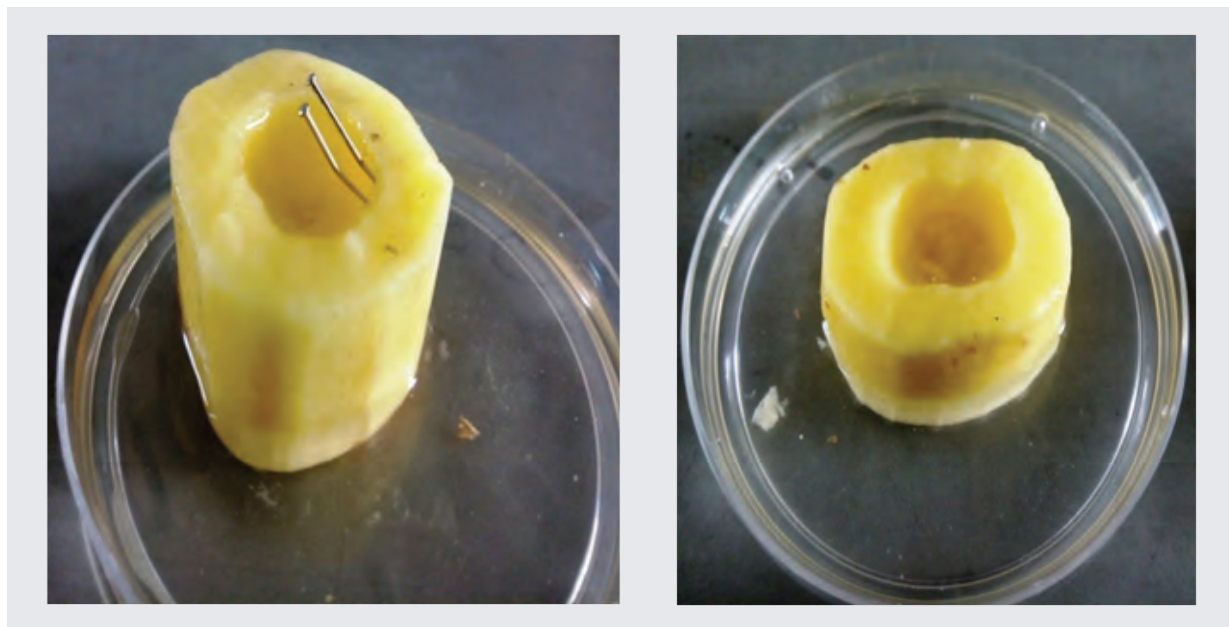


Fig. 1: Study of osmosis by potato osmoscope

6. Result

- i. The level of sugar solution in the first potato osmometer rises up due to the process of endosmosis.
- ii. The level of water in the second potato osmometer reduces due to exosmosis.

7. Precautions

- i. Potato tuber should be peeled off because its periderm is impermeable to water.
- ii. Bottom of the potato should be flat.
- iii. The cavity should be deep.
- iv. Mark the initial level carefully in both the potato osmometers.
- v. Both the potato tubers should be of almost the same size.

Suggested Reading(s)

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DEMONSTRATION OF ASCENT OF SAP



1. Aim

To demonstrate the ascent of sap using a dye

2. Introduction

Ascent of sap: The upward movement of water and the dissolved substances from roots to all the aerial plant parts through xylem is called ascent of sap. In vascular plants, water absorbed by roots is transported through the mature (dead) tracheary elements (xylem vessels and tracheids) of roots and stems. During the course of evolution, a transport system has been developed in plants, that relies on specific properties of water like surface tension, adhesion, cohesion and high tensile strength. The xylem sap in transpiring plants is under negative pressure. The summation of this negative pressure or tension along with the adhesion and cohesion is responsible for the long distance upward transport of water as explained by Cohesion-Tension theory. This is the most widely accepted theory that explains the easy movement of large quantities of water from the soil to the transpiring leaf surface with little input of metabolic energy. It is presumed that there is a continuous column of water which extends from roots to growing shoots. The water column is continuous and resists breaking because of cohesive and adhesive forces of water. Although, cohesion theory given by Dixon and Jolly gives the best explanation for the phenomenon but there are certain limitations to it. The problem of cavitation and embolism i.e. the vaporization of water and formation of gas bubbles, blocks the movement of water. However, later on, it was observed that surface tension and structure of xylem conduits (bordered pits) prevent air from being sucked and spread.

3. Materials Required

- 3.1. **Biological Materials:** Two fresh leafy shoots cut under water for example: Balsam, Australian Eucalyptus.
- 3.2. **Chemicals/Reagents:** Eosin solution (1%).
- 3.3. **Equipment:** Microscope.
- 3.4. **Glassware/Plastic ware:** Two beakers, slide, coverslip.
- 3.5. **Miscellaneous:** Razor or sharp-edged knife, needle, stands, distilled water.

4. Reagents Preparation

Eosin Stock Solution:

- i. Eosin Y - 1 g
- ii. Distilled water (100 mL)
- iii. Mix to dissolve.

4. Procedure

- i. Take a leafy shoot freshly cut under water and dip the cut end in Eosin solution taken in a beaker.
- ii. Adjust the leafy shoot in the stand & keep it for 30-40 minutes.
- iii. Cut a transverse section of the leafy shoot.
- iv. Mount the section in a drop of glycerin, put a coverslip and observe under the microscope (10 X and 40 X).

5. Observations

Red colored xylem elements indicate the path of colored solution.



Fig. 1: Ascent of sap in leafy shoot of rose

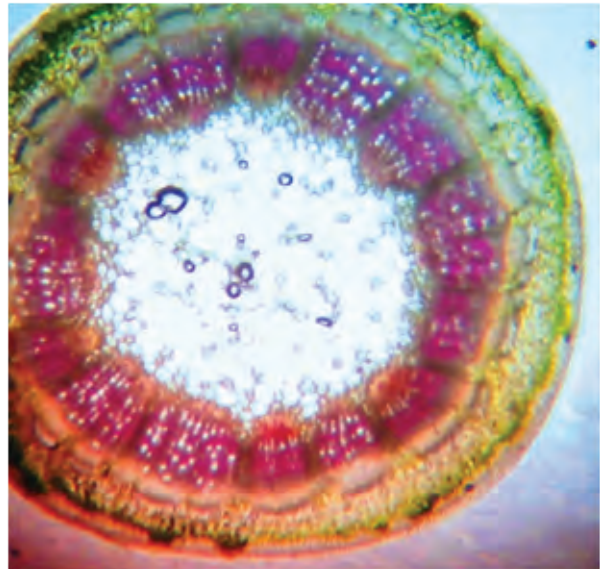


Fig. 2: T.S. of rose stem showing red colored xylem elements.

7. Result

With the above observations, it is clear that the phenomenon of ascent of sap takes place through xylem..

8. Precautions

- i. Leafy shoot should be cut in the water.
- ii. The T.S of the shoot should be uniformly thin.
- iii. Observations should be noted carefully.

Suggested Reading(s)

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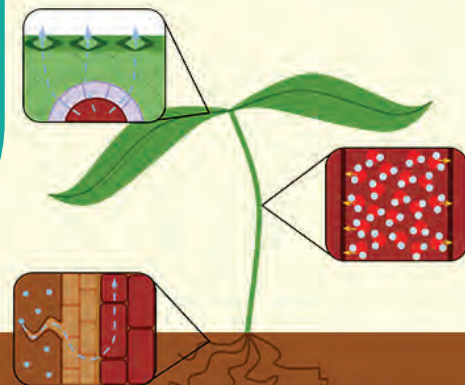
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DEMONSTRATION OF TRANSPIRATION PULL BY MERCURY METHOD



1. Aim

To demonstrate the transpiration pull by mercury method.

2. Introduction

Transpiration pull: A strain or tension developed in the water column in the xylem elements due to transpiration is called “transpiration pull” and is responsible for upward movement of water against gravity. Various theories have been proposed to explain this vital phenomenon viz. Capillarity theory, Imbibition theory, Atmospheric pressure theory, Root pressure theory, Vital theory and Cohesion theory. Among all these theories, cohesion theory is the most widely accepted theory. This theory was proposed by H. H. Dixon and J. Jolly in 1894. According to this hypothesis there are three basic elements for the ascent of sap: the driving force, adhesion or hydration and cohesion of water. The driving force is the gradient in decreasing (more negative) water potential from the soil through the plant to the atmosphere. Due to hydrogen bonding water shows adhesion, cohesion and high tensile strength and makes it possible to be pulled to the top of a tree by the driving force.

3. Materials Required

- 3.1. **Biological Material:** Excised plant twig (*Azadirachta indica*, *Polyalthia longifolia*)
- 3.2. **Chemicals/Reagents:** Mercury.
- 3.3. **Glassware/Plastic ware:** A long narrow glass tube wax, beaker
- 3.4. **Miscellaneous:** Stand, cork, water.

4. Procedure

- i. Cut a leafy shoot and fix it at one end of a narrow glass tube with water.
- ii. Apply wax to seal the joint.
- iii. Cover the other end of the glass tube with your finger and adjust it upright in a petri dish containing mercury.
- iv. Hold the set up with the help of a clamp stand.
- v. Set the apparatus in bright sunlight.
- vi. Observe the rise of mercury in the glass tube.

5. Observations

The length of water column in the glass tube decreases and mercury rises in the glass tube.

6. Result

Water is lost to the atmosphere due to transpiration by a leafy shoot. The suction created due to transpiration results in the rise of mercury in the narrow glass tube. This is called “transpiration pull” .

7. Precautions

- i. Take the leafy shoot from an actively transpiring plant.
- ii. Set the apparatus in bright sunlight.
- iii. The joint should be air tight.

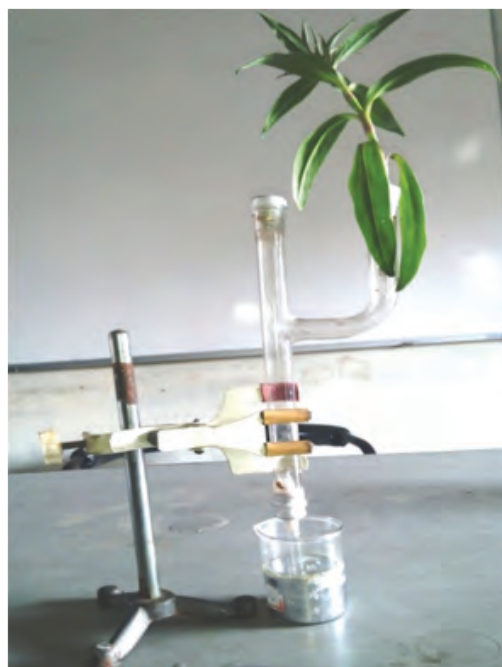


Fig. 1: Transpiration pull by mercury method

Suggested Reading(s)

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COMPARISON OF RATE OF TRANSPIRATION FROM LEAF SURFACE



1. Aim

To compare the loss of water from two surfaces of leaf by

- a) CoCl_2 method
- b) Four leaf method

2. Introduction

The process of water loss from the plants in the form of water vapors is called “transpiration”. If the diffusion of water vapor takes place through the stomatal pores it is known as “stomatal transpiration and accounts for 90 -95% of the water loss from leaves. If the loss is through cuticle then it is known as cuticular transpiration” and accounts for 5-10% of total water loss. About 0.1% of the total loss of water vapor occurs through lenticels present on fruits and woody stems and is known as “lenticular transpiration”. On the basis of distribution of stomata, there are five types of leaves:

- a. Amphistomatous- stomata present on both the surfaces of the leaves (e.g. maize, grasses).
- b. Epistomatous- stomata present only on the upper surface (e.g. floating aquatic leaves).
- c. Hypostomatous- stomata present only on lower surface (e.g. woody plants).
- d. Potato type – stomata present more on lower surface and less on upper surface (e.g. potato, tomato).
- e. Potamogeton type - stomata altogether absent or if present are vestigial (e.g. submerged aquatics). The rate of transpiration is directly proportional to the number of stomata present on the leaf surface.

A. CoCl_2 method

3. Materials Required

3.1. Biological Material: Potted plant (representative plant species of the particular region).

3.2. Chemicals/Reagents: Cobalt chloride solution (3%).

3.3. Equipment: Desiccators.

3.4. Glassware/Plastic ware: Beakers, glass slides.

3.5. Miscellaneous: Filter paper, thread.

4. Procedure

- i. Prepare 3% cobalt chloride solution in a beaker.
- ii. Dip filter paper strips in it, squeeze out excess solution.
- iii. Dry the strips & keep them in the desiccators.
- iv. Take a potted plant & select a healthy leaf.

- v. Place dry cobalt chloride treated filter strips on both surfaces of this leaf & immediately cover them with two glass slides (as shown in the Fig. 1).
- vi. Tie the slides firmly with thread.
- vii. Note the time taken for the change of color of strip on both sides.

5. Observation

The blue cobalt strip of lower epidermis turns pink first.



Unhydrated cobalt chloride Hydrated cobalt chloride

Blue in color

Pink in color

6. Result

The time taken for the change in color (from blue to pink) of the CoCl_2 strip is directly proportional to the number of stomata present. More the number of stomata, higher is the rate of transpiration. From the observations, the distribution of stomata on the leaf can be determined.



Fig. 1: Loss of water from two surfaces of leaf by CoCl_2 method

7. Precautions

- i. Keep the dried CoCl_2 strips in a desiccator.
- ii. Select a healthy and dry leaf for experiment.
- iii. Bind the slides immediately after placing strips on the sides of leaf.

B. Four leaf method:

3. Materials Required

3.1. Biological Material: Four leaves of *Ficus religiosa* (Peepal).

3.2. Equipment: Physical balance.

3.3. Miscellaneous: Vaseline, thread, iron stands, and forceps.

4. Procedure

- i. Take four healthy and young peepal leaves of almost equal size.
- ii. Apply vaseline to their cut ends.
- iii. Tie all these four leaves with a help of thread & label them as A, B, C and D.
- iv. Apply vaseline to the upper surface of leaf 'A', lower surface of leaf 'B', both surfaces of leaf 'C' and apply no vaseline on leaf 'D' (Equal amount of vaseline should be spread on the leaf surfaces).
- v. Note the weight of each of these four leaves one by one.
- vi. Hang these leaves with the help of iron stands and keep them in sunlight for few hours.
- vii. Observe the leaves carefully and note down their final weight.

5. Observations

S. No	Name of Leaf	Initial weight (gm)	Final weight (gm)
1	Leaf 'A'		
2	Leaf 'B'		
3	Leaf 'C'		
4	Leaf 'D'		

- The leaf 'D' wilts first followed by leaf 'A' and leaf 'B'.
- The maximum loss of weight is observed in leaf 'D' followed by leaf 'A'.
- The change in weight is almost nil in case of leaf 'C' and is negligible in leaf 'B'.



Fig. 2: Loss of water from two surfaces of leaf by four leaf method.

6. Results

- The number of stomata are more on the lower surface of leaf as compared to that of upper surface and so rate of transpiration is higher in leaf 'A' as compared to that of leaf 'B'.
- The vaseline blocks the stomata in leaf 'C' that does not show change in weight or wilting.
- The leaf 'D' shows maximum loss due to transpiration from both the surfaces.

7. Precautions:

- Apply vaseline smoothly and thoroughly on the assigned leaf surface.
- Block the cut end of the petiole with vaseline.
- Keep the leaves in bright sunlight.
- All leaves should be of about the same size. Vaseline should be uniformly spread on the surface of the leaves.

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DEMONSTRATION OF PHOTOTROPISM



1. Aim

To demonstrate phototropism.

2. Introduction

Phototropism is the movement of plant organs towards a unilateral source of light. Some parts of plant (viz. stem, leaves, branches, pedicels of flowers) move towards the source of light and are called positively phototropic while some other parts (viz. roots and rhizoids) move away from light and are termed negatively phototropic. When the plant parts move towards the direction perpendicular to the incident light, then it is called as diaphototropism. The response is due to the unequal growth rates on the two sides of the stem. The side facing the source of light shows less growth as compared to the side away from light. Phototropism was first studied by Charles Darwin (1880) in canary grass and oat coleoptiles. In later studies, F.W. Went suggested the involvement of a plant growth regulator, auxin, in this phenomenon.

Phototropic chamber: The phototropic chamber is an ordinary wooden box with removable top. It has a hole on one side for the supply of unidirectional light. The inside of the chamber is painted black to prevent the internal reflection of light.

3. Materials Required

3.1. Biological Material: A potted plant

3.2. Miscellaneous: Phototropic chamber (a chamber painted black from inside and with a hole on one side).

4. Procedure

- i. Take a well-watered potted plant.
- ii. Place it in the phototropic chamber and allow it to remain there for 2-3 days.
- iii. Keep the chamber near a window or provide an artificial source of white light and observe.

5. Observation

The stem has bent towards the hole & leaves got oriented at right angles to the light.

6. Result

- i. The stem is positively phototropic.
- ii. The leaves are diaphototropic.

7. Precautions

- i. Water the plant properly before keeping it in the chamber.
- ii. Place the box correctly so that plant receives proper sunlight through the hole.

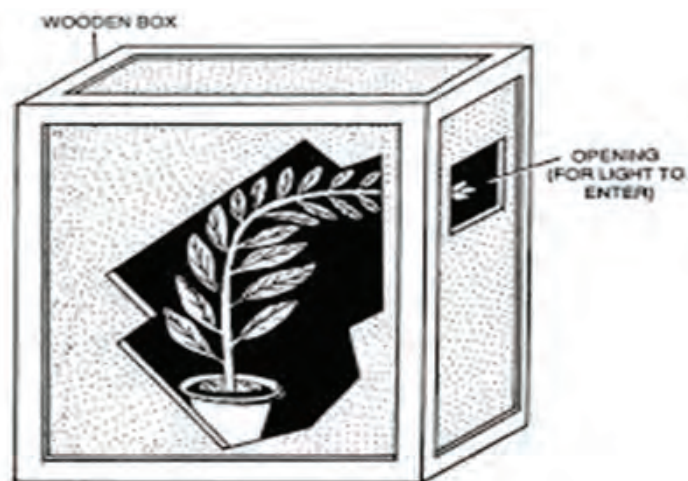


Fig. 1: Phototropic chamber

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STUDY OF SEED GERMINATION UNDER RED AND FAR RED RADIATION



1. Aim

To study seed germination under red and far red radiation.

2. Introduction

The pigment phytochrome (P) exists in two interconvertible forms one with absorption maximum in the red (R, 665 nm) region of the spectrum and one with an absorption maximum in the far red (FR, 730 nm). Absorption of red light by P_r would convert the pigment to the far red absorbing form while subsequent absorption of far red light by P_{fr} would drive it back to the red absorbing form. Phytochrome regulates a wide range of developmental responses in plants like seed germination, control of anthocyanins, de-etiolation etc. Through the present protocol the effect of light (red light, far red light) on seed germination of four plants can be studied.

3. Materials Required

3.1. Biological Materials: Seeds of Pea, Wheat, Mustard and Gram.

3.2. Glassware/Plastic ware: Petri dish.

3.3. Miscellaneous: Transparent paper.

4. Procedure

- i. Seeds of pea, wheat, mustard and gram are soaked in petri dishes.
- ii. 20 seeds are placed in each petri dish over filter paper and then covered with another petri dish.
- iii. Stimulations of red and far red light were done by placing the petri dishes under the red colored transparent paper.
- iv. One set was used as control set for each radiation.
- v. The experimental set up was placed in natural light near a south facing window.
- vi. The number of seeds germinated was noted every 6 days.

5. Observations

Sample: Pea Seeds

No. of days	Total seeds taken	Germination in normal light	Germination in red light	Germination in far red light

Sample: Mustard Seeds

No. of days	Total seeds taken	Germination in normal light	Germination in red light	Germination in far red light

Sample: Gram Seeds

No. of days	Total seeds taken	Germination in normal light	Germination in red light	Germination in far red light

Sample: Wheat Seeds

No. of days	Total seeds taken	Germination in normal light	Germination in red light	Germination in far red light

6. Result

The points are then plotted with the number of days in the X- axis and number of seeds germinated on the Y- axis.

7. Precautions

- i. Distilled water should be used to prevent microbial contamination.

Suggested Reading(s)

- i. Jain, V. K. (2000). *Fundamentals of plant physiology*. S. Chand limited. ISBN 9788121904629. <https://books.google.co.in/books?id=rDlenQEACAAJ>
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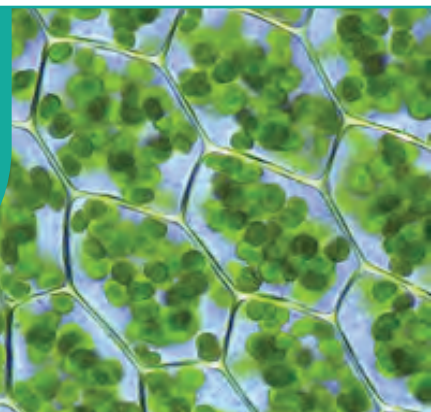
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DETERMINATION OF CHLOROPHYLL CONTENT



1. Aim

To determine the chl-a, chl-b and total chlorophyll content.

2. Introduction

Chlorophyll is the green pigment found in the leaves of aerial part of plants such as young stem, fruits and sepals of flower. Chlorophyll is a green pigment consisting of a tetrapyrrole ring with a central magnesium ion. It has a long hydrophobic phytol chain in its structure. Six different types of chlorophyll are known viz, chl-a, b, c, d, e and bacteriophyll. They are found to be distributed in the plant kingdom; of these chl-a and b are found in all higher green plants. The difference between these two chlorophyll molecules is that chl-a has a methyl group and chl-b has a formyl group. The ratio of chl-a to chl-b in higher plants is approximately 3:1. Chl-a is the primary pigment while chl-b is the accessory pigment that collects energy and passes it on to chl-a. Chlorophyll absorbs light mainly in the red (650 – 700 nm) and the blue - violet (400 – 500 nm) regions of the visible spectrum. Green light (~550 nm) is not absorbed but reflected giving chlorophyll its characteristic color. The chlorophyll content in an extract can be measured by using the following equation:

$$\text{Chlorophyll a (mg/g tissue)} = 12.7 (A_{663}) - 2.69 \times (A_{645}) \times V/(1000 \times W)$$

$$\text{Chlorophyll b (mg/g tissue)} = 22.9 (A_{645}) - 4.68 \times (A_{663}) \times V/(1000 \times W)$$

$$\text{Total Chlorophyll (mg/g tissue)} = 20.2 (A_{645}) + 8.02 \times (A_{663}) \times V/(1000 \times W)$$

Where,

A = Absorbance at specific wave length

V = Volume of chlorophyll extract in 80% acetone.

W = Fresh weight of the leaf tissue taken for the extraction.

3. Materials Required

3.1. Biological Material: Fresh leaves (spinach or any plant species of that particular region).

3.2. Chemicals/Reagents: 80% acetone.

3.3. Equipment: Spectrophotometer, centrifuge, mortar and pestle.

3.4. Glassware/Plastic ware: Volumetric flask (100 mL).

3.5. Miscellaneous: Water.

4. Procedure

- i. Weigh 1.0 g of fresh leaves after removing the midrib.
- ii. Grind the sample with the help of mortar and pestle using 20 mL of 80% acetone.
- iii. Centrifuge the mixture for 5 minutes at 5000 rpm.
- iv. Transfer the supernatant to a 100 mL volumetric flask.
- v. Repeat the process until the residue is colorless.
- vi. Make up the volume to 100 mL by adding 80% acetone to the mark of the flask.
- v. Shake the extract properly and take absorbance readings at 645 nm and 663 nm against the solvent blank.

5. Observation and Results

The concentration of different chlorophyll can be calculated from the table as follows:

Sample	Absorbance at 645 nm	Absorbance at 663 nm	Chl a (mg/g leaf tissue)	Chl b (mg/g leaf tissue)	Total chlorophyll (mg/g leaf tissue)

Chl-a,chl-b and total chlorophyll content can be calculated using the equations shown above.

6. Precautions

- i. Extractions are to be done carefully.
- ii. Calibrate the spectrophotometer with the blank before taking measurements of the sample.

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DETERMINATION OF MINIMUM QUADRAT SIZE

1. Aim

To determine the minimum size of quadrat for study of herbaceous vegetation in college campus, using species area curve method.

2. Introduction

Community ecology deals with groups of different kinds of population in the area. A group of several species (plants /animals) living in a natural area with mutual adjustment and beneficial interactions are known as Community or Biotic Community. Forest, grassland or a pond are examples of Natural Communities. Vegetation includes all plants of a particular area and comprises of small groups of population which ultimately form community. A community is characterized by various quantitative characteristics like frequency, density, abundance; dominance etc. and qualitative characteristic like phenology, species diversity, growth forms and structure, successional trend etc. Several methods have been used from time to time to assess these characters like Floristic methods, Physiognomic methods and Phytosociological methods. To study the composition, structure, species diversity, growth and trends of succession one can use Phytosociological methods which generally include three forms of sampling units: -Area, Line and Point. On the basis of nature of sampling units there are three popular methods of study of Communities. (1). Quadrat method (2). Transect method (3). Point method.

Quadrat Method:-A quadrat is the sampling unit which has an area of definite size. The shape of the quadrat varies depending upon the type of vegetation and purpose of study. The minimum size of the quadrat for a particular area can be defined as the size of quadrat in which maximum diversity of species can be recorded and can be determined by species area curve method.

3. Materials Required

3.1. Miscellaneous: Thread, scale, nails, graph paper.

4. Procedure

- i. A small quadrat (square) of 10 cm × 10 cm is laid in the college field area using thread, nails and a meter scale.
- ii. The number of plant species are counted in that area and noted down
- iii. The size of the quadrat is then further increased to 20×20 cm, 30×30 cm, 40×40 cm....100×100 cm (Table 1).
- iv. The number of species in each quadrat size is recorded.
- v. A graph is plotted between the size of quadrat (X axis) and number of species (Y axis).
- vi. The point of graph at which the curve starts flattening up is the minimum size of the quadrat required for sampling that field (Fig. 1).

5. Observations and Results

Size of Quadrat (cm x cm)	No. of Species
10x10	A
20x20	B
30x30	C
40x40	D
50x50	D

Table 1: Size of the quadrat and the no. of species.

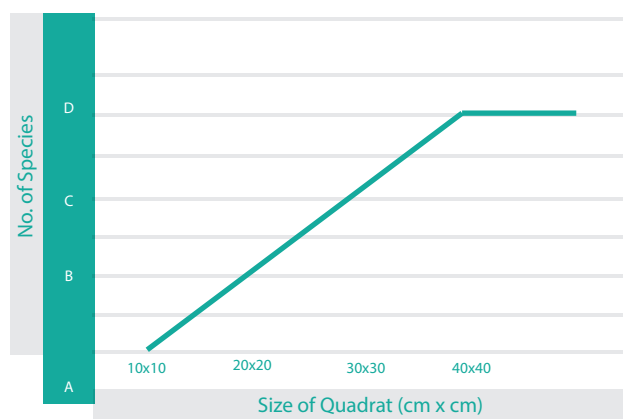


Fig. 1: No. of species and size of quadrat in cms.

From the above observations and graph, it is concluded that the minimum size of the quadrat is 40×40 cm which is the point at which the graph became straight line due to the constant number of species in the quadrats of higher size (viz. 50×50, and so on).

6. Precautions

- The number of species should be carefully recorded.
- The vegetation should not be uprooted.
- The site of study should be an undisturbed flora.

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CHEMICAL ANALYSIS OF SOIL SAMPLES



1. Aim

To analyse the chemical nature of soil by rapid spot tests.

2. Introduction

Soil may be defined as any part of earth's crust in which the plants root. The productivity of soil is dependent on its texture, nutrient composition and other physical characteristics. Biologically soil may be defined as the weathered superficial layer of the earth's crust in which the living organisms grow and also release the products of their activities like death and decay". Physical constitution of parent material influences the aeration, leaching rate and texture of the developing soil. The chemical composition of the soil is determined by the starting parent material and the climatic and other factors involved in pedogenesis. The soil complex contains almost all essential minerals as carbonates, sulphates, nitrates, chlorides and organic salts of Ca, Mg, Na, and K, etc.

3. Materials Required

3.1. Biological Material: Soil samples from different sites.

3.2. Chemicals/Reagents: Dilute HCl, diphenylamine, alcoholic solution of ammonium thiocyanate, H_2O_2 , sulphuric acid

3.3. Glassware/Plastic ware: White cavity tiles, test tubes.

4. Procedure

Rapid field tests to determine the chemical composition of the soil are performed as follows:

4.1. Test for Carbonate:

Take a pinch of soil in a white cavity tile and add dil. HCl to it. The acid reacts with carbonate and carbon dioxide gas escapes showing effervescence. Low carbonate content shows poor effervescence. Qualitatively, the degree of effervescence is divided on the scale of 1-4.

4.2. Test for Nitrate:

To a pinch of soil, in a white cavity tile, add few drops of 0.02% solution of diphenylamine in concentrated sulphuric acid. This produces blue colouration, the depth of which is directly proportional to the quantity of nitrate present. For the qualitative purpose, the nitrate content is expressed on the scale of 1-4.

4.3. Test for Base Exchange Capacity:

Deficiency of exchangeable bases like sodium, potassium, calcium and magnesium is easily determined by modified Comber's test. It is performed by vigorously shaking a pinch of soil with a standard alcoholic saturated solution of ammonium thiocyanate in a test tube and keeping it aside for the solids to settle down. Add a few drops of H_2O_2 to the clear supernatant liquid. Development of red colour in the solution indicates the base deficiency. It is noted qualitatively in 4 degrees of which very light red is 1 degree and deep red is 4 degrees.

4.4. Test for Reducing Capacity:

In this test, the colour produced before and after adding hydrogen peroxide gives the reducing capacity of soil. If by adding H_2O_2 the intensity of red colour increases, it indicates less reducing capacity of soil. In this condition a good proportion of exchangeable iron is present in the reduced ferrous state which, on getting oxidized with H_2O_2 intensifies the colour due to formation of ferric thiocyanate. The observations are recorded as below:

5. Observations

Soil	Carbonate content	Nitrate content	Base exchange capacity	Reducing capacity
Site I				
Site II				
Site III				
Site IV				

Table 1: Chemical properties of soil by rapid spot test

6. Results

- The given soil sample from Site I is composed of the _____ components.
- The given soil sample from Site II is composed of the _____ components.
- The given soil sample from Site III is composed of the _____ components.
- The given soil sample from Site IV is composed of the _____ components.

7. Precautions

- Concentrated acids should be handled carefully.
- Soil samples should ideally not be collected during or after rainy season.
- Soil samples should be oven dried before use.

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IDENTIFICATION OF TIMBER YIELDING PLANTS



1. Aim

Identification of timber yielding plants.

2. Introduction

Timber yielding plants are those which are exploited for timber that can later be used in many different ways. The timber yielding trees come from a wide range of families. It is easy to distinguish a tree especially when it is flowering as those are the diagnostic features available to identify a plant. However, when the wood is cut and kept aside, it becomes difficult to distinguish between different wooden pieces as the normal taxonomic features are not available. Many times, it happens that the wood which is sold in market may not be what it should be. Many timber merchants sell inferior quality timber passing it as high-quality timber. It is difficult for common man to distinguish between different types of wood. Wood from one plant species growing at different localities is also slightly different. Also, the number of local names and trade names for one type of wood increases the confusion. Therefore, it becomes very important to distinguish different types of wood material. Here plant anatomy can be used successfully to a good extent to distinguish between wood types.

Getting the actual plant twig containing some leaves, inflorescence and flower gives the basic picture of how an actual timber plant looks like in its vegetative and reproductive stages. This gives information about the arrangement of leaves, number, shape, size, color and the basic characteristics of flower (number of petals, size, color, and fragrance) and many other taxonomic features. Observing morphology of plant helps to relate the morphological and anatomical features. Observing the general characters of wood further narrows the search and gives information on some observable physical properties of wood. Observing the wood in cross section helps in determining the anatomical features, the size and arrangement of xylem vessels, parenchyma cells associated with it, type of wood, arrangement of tracheids and fibers. The features, characteristics and arrangement of rays are observed by taking transverse longitudinal section of mature stem. All these features together help in diagnosing the plant species of wood.

2.1. Teak



Fig. 1: Teak

- a) Botanical Name- *Tectona grandis*; Family- Verbenaceae
- b) Vernacular name- Sagwan (Hindi), Segan (Bengali), Adaviteeku, Peedateeku (Telugu), Tekku, Tekkumaran (Tamil), Tega (Kannada), Singua (Odishi), Saga, Sagach (Gujarati), Sag, Saga (Marathi).
- c) Tree description- Teak is large deciduous tree up to 35 m tall. It has grey brown branches. Leaves have 2-4 cm long petiole, papery leaves with hairs on lower surface. They are ovate-elliptic to ovate in shape, 15- 45 cm long and 8-22 cm wide with entire margin. The flowers are small, fragrant and white.
- d) Color- Sapwood white, pale yellow or grey and small. The heartwood when cut green has a pleasant aromatic fragrance and beautiful dark golden yellow which on seasoning soon darkens into brown darker streaks with a waxy feel. Sapwood and heartwood are sharply demarcated.
- e) Properties:
 - The wood is moderately hard and moderately heavy (650 kg/m³)
 - Strong and durable.
 - Straight, or sometimes wavy.
 - Texture- Coarse.
 - Very durable and highly resistant to termite damage.
 - Treatability- Extremely resistant; heartwood very refractory to treatment.
 - It is ring porous.
 - Growth rings are distinct, delimited with early wood vessels enclosed in paranchymatous tissues.
- f) Vessels- In late wood, they are medium to small, mostly solitary or in short radial multiples, round to oval in outline. Vessels in early wood are large, oval in outline and occasionally filled with tyloses and yellowish-white powdery deposits. Vessel lines of early wood zone one conspicuous on longitudinal surfaces.

- g) Parenchyma- It is paratracheal, vasicentric and in broad bands, distinct under the hand lens but distinct to the eye in early wood forming a continuous zone enclosing the vessel along with band of parenchyma delimiting growth.
- h) Rays- They are fairly wide spaced and distributed uniformly and moderately broad. They are distinct under the lens.
- i) Uses- In India, it is highly priced for construction, ship building, railway wagons, and sleepers, etc.

2.2. Shisham



Fig. 2: Shisham

- a) Botanical Name- *Dalbergia sissoo*. Family- Leguminosae (Fabaceae)
- b) Vernacular name- Shisham, Sisso (Hindi), Gette, Yette (Tamil), Sinsupa (Telugu), Birdi (Kannada), Sisam, Shishma (Gujarati).
- c) Tree description- The tree is 15-30 m tall, deciduous, often has crooked trunk and light crown. Bark is grey or brown, longitudinally and somewhat reticulately furrowed, thick. Leaves leathery, alternate, pinnately compound and about 15 cm, with 3-5 leaflets which are orbicular and abruptly acuminate. Flowers are whitish to pink, fragrant, sessile or short stalked in axillary panicles shorter than the leaves.
- d) Color- The sapwood is narrow and pale yellowish white in color often with the purple tinge. The heart wood ranges in color from golden brown through shades of light rose, purple to deep purple with nearly black lines darkening with age.
- e) Properties:
 - Hard to moderately heavy wood (820 kg/m³)
 - Narrowly interlocked grain.
 - Medium to coarse texture.
 - It is very durable and highly resistant to termites
 - Treatability- Extremely resistant
 - Wood is diffused porous. The timber is stronger and harder than teak. It resists any type of fungal attack.
 - Growth rings- They are distinct to indistinct, demarcated by a fine line of parenchyma but indistinct or inconspicuous.

- f) Vessels- They are large to small, few to moderately few, unevenly distributed, generally larger and comparatively more numerous in early wood; and small and few in late wood. They are mostly solitary and in radial multiples, open but occasionally filled with white or dark, gummy deposits, perforation simple, inter vessel pits vestured and small to medium sized.
- g) Parenchyma- It is aliform to aliform-confluent also forming straight to wavy narrow bands in late wood. Terminal or marginal, delimiting growth rings, strands are storied, fusiform often subdivided into crystalliferous locules.
- h) Fibers- Librifom, round to angular in cross section, often gelatinous, non-septate, storied, inter-fiber pits small, simple to narrow bordered.
- i) Rays- Uniseriate mostly but may be 2 or 3 seriate, weakly heterogenous composed mainly of procumbent cells.
- j) Uses- It ranks among the first wood for furniture and cabinet work. It is very suitable for carving, pattern making, in mathematical instruments, etc. it is also used for ammunition boxes, army wagons, gun carriage wheels, agricultural implements.

2.3. Babul

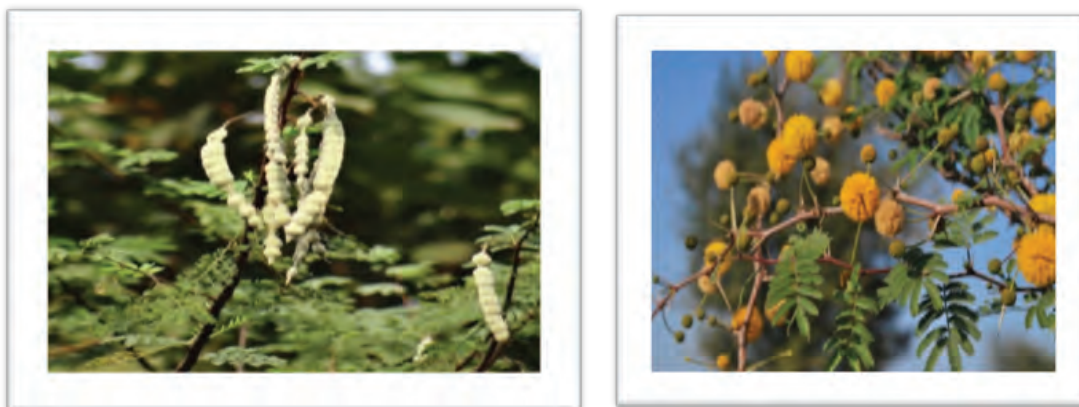


Fig. 3: Babul

- a) Botanical Name- *Acacia nilotica*. Family- Leguminosae (Fabaceae)
- b) Vernacular name- Babul, Babur (Hindi), Babla (Bengali), Karuvai, Karuvelam (Tamil), Nellatuma (Telugu), Karuvelam (Malayali), Fati, Karrijali, Jali, Meshwal, Gobli (Kannada), Kikar (Punjabi), Bawal (Gujrati)
- c) Tree description- It is small evergreen tree reaching a height of 10 m and diameter of 30 cm. It has short thick cylindrical trunk and a spreading crown. Bark has deep narrow longitudinal fissures running spirally. It is black or dark brown and rough. The stems and branches are dark to black in color. The stem has thin, straight, slightly recurved grey spines in axillary pairs, usually in 3-12 pairs, 3-6 cm long in young trees, mature trees are without thorns. The leaves often with petiolar glands, bipinnate with 3-6 pairs of leaflets. Flowers are in globose heads, 0.7-1.5 cm in diameter of a bright golden yellow color. Pods are strongly constricted, hairy, white grey, thick and softly tomentose.

- d) Color- The sapwood is yellowish white and usually wide. The heartwood when freshly cut is a pinkish or old rose color but it darkens on exposure to a dull red or reddish brown, it is often mottled with darker streaks, and lustrous. Heartwood is sharply demarcated from the sapwood.
- e) Properties :
- It is heavy (800 kg /m³), hard, tough wood.
 - Straight or slightly twisted to interlocked grain.
 - Medium to fairly coarse texture.
 - The sap wood is not durable. The heart wood is not durable to the same degree as teak and sal.
 - Treatability- Resistant
 - Wood is diffused porous. The wood is dull without taste or smell. It is nearly twice as hard as teak and has a very good shock resisting ability.
 - Growth rings are indistinct.
- f) Vessels- Medium to small, few to moderately few, mostly solitary or in radial multiples of 2, 3 or more, occasionally in clusters, filled with dark brown gummy deposits. Soft tissues are visible to the eye forming thick sheaths or halos round the pores.
- g) Parenchyma- Paratracheal vasicentric, fine lines delimiting growth rings.
- h) Rays- Moderately broad to fine, widely and irregularly spaced.
- i) Uses- It is popular for parts of carts, agricultural implements, tents, types of handles and is good for turnery wood.

2.4. Mango



Fig. 4: Mango

- a) Botanical Name- *Mangifera indica*; Family- Anacardiaceae.
- b) Vernacular name- Aam (Hindi), Maavu (kannada)
- c) Tree description- It is medium to large 15-30 m in height and 50-100 cm in diameter. Bark brown or dark grey, rough. The leaves of mango tree are evergreen, simple, oval-lanceolate, with slightly wavy margin. The length and breadth varies from 2-45 cm and 2-12 cm respectively. The color of young leaves generally varies from tan-red, pink. The color changes rapidly to dark glossy red then dark green as they mature. The flowers are produced in terminal panicles. Each flower is small white with 5 petals 5-10 mm long.

- d) Color- Yellowish white to greyish brown.
- e) Properties -
 - Moderately heavy (690 kg/m^3)
 - Straight to curly to somewhat interlocked grain.
 - Medium to coarse texture.
 - Non-durable.
 - Easily treatable.
 - Wood is grey or grayish brown wood, no taste or smell, medium weight, strong wood which retains its shape extremely well. It is somewhat lustrous. It is slightly lighter than teak, and in shock resistance and shear, it is equal or slightly better than teak.
 - Growth rings are fairly distinct.
- f) Vessels- Large to medium, few to moderately numerous, solitary or in radial multiples of 2-3 or more, often filled with tyloses.
- g) Parenchyma- Paratracheal- aliform to confluent, often delimiting growth rings.
- h) Rays- Fine to moderately broad, numerous, closely spaced.
- i) Pith- Flecks are usually present.
- j) Uses- It is used for rotary veneer work, ply wood making, chief furniture, floor ceiling boards, boat building, agricultural implement and parts of cart and ply wood manufacture

3. Materials Required

3.1. Biological Materials: Plant twigs, mature stems and square or rectangular wooden blocks of Mango, Babul, Shisham and Teak.

3.2. Chemicals/Reagents: Safranin, glycerin.

3.3. Equipment: Digital weighing balance.

3.4. Glassware/Plastic ware: Dropper, watch glass, slides.

3.5. Miscellaneous: Sharp razor, brush, needles, pencil, eraser.

4. Procedure

4.1. Recording the basic morphological features of plant:

- i Get a twig of plant preferably with flowers. Observe and compare the morphology of stem, leaf, and flower with the tree description given below.
- ii Observe the size, shape, color of stem, leaves, flowers and fruits.

4.2. Recording the general features of wood:

- i. Record the features like color, hardness, odour, luster, texture, grain pattern by observing the wooden blocks carefully with naked eye and hand lens.
- ii. Calculate the volume of wooden block and take the weight using digital balance. Use the values for calculating the density of wood (mass per unit volume expressed in g/cm³ or kg/m³).

4.3. Cutting transverse and longitudinal stem sections:

- i. Use a mature and old stem which shows secondary growth for both stem sections.
- ii. For Transverse Section, take a razor blade, mask one edge of blade with masking tape.
- iii. Hold a 2-3 cm long piece of stem material between thumb and first finger of your left hand.
- iv. Dip the top of the stem or wood material in water and cut transverse section by drawing razor blade across the top of the material to give the material a drawing cut i.e. at an angle of 45° in horizontal direction.
- v. Cut several sections in a watch glass containing water.
- vi. Transfer thinnest section with the help of brush in a clean watch glass containing water and 2 drops of safranin. Allow the section to stain for 3 minutes. Drain off stain and wash with water if necessary.
- vii. Put the section in the centre of the slide and put a drop of glycerin over the material. Cover with coverslip with the help of needle. Focus the slide under low power and then change to high power.
- viii. For Tangential Longitudinal Section- cut a section which is parallel to the long axis of the stem but cut part should be off the center along a tangent. It is similar to removing the slices of wood from a new unsharpened pencil from the surface. This can be done by holding the stem erect on the platform with the help of paper pad holder.
- ix. Follow rest of the procedure for staining and observing as given above.

5. Observations

Sr. No	Character	Observation
	Name of Plant	
	Morphological characters	
1	Length of leaves (in mean \pm S.D)	
2	Breadth of leaves (in mean \pm S.D)	
3	Length of petiole (in mean \pm S.D)	

4	Shape of leaves	
5	Number and shape of leaflets	
6	Any special character of leaves	
7	Color and fragrance of flower	
8	Size of flower (in mean \pm S.D)	
9	Number of sepals and petals	
Wood characters		
10	Color	
11	Density (weight/volume in gm /cm ³)	
12	Grain	
13	Texture	
14	Other special character	
Anatomical characters		
15	Wood type	
16	Growth rings	
17	Vessels	
18	Parenchyma	
19	Rays	
20	Other special character	

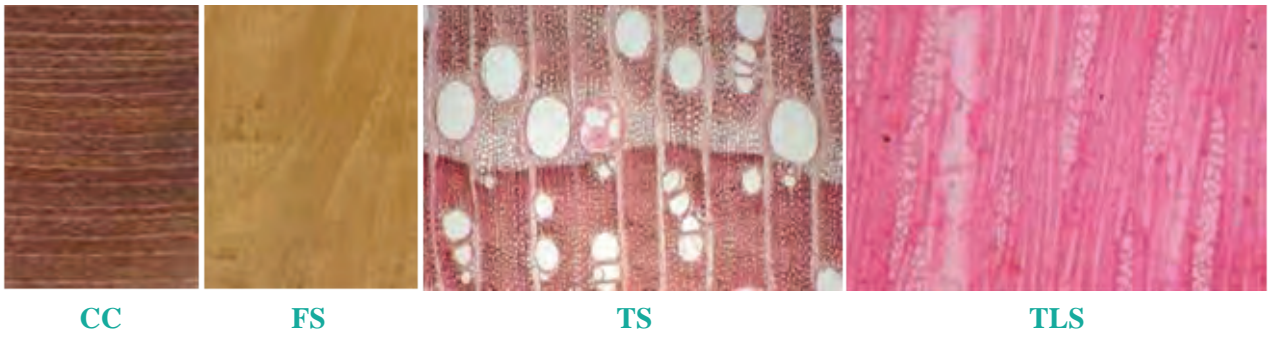


Fig. 5: Teak Wood

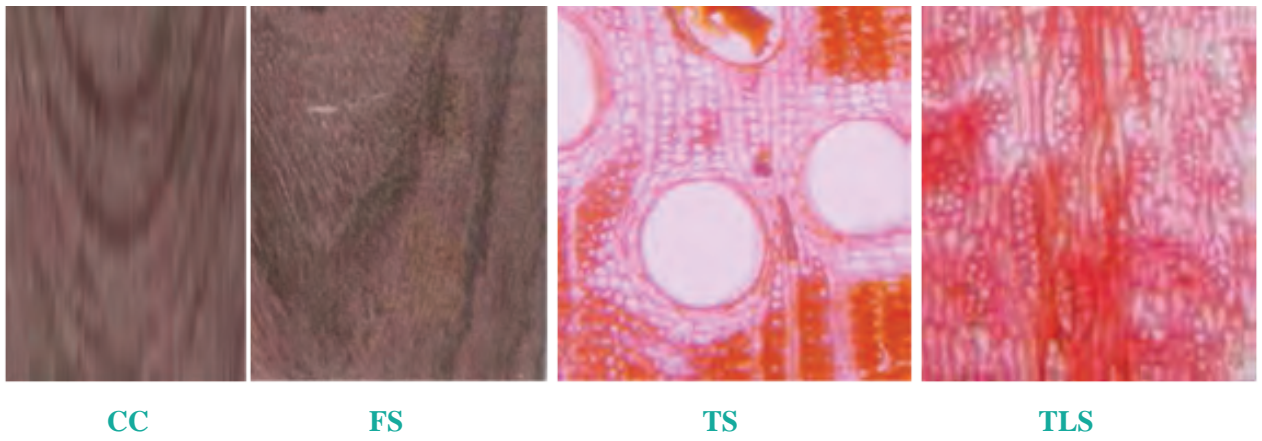


Fig. 6: Shisham Wood

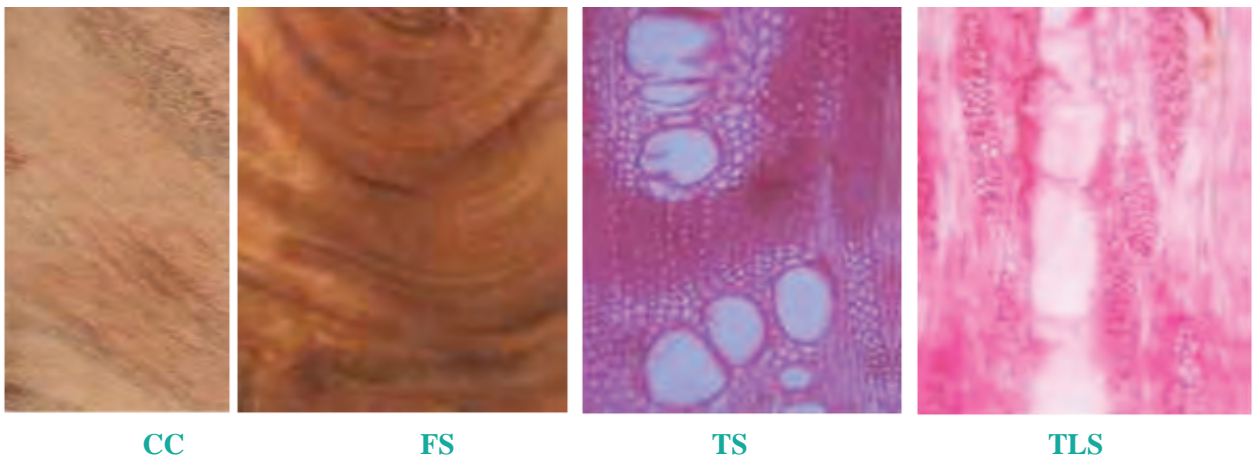


Fig. 7: Babul Wood

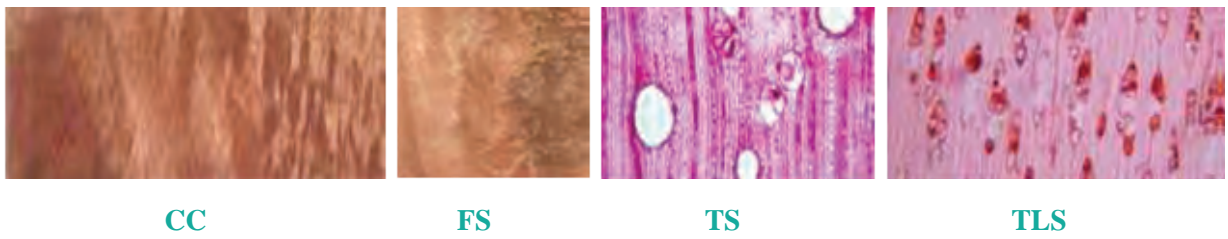


Fig. 8: Mango Wood

(CC- cross cut; FS- flat sawn; TS- transverse section; TLS- transverse longitudinal section)

Source of above pictures:- Timber Identification Manual - Manual of Timbers used by Wood Based Handicrafts Industry of Kerala, Uttar Pradesh and Rajasthan ('Complying with Lacey Act and the flegt action Plan in India', Dr. E.V. Anoop Dept. of Wood Science College of Forestry, Kerala Agricultural University, Thrissur, Kerala & MKS Pasha TRAFFIC India, New Delhi)

6. Result

The morphological characters of plant, the physical and anatomical characters of wood resemble the _____ tree species given in the theory text.

7. Precautions

- i. Stained sections should be washed with water to remove excess stain.
- ii. Avoid air bubbles in the section and while mounting the section.
- iii. Do not use needles for the purpose of transferring or handling the sections

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TISSUE CULTURE STUDIES IN MEDICINAL PLANT



1. Aim

To study organogenesis in *Bacopa monnieri*, a medicinal plant

2. Introduction

Plants are vital sources of medicine and play a key role in world health. The increasing demand for herbal synthetic drugs and antibiotics has highlighted the need for conservation and propagation of medicinal plants. Micro propagation is of special use for the conservation of these valuable genotypes with shoot cultures. Plant tissue culture is a collection of techniques used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. Regeneration of plants can be achieved through organogenesis and somatic embryogenesis. The steps of regeneration include initiation, proliferation, elongation, rooting and acclimatization.

Bacopa monnieri, commonly known as Brahmi, is an amphibious plant of tropics and normally grows on the banks of the rivers and lakes. It finds valuable application in ayurvedic medicine and is used to enhance memory, concentration, learning, as well as to cure mental illness. Besides, the plant has anti-inflammatory, analgesic, antipyretic, anticancerous, anticonvulsive and antioxidant properties. Research on anxiety, epilepsy, bronchitis and asthma, irritable bowel syndrome and gastric ulcers also supports the ayurvedic uses of Bacopa. It also has the ability to phytoremediate toxic heavy metals (e.g. cadmium, chromium and mercury) from aquatic bodies by absorbing and accumulating these metals in their shoots and roots. The conventional method of vegetative propagation is slow and inadequate to meet the demand of raw material of *B. monnieri*. The unsustainable collection of raw material from natural population has already placed the plant under threatened category. Thus, for constant supply of raw materials and reduction of pressure on natural/wild population, development of an efficient and reliable *in vitro* plant regeneration protocol for this wonder medicinal herb is essential.

3. Materials Required

- 3.1. Biological Material:** Shoot tip and nodal explants of *Bacopa monnieri* (Brahmi)
- 3.2. Chemicals/Reagents:** MS medium, sucrose, agar, 6-benzylaminopurine (BAP), 1N hydrochloric acid, 1N sodium hydroxide, teepol, 0.1% mercuric chloride, ethyl alcohol, double distilled water.
- 3.3. Equipment:** Magnetic stirrer, pH meter, autoclave, laminar airflow cabinet, plant growth chamber.
- 3.4. Glassware/Plastic ware:** Schott Duran bottle, beakers, conical flasks, petri plates, forceps, scalpel, spirit lamp, boiling tube, scissors.

4. Preparations of Reagents:

Cytokinins / (BAP): Weigh 20 mg of BAP, dissolve it in few drops of HCl and make the volume up to 100 mL using double distilled water. Store the stock solution in the refrigerator.

5. Procedure

5.1. Media Preparation:

- i. Dissolve 4.4 g/L MS medium (Himedia) in 800 mL of distilled water and add plant growth regulators (BAP 1 mg/L) from the stock already prepared and stored.
- ii. After that add 3% sucrose and make the volume up to 1 litre. Adjust the pH of the medium to 5.8 by adding 1N HCl / 1N NaOH before gelling with agar (0.8%)
- iii. Autoclave the medium at 121 °C at 15 lb pressure for 20 minutes. After that, pour the medium in conical flasks under laminar air flow cabinet.

5.2. Explant Culture:

- i. Take the shoot tip and nodal explants from fresh plant of *Bacopa monnieri*
- ii. Wash the explants under running tap water for 15 minutes, and treat them with 5% teepol for 10 minutes followed by rinsing with double distilled water for 4-6 times.
- iii. Sterilize the surface of the explants with 0.1% aqueous solution of HgCl₂ for 3 mins followed by washing with autoclaved double distilled water 2-3 times inside the laminar airflow cabinet.
- iv. Culture the surface sterilized explants on the MS medium supplemented with BAP (1 mg/L) for shoot initiation.
- v. Keep the cultures in the culture room at 26±1 °C, with a 16 hrs. photo period provided with cool white fluorescent tubes.
- vi. After four weeks of culture, subculture the swollen nodal explants with elongated shoots and large number of shoot buds by either cutting them transversely or as such with the aim of further shoot and root proliferation.
- v. After 4-5 weeks, take the *in vitro* plantlets from the culture flasks and remove the agar carefully from the roots under running tap water. Then transfer the plantlets directly to the pots containing garden soil and keep in culture room for 2 days.
- vi. Transfer the acclimatized plants to the earthen pots in botanical garden.

6. Observations

It was observed that after one week of culture, the nodal explants got swollen and numerous shoot buds were initiated, of which only few shoot buds were elongated. The enlarged nodal explants with induced shoot buds were transversely cut and sub-cultured on MS supplemented with 1.0 mg/L BAP for elongation of shoot buds. An average of 7 shoots /explant with an average length of 4 cm was recorded after 3 weeks of sub-culture. All the regenerated plants were successfully acclimatized.

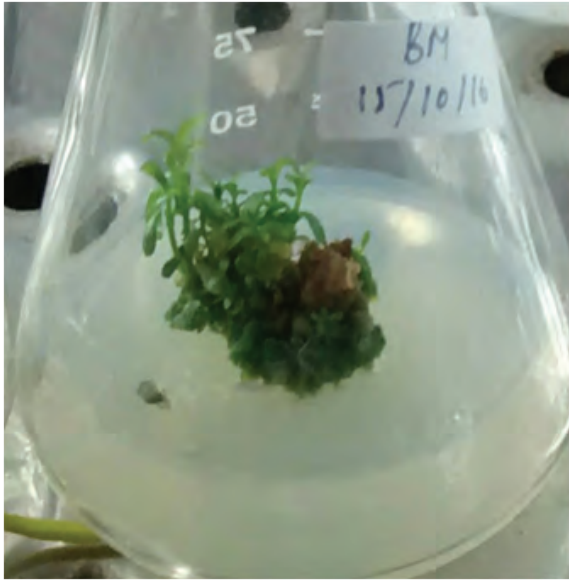


Fig. 1: Nodal explant cultures of *B.monneri* on MS medium modified with 1 mg/L BAP

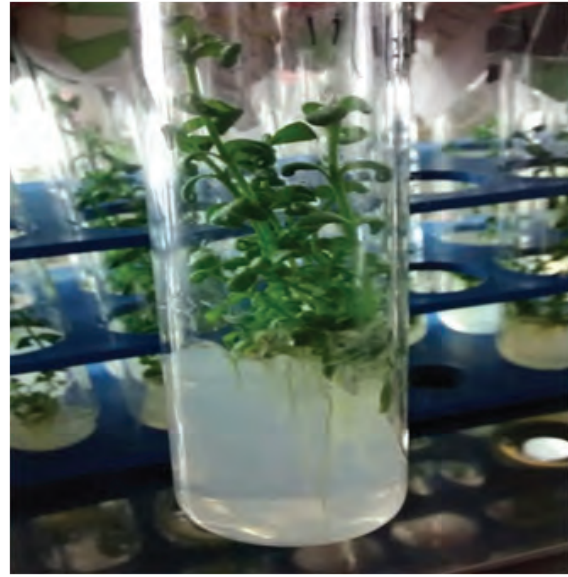


Fig. 2: Sub cultured nodal explants with induced shoot buds after 3 weeks of culture



Fig. 3: Plantlets transferred to pots for acclimatization after 4 weeks.

7. Results

An efficient and reproducible regeneration protocol has been standardized for micro propagation of an important medicinal plant; *Bacopa monnieri*. BAP was used for shoot regeneration; the stability of ribosides and nucleotides occurring naturally in BAP compared to other cytokinins possibly give BAP an edge which may be the cause of enhanced shoot multiplication response in a number of plant species including *B. monnieri*. The results obtained here suggest that a single step rooting and acclimatization process reduces the cost and time for *in vitro* plant regeneration and is a better protocol.

8. Precautions

- i. All the glassware were disinfected with teepol and wiped with ethyl alcohol to avoid contamination.
- ii. The explants were carefully cut and washed and should not be exposed to ultra violet radiation under any conditions.
- iii. The measurements and data should be recorded with precision.

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ZOOLOGY



Separator Photo Credit

Raochestes akroparallagi at Kakkayam, Kerala
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CULTURE OF HYDRA IN THE LABORATORY



1. Aim

To culture Hydra and study its behaviour.

2. Introduction

Hydra is a coelenterate free-living polyp found in freshwaters. They are small cnidarian polyps that under controlled feeding and temperature can reach a size of 5–20 mm depending on species and strain. They are considered a pest in freshwater aquaria. Armed with tentacles and nematocysts. They are interesting model systems to study various biological aspects. Studies using Hydra span biology from ecology and evolution to molecular biology. Insights into mechanism of wound healing, development and regeneration are other areas where Hydra as a model system is popularly used by scientists.

On the basis of anatomy of the nematocysts, Hydra is classified into four main groups - *H. vulgaris*, *H. oligactis*, *H. braueri* and *H. viridissima*. Commonly collected strains are Brown Hydra (*vulgaris* or *oligactis*) and Green Hydra (*viridissima*). Green Hydra harbour symbiotic intracellular Chlorella-like algae found only in the endoderm. *H. oligactis* (unlike *H. vulgaris*) is confined to the Northern Hemisphere and it is considered a cold-water species.

3. Materials Required

3.1. Biological Materials: Hydra from pond water, *Daphnia*, *Nauplius* of *Artemia*.

3.2. Chemicals/Reagents: CaCl₂, NaCl, KCl, Tris base, MgSO₄, HCl, dechlorinated tap water / potable water, distilled water, absolute alcohol for cleaning glassware.

3.3. Equipment: Magnifying hand lens, dissecting microscope / stereo microscope, camera and image capture software for microscope (optional), autoclave for sterilisation.

3.4. Glassware/Plastic ware: Pyrex dishes or glass bowls as culture vessels, loose covers for the culture vessels to permit gas exchange.

3.5. Miscellaneous: Pasteur pipettes with tips fire polished to remove sharp edges, larger glass pipettes (5 mL and 10 mL) with rubber bulbs for transferring Hydra, clean muslin cloth, tungsten bulb (CFL lamps may also be used).

4. Procedure

4.1. Preparation of culture medium:

- Solution 1:** Prepare a solution consisting 0.5 M CaCl₂, 0.5 M NaCl, and 0.05 M KCl in distilled water.
- Solution 2:** 0.5 M Tris base (pH 7.7 adjusted with HCl) in distilled water
- Solution 3:** 0.05 M MgSO₄ in distilled water.
- Autoclave the stock solutions for 15 minutes and store them at room temperature.

- v. Prepare Hydra Culture Medium: Take 900 mL distilled water in one litre standard flask and add 2 mL solution 1, 2 mL solution 2 and 2 mL solution 3. Stir for at least 10 minutes. Dilute to 1000 mL mark with additional distilled water. Mix well.
- vi. The final culture medium will contain 1 mM Tris, 1 mM NaCl, 1 mM CaCl₂, 0.1 mM KCl, and 0.1 mM MgSO₄, with a pH 7.6.

NOTE: Short – term cultures of Hydra can be successfully maintained in dechlorinated tapwater itself. With little care, the colony can be maintained for several days.

4.2. Collection of Hydra from wild:

- i. Take a bucketful of potable tap water and keep it overnight to release all the chlorine. Use this water to collect Hydra.
- ii. Hydra can be found in any reasonably unpolluted body of freshwater in any season. collect the Hydra as explained below;

Method a)

- i. Collect submerged sticks, leaves or submerged vegetation from a pond. Place these in the same pond water collected in a glass or enamel pan. Keep the pan overnight and carefully examine under a low power dissecting microscope. Hydra is usually found.
- ii. Take a clean 5 mL pipette. Attach a rubber bulb. Use this pipette to suck fresh pond water very close to submerged vegetation, under part of submerged leaves etc. Transfer the water to a bowl or enamelled pan.
- iii. Take the water back to the laboratory and examine the water under dissecting microscope, to locate hydra.

Method b)

- i. Take a fresh soft tooth brush. Clean it with flowing clean tap water. Rinse the brush in dechlorinated tap water. Use this brush and scrap on rocks, under surface leaves submerged stem, sticks, twigs etc. Dip the brush in a beaker of dechlorinated potable water to release all the scrapped material. Repeat this several times. Take the water in the beaker to the laboratory and examine under the dissecting microscope to locate Hydra.

Method c)

Tranfering individual Hydra

- i. Once the Hydra is located, use a good Pasteur pipette and gently suck the Hydra into the pasture pipette tube. Transfer the Hydra into a collection bowl, one by one. The collection bowl can be filled with dechlorinated tap water.
- ii. If the Hydra are attached to any surface, gently squirt a stream of water on the Hydra (without air bubbles) using the Pasteur pipette. The Hydra will get dislodged and they can be collected as explained before.
- iii. Hydra is extremely sensitive to detergent and heavy metals (e.g. even tap water is toxic) so new Pyrex dishes or autoclaved glass bowls are recommended to keep the water collected.
- iv. The Hydra polyps are rarely visible immediately, because they shrink due to the stress of collection and agitation. As the water becomes stationery careful observation with a microscope or magnifying glass will usually reveal the extended polyps the next day.

4.3. Food for Hydra:

- i. Hydra polyps can be fed either with *Daphnia* or *Nauplius* larvae of *Artemia*. It is important to feed *Daphnia* or *Nauplius* larvae of *Artemia*, from monocultures to prevent contamination.

Daphnia

- i. Obtain *Daphnia* colony from an aquarist and isolate *Daphnia*.
- ii. Keep them in a 500 mL beaker filled with 300 mL of dechlorinated tap water. Aerate the colony with an aerator.
- iii. Feed the *Daphnia* with a drop of *E. coli* culture or a drop of pasteurised milk.
- iv. Avoid adding excess feed.
- v. Maintain the monoculture of *Daphnia* for feeding Hydra.

Nauplius of Artemia

- i. Prepare sea water by dissolving 33 g of NaCl in 1000 mL of dechlorinated tap water (33% solution).
- ii. Obtain lyophilized stocks of *Artemia* eggs.
- iii. Take a glass beaker or wide mouthed glass bottle and fill it 3/4th with sea water.
- iv. Sprinkle a pinch of eggs on the surface of sea water.
- v. Cover the container with permeable film and place it at room temperature in a cool place (about 23 °C).
- vi. Aerate the water such that the air bubbles rise up from the bottom of the container.
- vii. Place an incandescent bulb, near the culture vessel, shining on the container.
- viii. The eggs will hatch in two days.
- ix. *Artemia* are attracted to light and will congregate along the sides of the container close to the light source.
- x. Use a 10 mL pipette with a rubber bulb to collect the *Nauplius* larvae into a container pre-filled with hydra culture medium or dechlorinated tap water.
- xi. Filter the larvae through muslin cloth and transfer them gently but, immediately into a fresh container with dechlorinated tap water.
- xii. Repeat the washing once more to remove the salt from the *Nauplius* larvae.
- xiii. Use a clean Pasteur pipette to pick up the larvae and feed to the Hydra. A single Hydra may eat up to two *Nauplius* larvae at a time. Refrain from overfeeding.
- xiv. The Pasteur pipette and the muslin cloth should be rinsed thoroughly in dechlorinated water or distilled water before and after each use.

4.4. Culture of Hydra:

- i. Autoclave clean glass bowls or pyrex square dishes for use as culture vessels.
- ii. Partially fill the culture vessels with culture medium.
- iii. Gently transfer few Hydra polyps, using pasture pipette into the culture vessels. About 10 – 15 hydra polyps in 100 mL of medium is adequate.
- iv. Keep the culture vessels at 22 °C (air-conditioned room) or in a BOD incubator.
- v. Allow the hydra to settle by keeping the culture undisturbed.
- vi. Feed the Hydra with *Daphnia* (one – two *Daphnia* per polyp) or *Nauplius* of *Artemia* (one – two larvae per polyp).



Fig. 1: A healthy Polyp of Hydra

- vii. Use a Pasteur pipette dedicated to the feeding and change it frequently to minimize contamination of Hydra with pathogens.
- viii. In laboratory cultures, Hydra floats when hungry and attach again after they have been fed.

4.5. Cleaning the Culture:

- i. Clean the Hydra culture about two – three hours post-feeding.
- ii. Remains of unfed food, dead food and the egestion of Hydra after feedings will lead to contamination of culture with sub culture of fungus and bacteria. This will be fatal to the Hydra colony.
- iii. After feeding, the Hydra will become sluggish and will remain attached to the sides of the culture vessel.
- iv. Gently decant the culture medium without any jerks and disturbance to the colony.
- v. After the medium is fully drained, add fresh medium by gently pouring from the sides of the culture vessels.
- vi. Feeding can be made once in two days in the daytime so that post-feeding cleaning can also be completed.
- vii. Once a week or twice a week (depending on the contamination), the hydra must be transferred to fresh culture vessel with fresh culture medium.
- viii. To transfer the Hydra, use a pasteur pipette and gently squirt the culture medium on the base of the Hydra (without blowing air bubbles). The Hydra will be dislodged.
- ix. Pick up the dislodged Hydra by sucking it gently into the tube of the Pasteur pipette.
- x. Release the Hydra gently into another culture vessel filled with fresh culture medium.

5. Observations

5.1. Feeding behaviour:

- i. Take a small petri dish (2" diameter) or 6-well culture microplates and fill it partially with culture medium.
- ii. Gently, transfer a single Hydra polyp into the petri dish or microplate.
- iii. Observe the polyp under a low magnification microscope or stereo microscope (If possible, attach a image recording device so that the polyp can be easily observed).
- iv. Introduce one or two *Daphnia* or *Artemia Nauplius* into the culture.
- v. Observe the behaviour of the Hydra.
- vi. Record the behaviour; reaction of the polyp, tentacular movements, time taken for the prey to be engulfed, movement of the mouth, time taken for complete feeding etc.

5.2. Budding:

- i. Transfer a Hydra polyp that has bulging on its side into a petri dish or microplate.
- ii. Observe under the microscope and record the bud and also stages of bud development.
- iii. The testis and ovaries (during sexual reproduction) can also be identified under the microscope.

5.3. Staining the nerve net:

- i. Transfer a hydra polyp into a clean watch glass along with little culture medium.
- ii. Add dilute aqueous methylene blue (0.1%) stain.
- iii. Allow the Hydra to stain for few minutes (stain several polyps for varying time periods)
- iv. Make a temporary mounting in glycerine.

- v. Observe under the compound microscope, the nerve ganglia appear as blue dots.
- vi. Note the distribution of nerve ganglia in the body of the polyp. Note the abundant supply of ganglia around the hypostome.

6. Precautions

- i. The culturing method may need slight modifications to establish the colony to local ambient conditions.
- ii. The hydra polyps are quite sensitive to traces of detergents, residual toxins, metal ions and chlorine etc. Use only thoroughly cleaned culture vessels.
- iii. Contamination with sub cultures of fungus and bacteria must be prevented. Use dedicated Pasteur pipettes that are cleaned and autoclaved for feeding and transferring.
- iv. The hydra polyps are very sensitive to jerks and sudden movements, even those caused by the flow of medium while pouring.
- v. Avoid overfeeding, the polyps have tendency to overfeed and gorge themselves to death.
- vi. The hydra polyps will start budding and forming new young polyps within one week of good maintenance and feeding.

7. Extension Activities

- i. Hydra polyps can be used for several interesting behavioural studies like phototaxis, response to coloured light, regeneration and bioassays with toxins and drugs.
- ii. Procedures for these studies can be developed based on the references available.

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SHELL-LESS CULTURE SYSTEM FOR CHICK EMBRYOS



1. Aim

To study development of chick embryo using a shell-less *ex-ovo* culture.

2. Introduction

Observation on development of chick is difficult because it happens inside an opaque egg membrane. Several techniques have been standardised to establish *ex-ovo* culture of the embryo without the shell. Shell-less culture of chick embryo is possible in glass bowls, petri dishes etc. In a shell-less culture, a chick embryo is taken from an eggshell and cultured in an artificial environment outside the shelled egg. This is an important technique for various embryonic manipulations. A simple culture system with a high hatchability can be set up using an artificial vessel and a plastic film.

3. Materials Required

3.1. Biological Materials: Fertilized chicken eggs pre incubated for two days.

3.2. Chemicals/Reagents: 0.01% benzalkonium chloride in distilled water, 70% ethanol / providone iodine solution, calcium lactate pentahydrate powder, sterile distilled water / sterile water for injection (available with Chemist).

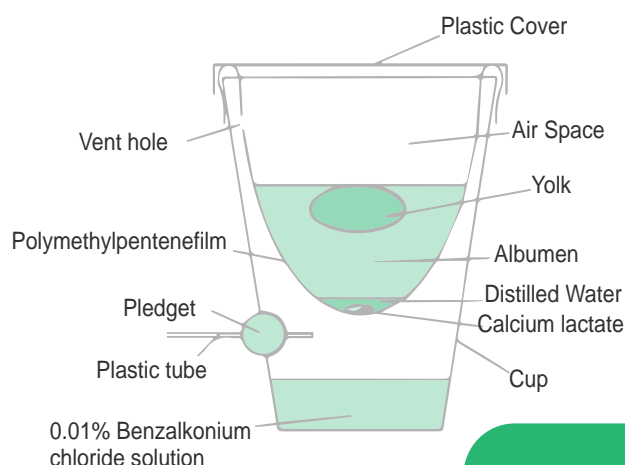
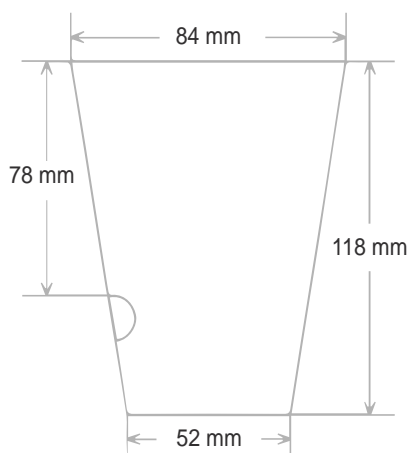
3.3. Equipment: Incubator, aseptic working area.

3.4. Glassware/Plastic ware: Sterile petri dishes, polystyrene plastic cup / paper cup (400 mL), glass rod, TPX film wrap / polyethylene sheet (permeable to gases), plastic straw.

3.5. Miscellaneous: Egg, candle, sterile forceps, scissors, needles, surgical cotton, elastic rubber bands, sterile gloves.

4. Procedure

4.1. Preparation of Culture Vessel:



- i. Take a clean and fresh polystyrene plastic / paper cup (400 mL capacity)
- ii. Make a circular window of 1 – 1.5 cm diameter on one side about 2 cm from the bottom
- iii. Make a loose ball of fresh surgical grade cotton and plug this window. The cotton will serve as a filter.
- iv. Take a clean plastic straw and cut it to a length of 5cm.
- v. Insert the straw gently between the cotton plug and the edge of the circular window into the inside of the cup. The straw will serve as a conduit for aeration.
- vi. Add about 20 – 30 mL of 0.01% benzalkonium chloride in distilled water to the cup.
- vii. Loosely wrap a polymethylpentene (TPX) film (available as food wrap films) or a clear polyethylene sheet, on the mouth of the cup.
- viii. Secure the sheet with elastic rubber bands on the rim of the plastic or paper cup.
- ix. Using a gloved hand or an egg (cleaned with 70% alcohol) held with the blunt end downwards, gently make a depression in the loose plastic sheet such that the sheet sags down a little, into the cup, to form a shallow depression. Take care to avoid creases. This shallow depression will accommodate the contents of egg.
- x. Add 250-300 mg calcium lactate pentahydrate powder into the depression and add 2.5 – 3 mL of autoclaved distilled water (calcium supplementation is essential for shell-less cultures).
- xi. Heat a glass rod and make eight ventilation holes on the upper surface of the film just below the rims such that the embryo does not come in direct contact with the holes.
- xii. The culture vessel can be sterilized by exposing to UV lamp for 20 minutes.

4.2 . Transfer of Chick Embryo:

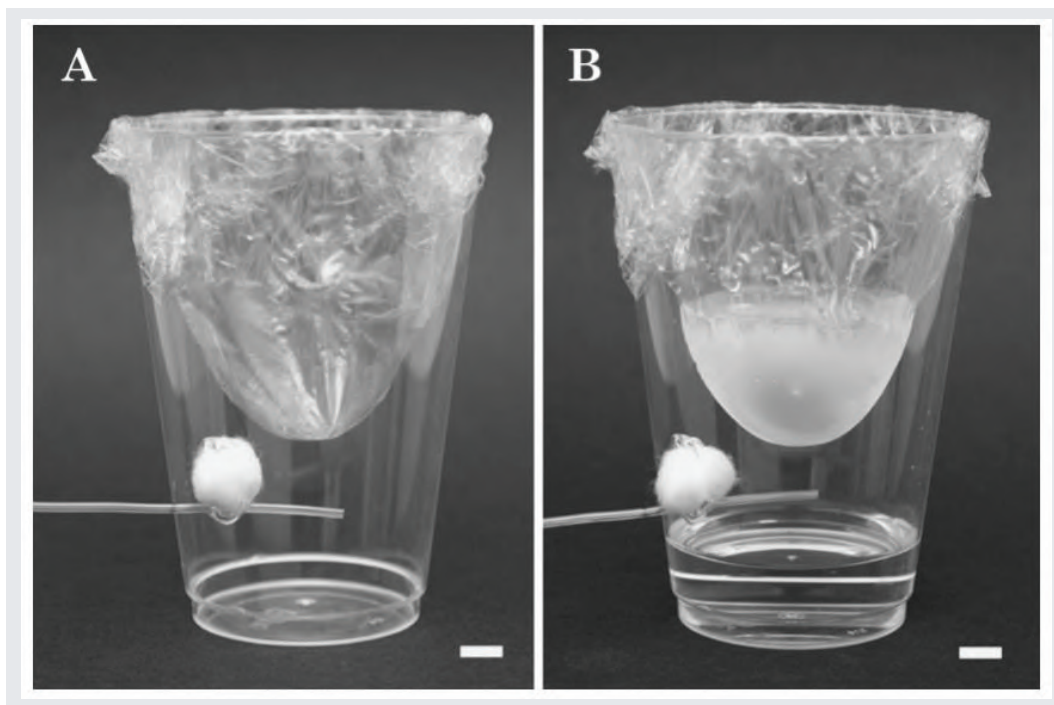


Fig. 1: A – The culture system, B – Culture system after transfer of embryo

- i. Preincubate the eggs in an incubator at 37 °C till day 3.
- ii. On day 3, take out the eggs from the incubator.
- iii. Candle the eggs and mark the position of the embryo.
- iv. Wipe the egg surface with 70% alcohol or Providone Iodine solution.
- v. Air dry the eggs by placing it horizontal, with the embryo side facing upward, for three minutes.
- vi. Pierce the blunt end of the egg to release the air from air sac.
- vii. Crack the shell in the middle such that the egg shell breaks into two halves.
- viii. Very gently release the egg contents into the depressed sheet such that the albumin flows first and then the yolk.
- ix. Ensure that the embryo faces upwards as the yolk is released into the cup.
- x. Remove carefully, any broken pieces of the egg shell dropped inside the culture vessel, with sterile forceps.
- xi. Cover the brim of the cup loosely with a sterile (autoclaved) petri dish lid.
- xii. Label the petri dish cover appropriately with date.
- xiii. Place the shell-less culture in an incubator at 37.5 °C and 65-75% humidity.
- xiv. Observe the embryo daily once and note down the stages of growth.
- xv. Compare with a standard chart and identify the stage of development as per Hamburger and Hamilton, 1951.

5. Precautions

- i. Use of embryos preincubated for less than two days may lead to mortality within 7 – 8 days.
- ii. Any embryo with broken yolk should not be used for culture.
- iii. Only use those embryos, where the embryo is positioned on top of the yolk.
- iv. Some food wraps are impermeable and may lead to mortality of embryos. It is therefore; preferable to use those which are permeable (Generally they are cheaper. Trials with various types of plastic sheet is recommended to select the best one)
- v. Opening and shutting of incubator doors should be limited to once or twice a day to minimize stress to the fragile embryos.
- vi. Place trays of distilled water with paper towel wicks in the incubator to increase humidity.
- vii. The embryo should not be kept out of incubator for more than five minutes.
- viii. Transfer of chick embryo can be done on a clean work station but for better results work between bunsen burners or in Laminar flow unit.

6. Extension Activities

- i. Successful hatching is possible with well-maintained shell-less systems.
- ii. A short-term culture system can be developed by using an autoclaved glass bowl and covering it with a loose glass plate. The embryos in such systems will survive up to eight days. Thin albumen from unfertilized eggs is first poured into a sterile glass bowl. This albumen acts as a shock absorber, provides a cushion for the culture and it limits desiccation. The fertilized eggs are then cracked from above and the contents of the eggs are gently released over the albumen cushion in the bowl. Each bowl is then covered with a lid, and cultures are incubated at 37.5 °C and 80% humidity.
- iii. Shell-less systems can be effectively used to study effect of various xenobiotics on the development of the embryo. Dosing can be done by placing small volumes (<0.5 mL) of solutions on the albumin near the embryo.
- iv. Embryos can be exposed to different concentrations of glucose in sterile saline, to study effect of diabetes on embryonic development.

- v. Embryos can be exposed to xenobiotics like lithium chloride, nicotine, lead etc. by directly dosing solutions in sterile distilled water.
- vi. Embryos can be exposed to drugs like antidiabetics, beta blockers, antipsychotics etc. to study their effect on development of eye, retina, brain, heart, spinal cord etc.
- vii. The volume of the egg contents is approximately 35 mL; Dosages can be calculated by equating this volume to the average adult human body weight of 50 kg.

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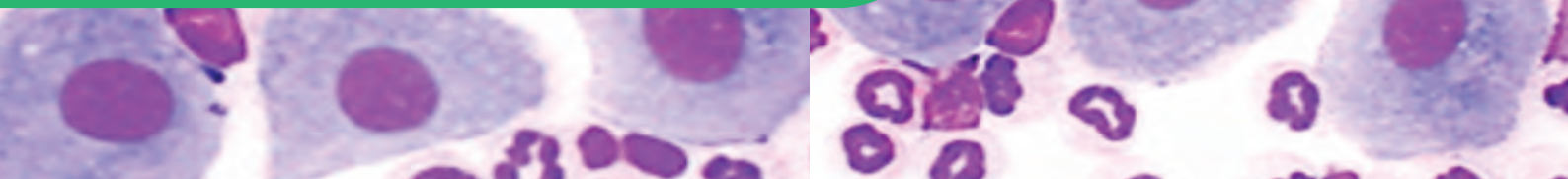
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STUDY OF ESTROUS CYCLE IN RAT



1. Aim

To determine the stages of estrous cycle in rat by studying the vaginal smear.

2. Introduction

During the reproductive life span of female mammals, the ovary exhibits cyclic changes under the influence of pituitary gonadotropins and expels ova periodically. The cyclic secretion of estrogens from the ovary affects the entire reproductive tract (uterus and vagina) producing cyclic changes in the vagina. In most of the therian mammals (placental mammals and marsupials) the reproductive cycle is referred to as the estrous cycle. Higher primates including human, some bats and elephant shrew show menstrual cycle. The estrous cycle is so called because of a definitive period of estrous or psychic heat. The cycle may involve only one estrous period in a year or season (monestrous, eg. dog, fox etc.) or many in year (polyestrous eg. rat, mouse etc.). The main difference between estrous cycle and menstrual cycle is that in menstrual cycle, if pregnancy does not occur, the endometrium is shed as menstrual discharge whereas in estrous cycle, the endometrium is resorbed. During the estrous cycle, the vaginal epithelium shows marked changes resulting in corresponding changes in the luminal cell populations. These changes provide a reliable index of ovarian activity to determine the reproductive stage of the animal. This was reported for the first time by Stockard and Papanicolaou (1917) in guinea-pig. The estrous cycle of female rat, is characterized as proestrous (~12 hours), estrous (~30 hours), metestrous / diestrous I (~6 hours) and diestrous / diestrous II (~55 hours). The ovulation occurs from the beginning of proestrous to the end of estrous. The short length of the estrous cycle of rats makes them ideal for investigation of changes occurring during the reproductive cycle. In correlation with the reproductive cycle changes that occur in the vaginal wall, cyclic changes in the cell types are seen in the vaginal lumen. Exfoliated vaginal cells are thus, obtained in the vaginal secretions. Vaginal smears can be prepared to observe these exfoliated vaginal cells. The estrous cycle in the rat is of four days and may be determined according to the cell types observed in the vaginal smear. Using the 10X objective lens, it is possible to analyze the proportion among the three cellular types, which are present in the vaginal smear. Using the 40X objective lens, it is easier to recognize each one of these cellular types.

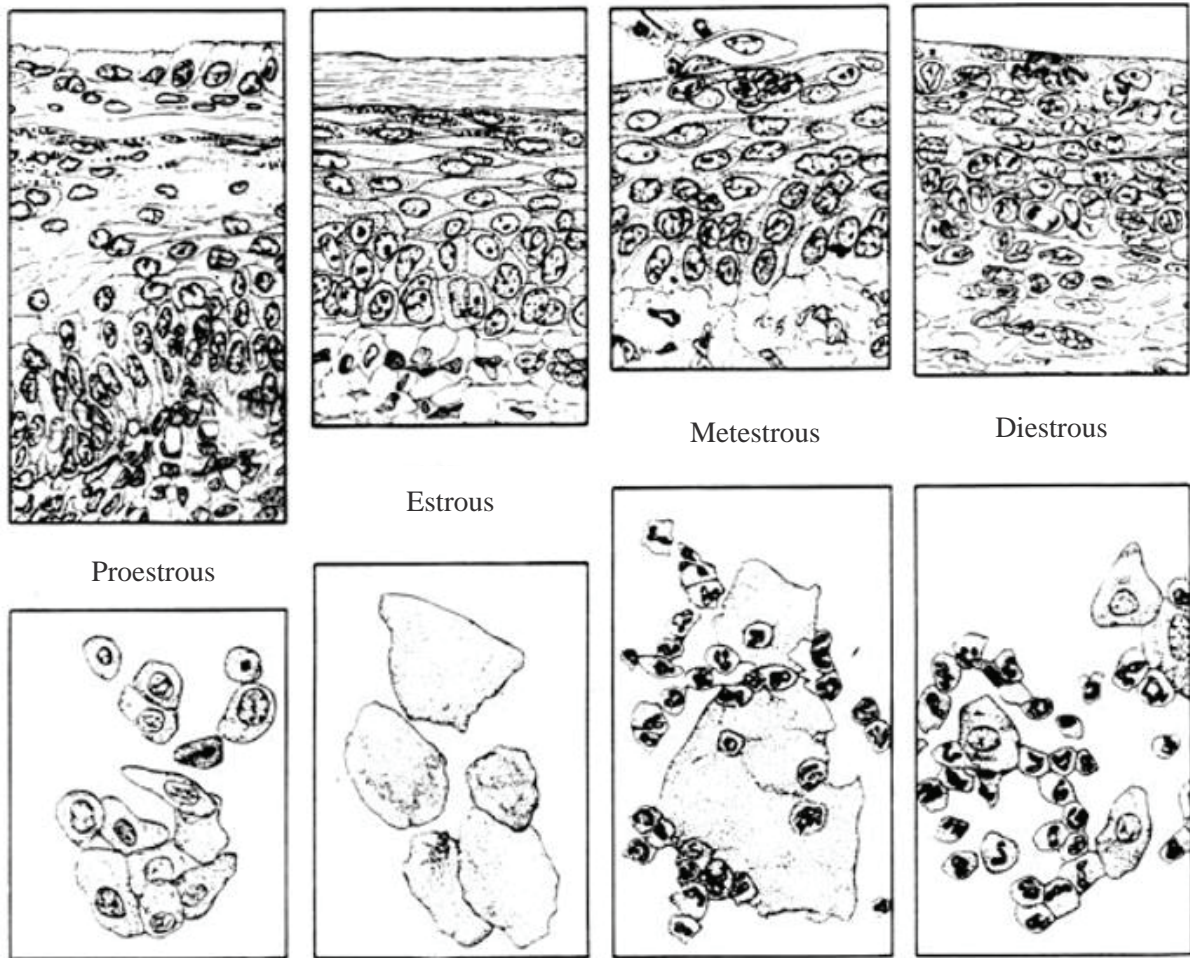


Fig. 1: Changes in the proportion of exfoliated cells from vaginal wall (bottom panel) and corresponding to the changes in the vaginal wall top panel

(Source: Marcondes et al; 2002)

During the estrous cycle, prolactin, LH and FSH remain low and increase in the afternoon of the proestrous phase. Estradiol levels begin to increase at metestrous, reaching peak levels during proestrous and returning to baseline at estrous. Progesterone secretion also increases during metestrous and diestrous with a decrease afterwards. Then the progesterone value rises to reach its second peak towards the end of proestrous.

3. Materials Required

3.1. Biological Materials: Female Wistar rats (*Rattus norvegicus*), three months old, weighing 200 to 300 g.

3.2. Chemicals/Reagents: Sterile normal saline 0.9% (available with chemist as vials for injection), deionized water, giemsa stain, absolute methanol

3.3. Equipment: Microscope (with camera attachment, optional)

3.4. Glassware/ Plastic ware: Petri dish, coplin jar

3.5. Miscellaneous: Distilled water, filter papers, surgical cotton, cotton ear buds, plastic dropper, micropipette, gloves, glass slide.

4. Procedure

- i. Do not carry out the procedure in the presence of rats from other cages to avoid stress and increase of aggressiveness of females that will be caught afterwards.
- ii. Carry each animal cage, with a single female rat to the experimental room.
- iii. Grasp each rat either by its dorsal neck skin fold or by a gloved hand holding the head and shoulder areas immobile.
- iv. Allow the animal to calm down and hold the animal upright with tail dangling down and the vaginal opening exposed. If the female is aggressive then, hold the rat pinned down on the table with all its feet firmly on the table. Lift the tail and hold it folded on the dorsal side of the rat, to expose the anal and vaginal openings.

4.1. Collecting vaginal fluid with Dropper / pipette (lavage):

- i. Take plastic dropper or a small pasteur pipette or a micropipette filled with 10 μ L of sterile normal saline (NaCl 0.9%)
- ii. Insert the tip of the dropper or pipette into the vaginal opening such that about 3 – 4 mm of the tip is inside the vaginal opening.
- iii. In a smooth action, release the saline into the vaginal opening and suck back the saline along with the vaginal secretion.
- iv. Place the sucked fluid on a clean glass slide.
- v. Spread the fluid with the tip of the dropper / pipette to make a circular smear (2cm diameter).

4.2. Collecting vaginal fluid using a cotton bud:

- i. Take a clean cotton bud (cotton ear buds available with the chemist) and soak it with sterile normal saline (NaCl 0.9%).
- ii. Gently insert the cotton bud into the vaginal opening such that 3/4th of the bud is inside the vagina.
- iii. Rotate the bud two times and withdraw the bud gently.
- iv. Tap the cotton bud on a clean glass slide such that a circular smear (2 cm diameter) is formed on the slide. (Do not rub the cotton bud on the slide).

4.3. Processing the smear on the slide:

- i. Observe the smear under the low power (10X) of the microscope and observe the cells.
- ii. Air dry the smear by placing in a petri dish for 5 min. Do not cover the petri dish.
- iii. With a pipette, add two to three drops of methanol over the dried smear.
- iv. Air dry the smear for 5 minutes till the methanol is evaporated and the cells are fixed to the glass slide.
- v. With a pipette add two to three drops of Giemsa stain (undiluted) such that the stain fully covers the smear area.
- vi. Immediately, cover the petri dish and leave for 2 minutes for staining.
- vii. With a dropper or pipette, gently add few drops of deionised water on the stain, such that the diluted stain spreads till all edges of the slide but does not flow out from the slide.
- viii. Notice a green shiny scum formed over the surface of the diluted stain.
- ix. Gently cover the petri dish and keep aside undisturbed for 15 minutes.
- x. Open the petri dish and lift the slide by one of its narrow ends and drain the diluted stain into the petri dish.
- xi. Place the slide, for 2 minutes, in a coplin jar into which a gentle stream of running water is directed. Do not allow the stream of water to fall directly on the smear.

- xii. Place the slide on a strip of filter paper such that the smeared surface is facing upwards.
- xiii. Label the slide on the smeared surface with a diamond glass marker pen.
- xiv. Observe the slide first under the low power of the microscope (10X) and then under the high power (40X).
- xv. Record the cell types seen and identify the stage of the estrous cycle depending on the proportion of cell types seen.

5. Observations

Three types of cells are seen in the vaginal smear:

- i. Round polygonal cells with dark blue nucleus and pinkish cytoplasm – nucleated epithelial cells (NE);
- ii. Irregular ones with no nucleus but pinkish cytoplasm – cornified epithelial cells (CE);
- iii. Small round ones with pinkish cytoplasm and irregular dark blue nucleus – leukocytes (LC).

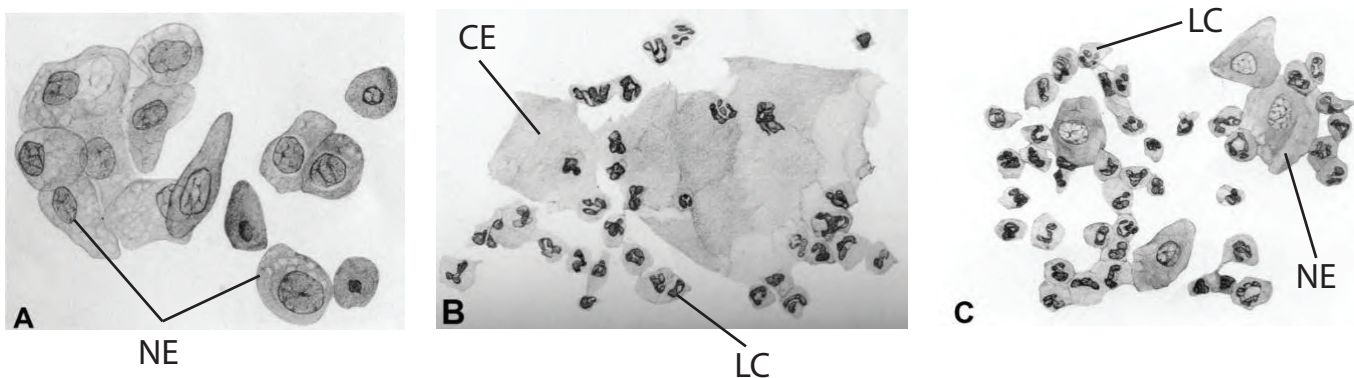


Fig. 2: A – Proestrous, B – Metestrous and C – Diestrous

(Source: Michelle et al., 2015)

A proestrous smear consists mainly nucleated epithelial cells; an estrous smear consists mainly of non-nucleated cornified epithelial cells; a metestrous smear consists of many leukocytes along with cornified non-nucleated epithelial cells and a diestrous smear predominantly consists of leukocytes along with nucleated epithelial cells. Intermediate stages will show varying predominance of the corresponding exfoliated cells for example; an intermediate stage between metestrous and diestrous may show leukocytes, cornified epithelial cells along with few nucleated epithelial cells.

6. Conclusion

The vaginal smear of female rat shows the presence of following exfoliated vaginal cells;

Cell Type	Nucleated Epithelial cells	Non-nucleated cornified epithelial cells	Leucocytes
Score for Abundance of cells (+ / -)			

From the proportion of cell types seen in the vaginal smear, the female rat is in _____ stage of estrous.

7. Precautions

- i. The tip of the dropper or pipette should not be inserted deep into the vagina.
- ii. The cotton bud should not be rotated more inside the vagina or should not cause any scrapping of vaginal wall. This will injure the vaginal wall and cause leucocytes to be released.
- iii. Ensure that the cells are fixed to the slide using absolute methanol to avoid the cells from falling off while staining.
- iv. Use A. R grade Giemsa stain.
- v. Do not keep the petri dish open while staining with Giemsa, to avoid deposition of precipitates on the preparation.
- vi. If Giemsa stain is not fresh, then filter the stain through Whatman filter paper No. 41 before use.
- vii. Pour deionised water over the stain, very carefully, to hold the diluted stain over the slide due to surface tension. If the diluted stain drains off from the slide surface, staining will not be proper.

8. Extension activities

- i. The vaginal smear technique can be replicated using female mouse.
- ii. Keep a male rat individually housed in a cage, in the same room where two three female rats are separately housed. Keep the cages away from each other. Change the bedding of the cage of the male rat, every fourth day. Take vaginal smear before introducing the male rat and daily for ten days after the introduction of the male. Obtain vaginal smears daily in the morning using micropipette and normal saline. Note down the results.
 - a. What are the changes seen after 10 days?
 - b. Does the result support the theory of Bruce Effect?

Suggested Reading(s)

- i. Michelle, C. C., Linda, K., & Greg, T. (2015). Vaginal Cytology of the Laboratory Rat and Mouse: Review and criteria for the staging of the estrous cycle using stained vaginal smears. *Toxicol. Pathol.*, **43**: 776-793.
- ii. Marcondes, F. K., Bianchi, F. J., & Tanno, A. P. (2002). Determination of the estrous cycle phases of rats: Some helpful considerations. *Braz. J. Biol.*, **62**(4A): 609-614.

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Title picture: Devall, D. A. and T. A. Lovick, Differential activation of the periaqueductal gray by mild anxiogenic stress at different stages of the estrous cycle in female rats, *Neuropsychopharmacology*, American College of Neuropsychopharmacology, April 2010.

DIFFERNT STAGES OF MEIOSIS



1. Aim

To study the different stages of meiosis in grasshopper testis cells.

2. Introduction

Grasshopper testis is an ideal material for studying various stages of meiosis. Grasshoppers are insect pests of family Acrididae. They are easily available, with morphologically distinguished males and females. Chromosomes in the grasshopper testis provide many advantages to cytologists because they are large and few in number (17 or 19 or 21 chromosomes in males; odd number is because grasshoppers have XX/XO sex chromosome system). Each bivalent can be easily identified according to length. Chiasmata are clearly visible during diplotene and diakinesis thus facilitating the study of their structure, frequency, distribution and movement. Moreover, in the early diplotene, position of the centromere is marked by relatively denser staining, and is therefore easily identified. Besides these cytological advantages, the techniques involved in the preparation of slides of this material are quick and simple and therefore it is ideal for demonstrating the stages of meiosis to students.

3. Materials Required

3.1. Biological Material: Male grasshopper

3.2. Chemicals/Reagents: Insect saline (0.67% NaCl), 1:3 acetomethanol fixative, 70% and 90% ethanol, 2% aceto carmine stain (2 gms of carmine mixed with 100 mL of 45% acetic acid and boiled using a reflux condenser for 1 hr. to dissolve carmine, 45% acetic acid, methanol).

3.3. Equipment: Microscope.

3.4. Glassware/ Plastic ware: Beakers (different size), glass slides, cover slips, spirit lamp.

3.5. Miscellaneous: Distilled water, filter papers, sealing wax or nail polish, gloves, fine forceps, fine scissor.

4. Procedure

- i. Wash hands and put on gloves.
- ii. In order to locate the testis, dissect a chloroformed male grasshopper in insect saline. Give a small vertical incision on the dorsal side of segment 5 to 6, at the junction of thorax and abdomen. Press the abdomen gently.
- iii. The testis covered in yellow fat bodies will pop out. Clear out the yellow fat and the white coloured tubules with the help of forceps and needles as much as possible.

4.2. Fixation:

- i. Keep the testis material in the 1:3 acetic alcohol for at least five minutes before staining procedures are carried out.
- ii. The testis material may also be stored for further use (up to one year) either in the fixative or by transferring it to 70 per cent ethyl alcohol before being stained or after fixation.
- iii. Store the material at room temperature or in a refrigerator.

4.3. Slide preparation:

- i. Place a small amount of fixed testis material on a clean glass slide.
- ii. Add few drops of Aceto-carmin stain and tease the material with a needle.
- iii. Keep the teased material in stain for a minute.
- iv. Put a cover glass over it and warm the slide on a hot plate (60 – 80 °C) for 10 seconds.
- v. Place 2 filter papers on the cover glass and gently apply uniform pressure by pressing with thumb to spread the tissue.
- vi. Observe the preparation under a microscope.
- vii. Observe the spermatocytes under low power (10 X) and then under high power (40 X or 100 X) of the microscope.
- viii. Observe different stages of meiosis as shown in Fig.1.

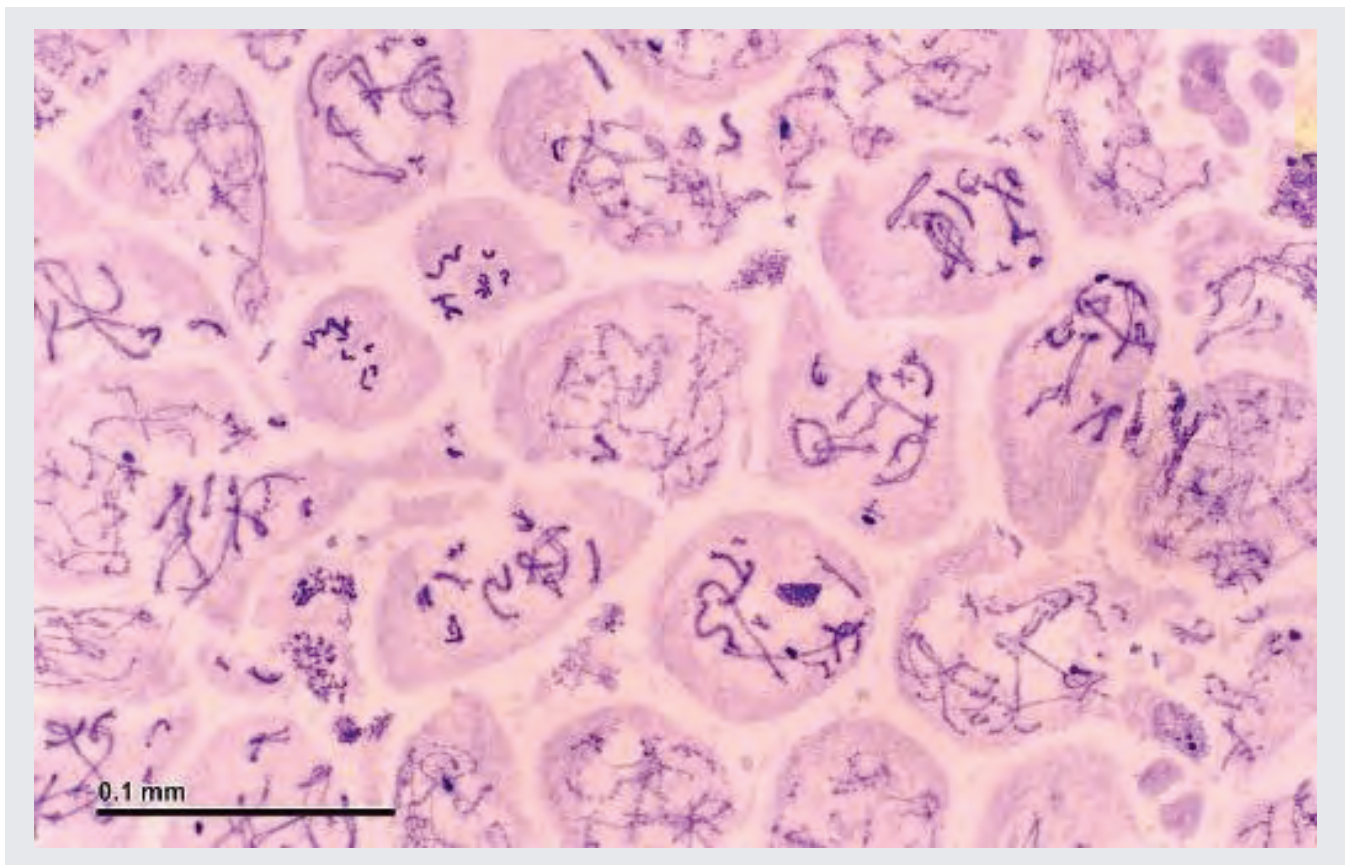


Fig. 1: Meiosis in grasshopper testis (40X)

5. Precautions

- i. Use good quality glacial acetic acid for preparing the stain.
- ii. Do not allow the stain to boil while heating on a hot plate.

Suggested Reading(s)

- i. Celis, J. E., (1998). *Cell biology: A laboratory handbook, (volume 1)*. The University of Michigan: Academic Press. ISBN 0121647250, 9780121647254. https://books.google.co.in/books/about/Cell_Biology.html?id=2bZqAAAAMAAJ
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Title picture: <https://planet-vie.ens.fr/content/meiose-plante-a-fleur>

STUDY OF DERMATOGLYPHICS OF FINGERS AND PALM



1. Aim

To study dermatoglyphics of fingers and palm.

2. Introduction

Dermatoglyphics (skin writings) is a collective name for all the skin patterns of the fingers, toes, palms and soles. It was two Indian police officers, Sub inspectors Azizul Haque and Hem Chandra Bose, who played key roles in the advancement of the science of fingerprints. The so-called Henry's System of Fingerprint Classification was actually worked out by them. The world's first fingerprint bureau was set up at Calcutta (now Kolkata) in 1897, mainly by their efforts. Their efforts in inventing a fingerprint classification system, however, were not officially recognized. In 1890, Francis Galton suggested fingerprints as a useful tool in personal identification. Over the years, the patterns of epidermal ridges and flexion creases on the fingers, toes, palms of the hands and soles of the feet have become a subject of scientific interest. Dermatoglyphics, a term coined in 1926 by Harold Cummins, is the study of the epidermal ridges and includes other aspects of hand, finger and foot prints. Dermatoglyphics is being studied empirically to find out its significance in clinical conditions especially those related to chromosomal abnormalities like Down's syndrome. The areas of skin which show patterns on surface are arranged in ridges that are separated by narrow grooves. Fingerprint patterns of dermal ridges can be classified into three major groups: arches, loops and whorls. The total ridge count (TRC) can be recorded for each fingerprint. The TRC for arch pattern is zero while those for whorl and loop pattern are 12 and 15, respectively. The fingertip patterns show differential incidence in populations like loops (70%), whorls (25%) and arches (5%). Perkinje in 1923 gave the methodology for studying fingerprints. Fingerprint patterns are individualistic for each person but they are of following basic types as shown below:



Fig. 1: i) Arch,
(TRC is zero)



ii) Whorl,
(TRC is 12)



iii) Loop,
(TRC is 15, Highest Value)

The arch is the simplest and least frequent pattern. It may be subclassified as "plain" when the ridges rise slightly over the middle of the finger or "tented" when the ridges rise to a point. The loop pattern has a triradius and a core. A triradius is a point at which three groups of ridges coming-from three directions meet at angles of about 120 degrees. The core is essentially a ridge that is surrounded by fields of ridges which turn back on themselves at 180 degrees. Loops can be either radial or ulnar. Ridges are counted from the triradius to the core along a straight line.

3. Materials Required

3.1. Chemicals/Reagents: Xylene (carcinogenic, to be handled with care).

3.2. Miscellaneous: Ink, ink pads, bond paper, 2B pencil, scale, a sheet of rubber pad, a USB fingerprint scanner (optional) etc.

4. Procedure

4.1. Fingerprints using stamp pad:

- i. Wash hands thoroughly with soap and dry them well.
- ii. Apply ink with ink pads on the fingers of left hand. (Note: the ink may not be easily removed from the fingers).
- iii. Place the white bond paper on a rubber sheet pad
- iv. Firmly press the fingertips to take the impression of fingertips on the bond paper and observe the patterns.
- v. Similarly repeat the process for right hand.
- vi. Note fingertip patterns.

4.2. Fingerprints using lead pencil and scotch tape:

- i. On a piece of white paper, make a square of 3 cm X 3 cm.
- ii. Using a 2B lead pencil, shade the entire square uniformly.
- iii. Wash hands thoroughly with soap and dry them well.
- iv. Rub one of your fingers on the graphite square, making certain you have covered all the triradii on the finger print.
- v. Now carefully place a piece of Scotch Tape onto your blackened finger so that the tape comes in contact with the entire print.
- vi. Make certain you include any triradii on the outer edges of the finger by rolling the finger over the tape in one smooth motion.
- vii. Peel away the tape and affix it to the appropriate place on your record sheet.
- viii. Repeat this process, preparing a print of each of your 10 fingers.

4.3. Finger prints using a USB fingerprint scanner

- i. Wash hands thoroughly with soap and dry them well.
- ii. Mope the finger tips with a fresh tissue paper.
- iii. Place the finger on the scanner and record the pattern on the computer. Obtain prints of all ten fingers.

4.4. Palm prints using stamp pad ink:

- i. Wash hands thoroughly with soap and dry them well.
- ii. Apply ink evenly on the palm of left hand. (Note: the ink may not be easily removed from the palm).
- iii. Place the white bond paper on a rubber sheet pad
- iv. Firmly press the left palm to take the impression of palm on the bond paper and observe the patterns. Obtain the pattern of the wrist also.
- v. Press down the back of the hand to get a good impression.
- vi. Similarly repeat the process for right hand.
- vii. Use a ballpoint pen and draw straight lines from the triradii at the base of index and little finger to the axial triradi.
- viii. Measure the “atd” angle formed between lines drawn.
- ix. Note the “atd”, “tda” and “dat” angles.



Fig. 2: Measurement of “atd”, “tda” and “dat” angles.

Record the fingerprint pattern data, total ridge count, sex and the “atd”, “dat” and “tda” angles in the observation table. Use the class data to construct a histogram in which frequencies are plotted against total ridge count and “atd” angles.

5. Observation Table

Age: _____, Sex: _____,

Handedness: LEFT / RIGHT

Analysis of hand: LEFT / RIGHT

Finger	Thumb	Index	Middle	Ring	Little	Remark
Type of Pattern						
Ridge Count						
TOTAL RIDGE COUNT						
Ridge Count	“atd”		“tda”		“dat”	

6. Precautions

- i. Handle xylene carefully (carcinogenic).
- ii. Use less ink while taking finger prints.
- iii. Use cotton wetted with xylene to clean ink from hands

Suggested Reading(s)

- i. Schaumann, B., & Alter, M. (1976). *Dermatoglyphics in medical disorders*. New York: Heidelberg, Springer-Verlag.
- ii. Margaret, W. M., David, R. B., Daniel, L. P., & Susan, J. B. (2011). *Handbook of dermatology a practical manual*. Blackwell Publishing.
- iii. Gordon, M., Thomas, M. & Jon, H. (1989). Fingerprint Ridge Count: A polygenic trait useful classroom instruction. *The American Biology Teacher*, 51(4).
- iv. Thomas, F. (1990) Using Dermatoglyphics from Down Syndrome and Class Populations to Study the Genetics of a Complex Trait. *Association for Biology Laboratory Education* (ABLE). <http://www.zoo.utoronto.ca/able>.

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Title picture: <http://geniusacademy.com.np>

APPENDIX

Types of finger patterns:

a) Loops: In loops ridges traverse from one side and recurve on the same side back. They are of two types.

b) Arches: These are of two types - (i) Simple or parallel arches. (ii) Tented arches.

c) Triradii: Are represented by a, b, c, d and t- regions.

d) Whorls: Start from one point and curve back. They may be clock wise or anticlock wise in direction. They are either simple whorls, concentric whorls, double loop or central pocket.

The ridge pattern on the palms falls into six chief areas. An important diagnostic feature in dermatoglyphics is the tri-radius which can be defined as the meeting point of three spokes that demarcate three regions, each region containing a system of almost parallel ridges. On a fingertip, one triradius always accompanies a loop pattern while two triradii always, accompany a whorl. On the palm there are normally four triradii, one at the base of each finger, called a,b,c and d and another, known as t, near the base of the fourth metacarpal bone or at some point on its axis.

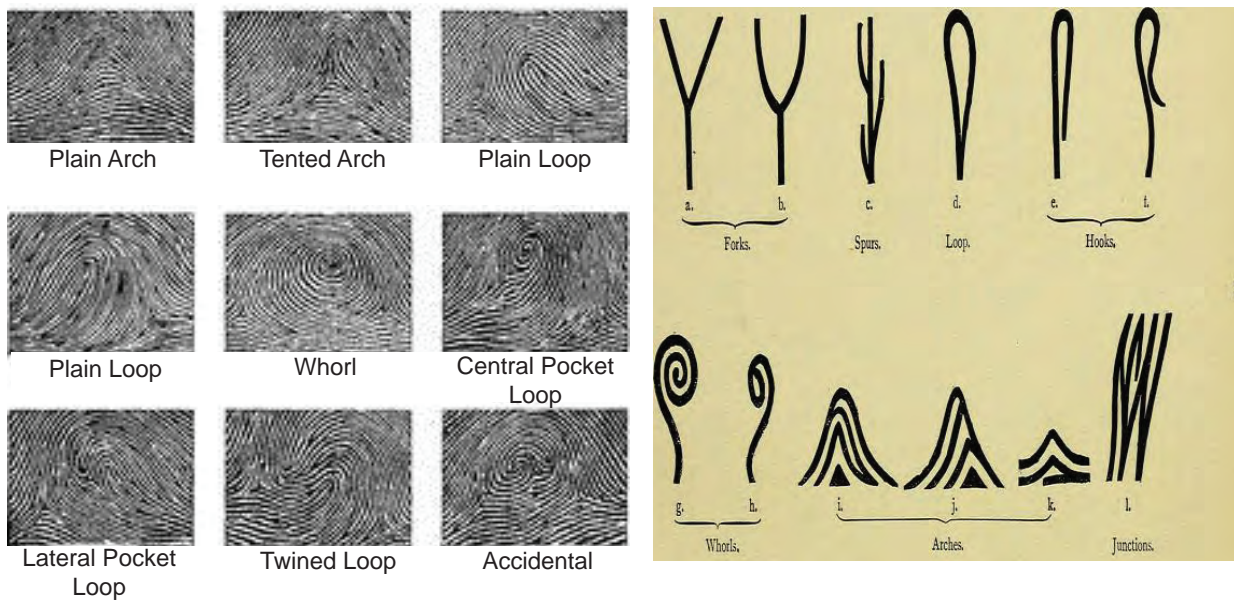


Fig. 1: Fingerprint patterns of dermal ridges

While the formation of the epidermal ridge pattern and the total ridge count are polygenic, they are also influenced by environmental factors and thus may be said to be multifactorial. Polygenic traits tend to be neglected in the classroom and laboratory despite the fact that in a variety of organisms many significant traits are inherited in this manner. The inheritance of many significant human behavioral, anatomical and physiological characteristics is best explained by a polygenic model of transmission. The inheritance of polygenic traits cannot be analyzed by the pedigree method used for single gene traits, nor by chromosome studies as might be done in the case of suspected chromosomal anomalies. Polygenic traits, in contrast to single gene traits and chromosome abnormalities, exhibit a wide and continuous range of expression and are measurable in nature. Expression of polygenic traits is often markedly affected by the environment, causing them to be referred to as multifactorial traits.

The embryology of the epidermal ridges offers clues to the prenatal environmental influence on their pattern of development. Fetal fingertip pads are observable around the sixth week of gestation and reach their maximum size by week 12 or 13, after which they regress, giving rise to elevated dermal ridges.

The ridges, once formed, are very resistant to later prenatal or postnatal influences, thus making them an ideal trait for genetic studies as well as for identification of individuals. While the formation of the epidermal ridge pattern and the total ridge count are polygenic, they are also influenced by environmental factors and thus may be said to be multifactorial. The embryology of the epidermal ridges offers clues to the prenatal environmental influence on their pattern of development. Fetal fingertip pads are observable around the sixth week of gestation and reach their maximum size by week 12 or 13, after which they regress, giving rise to elevated dermal ridges. The ridges, once formed, are very resistant to later prenatal or postnatal influences, thus making them an ideal trait for genetic studies as well as for identification of individuals.

Fingerprint patterns of dermal ridges can be classified into three major groups: arches, loops and whorls (see Fig.1). The arch is the simplest and least frequent pattern. It may be subclassified as "plain" when the ridges rise slightly over the middle of the finger or "tented" when the ridges rise to a point. The loop pattern has a triradius and a core. A triradius is a point at which three groups of ridges coming from three directions meet at angles of about 120 degrees. The core is essentially a ridge that is surrounded by fields of ridges which turn back on themselves at 180 degrees. Loops can be either radial or ulnar. A finger possesses a radial loop if its triradius is on the side of the little finger for the hand in question and the loop opens toward the thumb. A finger has an ulnar loop if its triradius is on the side of the thumb for that hand and the loop opens toward the little finger. The whorl pattern has two triradii with the ridges forming various patterns inside.

Total Ridge Count:

The focus of this investigation is the polygenic trait called the total ridge count (TRC), the sum of the ridge counts for all 10 fingers. Holt (1968) found that the average TRC for males is 145 and for females, 126. The ridge count on a finger with a loop is determined by counting the number of ridges between the triradius and the center or core of the pattern. For an arch, the ridge count is zero. For a whorl a ridge count is made from each triradius to the center of the fingerprint, but only the higher of the two possible counts is used.

“atd” Angles:

Dermatoglyphics is the scientific term used for study of epidermal ridges and their configuration on the palmer region of hand and planter region of foot and toes. Many articles have been published in medical journals around the world, and dermatoglyphics has been used in such diverse field as pediatric medicine, genetic research, criminology, psychiatry and anthropology. Empirical studies show that, different diseases have different finger prints associated with them. “atd” angles are used to study the patterns on palm. These angles have been used to empirically relate palm patterns with certain disease conditions like mental retardness, obesity, diabetes etc.

BEHAVIOURAL STUDIES ON SIAMESE FIGHTING FISH : A LAB WORK IN ETHOLOGY



1. Aim

To study behaviour of fish using *Betta splendens* (family Anabantidae) as a model.

2. Introduction

Betta splendens, (The Siamese fighting fish, family Anabantidae), also known as the *Betta*, is a popular species of freshwater aquarium fish. The wild ancestors of this fish are native to the rice fields of Thailand, Malaysia, Cambodia and Vietnam. They are called *pla-kad* (biting fish) in Thai. The name of the genus is derived from *ikan bettah*, taken from a local dialect of Malay. These fish are seen inhabiting shallow waters and puddles. They can survive in low oxygen waters since they have accessory respiratory organs which help them to breathe air directly. The fish surface intermittently to gulp air. If they are denied access to surface air, they will drown.

Male *Bettas* are extremely territorial and aggressive towards each other. These fish have historically been the objects of gambling; two male fish are pitted against each other in a fight and bets are placed on which one will win. One fish, sometimes, may get killed in the fight. The behavior of Siamese fighting fish has been a subject of interest to many behavioral scientists and comparative psychologists.

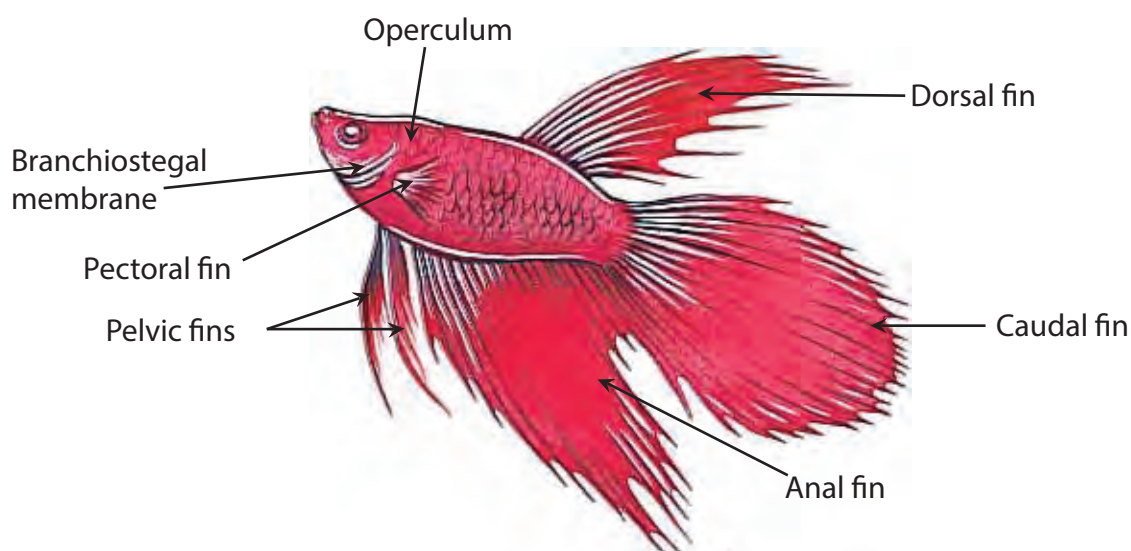


Fig. 1: Male Siamese fighting fish are available with aquarists in various colours and various shapes and sizes of the fins. Females lack the elaborate fins and generally are less bright in colour.

3. Materials Required

3.1. Glass Aquarium: Design and construct an aquarium of glass with three separate compartments with a dimension of 50 cm (length) X 15 cm (breadth) X 15 cm (height), as shown below (Fig. 2):

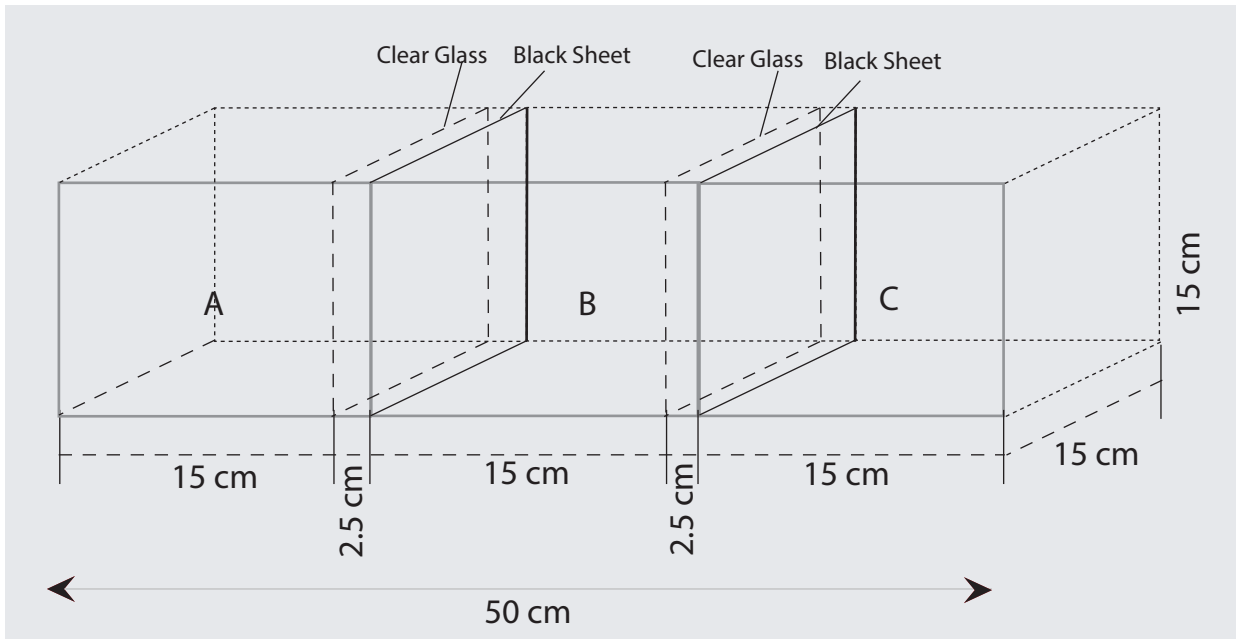


Fig. 2: Dimensions of the aquarium

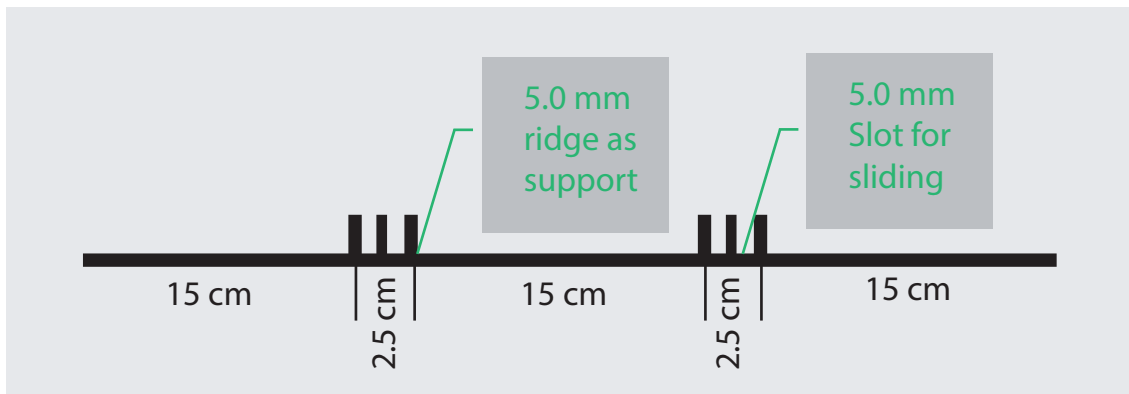


Fig. 3: Top view of side wall of Aquarium

Each compartment will be of 15 cm X 15 cm X 15 cm. The compartments will be separated by removable partitions of transparent glass, black plastic sheet or a plain mirror. To slide these removable partitions, two slots of 5mm will be provided with three ridges on the top and bottom of the side walls of each partition. See the diagram Fig. 3 for details.

NOTE: The black partition should be completely opaque. A thick stiff plastic sheet or a fully black acrylic sheet will be sufficient.

- 3.2. Live Siamese fighter fish:** Obtain one female and two male Siamese Fighter Fish from the aquarist. Fill the aquarium with potable water (if tap water is used then, keep the water filled in a bucket overnight to allow the chlorine to escape). Keep the three fish separate in the three compartments separating each from the other by a black plastic sheet. Keep the males in the adjacent compartments (“A” and “B”) and the female in the third compartment (“C”). Cover the top of the aquarium with a glass sheet or any flat cover such that, a small air space is left along the top edges of the aquarium. Keep the aquarium in a well-ventilated room. Aeration is not required.
- 3.3. Partitions:** Two plastic black partitions, two transparent glass partitions, one plane mirror partition.
- 3.4. Miscellaneous:** A framed fish net with handle that will fit inside the compartment (this will help in catching fish, if needed), a timer, a video camera to record fish behaviour (if needed), note book and pen.

4. Procedure

4.1. Set up for the experiment:

- i. Set the aquarium with three compartments with a single fish in each compartment (compartment A – male fish, compartment B – male fish and compartment C – female fish).
- ii. Separate the compartments by two plates; one transparent clear glass plate and one opaque black sheet, as shown in the diagram. Ensure that if the glass plate is removed the fish cannot see the occupant in the adjacent compartment because of the opaque black sheet.
- iii. Watch all the three fishes and try to locate the body parts as shown in the diagram.
- iv. Keep the observation table and writing material ready to record the observations.
- v. Read the observation table and get acquainted with the observation parameters so that it will be easier to record the behaviour.
- vi. Do not touch the aquarium or shake the table.

4.2. Male in front of the mirror:

- i. Gently remove the clear glass partition between the “A” and “B” compartments and replace it gently with the plane mirror such that the mirrored side faces the male fish in compartment “A”.
- ii. Watch the male fish in compartment “A” and note down the observation in the table provided.
- iii. Do not allow the fish to respond for more than a minute, otherwise it will be stressed and tired.
- iv. Gently replace the plane mirror with clear glass partition and wait for 5 minutes.

4.3. Male in presence of another male:

- i. Now gently remove the black partition between compartments “A” and “B” so that both the male fish can see each other.
- ii. Carefully observe the behaviour of male fish in tank “A” and also see the corresponding response of male fish in tank “B” to the actions shown by the male fish in tank “A”.
- iii. Record your observations in the table provided.
- iv. Do not allow the fish to respond for more than a minute, otherwise they will be stressed and tired.
- v. Gently replace the black sheet partition and wait for 5 minutes.

4.4. Male in presence of female:

- i. Now gently remove the black sheet partition between compartments “B” and “C” so that the male and female fish can see each other.
- ii. Carefully observe the behaviour of male fish in tank “B” and also see the corresponding response of female fish in tank “C” to the actions shown by the male fish in tank “B”.
- iii. Record your observations in the table provided.
- iv. Do not allow the fish to respond for more than a minute, otherwise they will be stressed and tired.
- v. Gently replace the black sheet partition.



Fig. 5: Fighting Fish

Source: <http://petstop.com/wp-content/uploads/2016/03/fighter-1.jpg>

5. Observation Tables

5.1. Male in front of Mirror:

Behavior	Whether seen or not seen	Remarks (if any)
Swimming side ways to show the lateral side	YES / NO	
Operculum extended out	YES / NO	
Dorsal Fins flared	YES / NO	
Quivering (wavy) body movements	YES / NO	
Swimming fast	YES / NO	
Exposing the ventral side to the other fish	YES / NO	
Swimming straight in a frontal approach (facing)towards the other fish	YES / NO	
Change in colouration	YES / NO	
Caudal fins flared	YES / NO	
Pectoral fins flared	YES / NO	
Swimming backward	YES / NO	
Moving vertically facing the other fish	YES / NO	
Biting	YES / NO	
Branchiostegal membrane extended	YES / NO	
Operculum fluttering	YES / NO	
Swimming away	YES / NO	
ANY OTHER BEHAVIOR / ADDITIONAL NOTES		

5.2. Male in presence of another male:

Behavior of male in Tank "A"	Whether seen or not seen	Corresponding behavior of male in Tank "B"
Swimming side ways to show the lateral side	YES / NO	
Operculum extended out	YES / NO	
Dorsal Fins flared	YES / NO	
Quivering (wavy) body movements	YES / NO	
Swimming fast	YES / NO	
Exposing the ventral side to the other fish	YES / NO	
Swimming straight in a frontal approach (facing)towards the other fish	YES / NO	
Change in colouration	YES / NO	
Caudal fins flared	YES / NO	
Pectoral fins flared	YES / NO	
Swimming backward	YES / NO	
Moving vertically facing the other fish	YES / NO	
Biting	YES / NO	
Branchiostegal membrane extended	YES / NO	
Operculum fluttering	YES / NO	
Swimming away	YES / NO	
Note any other behavior with its corresponding response of the other fish		

5.3. Male in presence of female:

Behavior	Whether seen or not seen	Remarks (if any)
Swimming side ways to show the lateral side	YES / NO	
Operculum extended out	YES / NO	
Dorsal Fins flared	YES / NO	
Quivering (wavy) body movements	YES / NO	
Swimming fast	YES / NO	
Exposing the ventral side to the other fish	YES / NO	
Swimming straight in a frontal approach (facing)towards the other fish	YES / NO	
Change in colouration	YES / NO	
Caudal fins flared	YES / NO	
Pectoral fins flared	YES / NO	
Swimming backward	YES / NO	
Moving vertically facing the other fish	YES / NO	
Biting	YES / NO	
Branchiostegal membrane extended	YES / NO	
Operculum fluttering	YES / NO	
Swimming away	YES / NO	
ANY OTHER BEHAVIOR / ADDITIONAL NOTES		

6. POST-LAB Evaluation Exercise

Answer the following based on your observations:

6.1 From the above observation tables list the actions observed in the fish as those associated with expression of aggression and those associated with expression of courtship

Aggression	Courtship

6.2 From the table obtained as above (Question No. 1) list those actions which are specific to aggression and those which are specific to courtship.

Aggression	Courtship

6.3 Choose the most appropriate alternative(s).

6.3.1 If the three experiments that were performed are named as Part A (male in front of mirror), Part B (male in presence of another male) and Part C (male in presence of female) then experiments that evaluate the aggressive behaviour in Siamese fighter fish are :

- a) Part B and Part C
- b) Part A and Part B
- c) Part A and Part C
- d) Part A only
- e) Part B only
- f) Part C only

6.3.2 Flaring of opercula in the fish probably helps the fish to

- a) breath better while display
- b) appear bigger in size
- c) appear aggressive
- d) obtain better buoyancy while display

6.3.3 The behavior of Siamese fighter fish can be classified as innate behavior because

- a) the actions are repetitive
- b) both males show similar actions
- c) male and females show similar actions
- d) they are genetically inherited

7. Precautions

Siamese fighting fish are quite curious and will watch humans going about their business near the tank. Like other fish, they may respond to the presence of humans and get distracted or become alert to actions such as a hand placed over the water's surface or tapping the aquarium walls or jerks to the tank or sudden movements by persons near the tank. So, carry out the experiment by taking care to affect the normal behavior of the fish, the least.

8. Extension Activities

- i. The experiments can be repeated by exposing the male fish to other fish species like guppies with coloured fins or coloured swordtail fish etc. Observations can be made to study the effect of colour, shape, size and species of the fish for interesting evaluations.
- ii. The behaviour of the fish can be recorded in a video for better evaluation and more in-depth studies.
- iii. If the three fish are kept in the tank for three to four days, undisturbed, the male fish may start making a bubble nest to attract the female. This may be attributed to the chemical signals released by the female fish into the water. So, even if the male fish is unable to see a female fish, the chemical signals could trigger nest making. This could be another interesting observation of behaviour.

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MICROTOMY FOR HISTOLOGY



1. Aim

To fix tissue, prepare paraffin block section and stain slide for histology.

2. Introduction

The purpose of fixation is to preserve tissue permanently in as life-like state as possible. Fixation should be carried out as soon as possible after removal of the tissues (in case of the surgical pathology) as soon after the death (with autopsy) to prevent autolysis. Once the tissue has been fixed, it must be processed into a form in which it can be made into thin microscopic sections. Tissue is embedded in paraffin, which is similar in density to tissue. The technique of embedding fixed tissue into paraffin is called tissue processing. Paraffin embedded tissues are then sliced into micrometer thin sections using a microtome. The paraffin supports the soft tissue during the process of sectioning. This embedded paraffin must be gradually removed from the tissue sections while staining in order to get the paraffin wax out of the tissue & allow water and alcohol soluble dyes to penetrate tissue sections. Hematoxylin and Eosin combination is the most common stain used in histology. Hematoxylin being a basic dye has an affinity for the nucleic acid of cell nucleus. Eosin is an acidic dye with an affinity for cytoplasmic component of cell. In the stained sections, Eosin gives a pink background to the bluish-purple nuclei. Hematoxylin itself (or hematein, its oxidation product) is a weak stain and requires a mordant to increase its attraction for tissues. A mordant is a high-molecular weight metal salt that forms a complex body with tissue and with the dye and acts as a strong bonding agent. There are several fixatives that are used in the pathology laboratories and research laboratories based upon the nature and type of tissue and the histologic details to be demonstrated.

3. Materials Required

3.1. Chemicals/Reagents: Aldehyde fixatives (formalin, 10% neutral buffered formalin (NBF), 4% paraformaldehyde (PFA), glutaraldehyde etc.).

3.2. Glassware/Plastic ware: Large size cover glass (20-22mm), slides, glass vials, Coplin jars.

3.3. Miscellaneous: Microtome, blade, tissue, paraffin wax (melting point 57-58°C).

4. Procedure

4.1. Tissue fixation:

- i. Excise the tissue from a freshly sacrificed animal. Gently blot the tissue of excess blood and slice the tissue with a sharp surgical blade. Drop the sliced pieces into a glass vial containing 10% formalin solution. Fixative volume should be 20 times that of tissue on a weight per volume; use 2 mL of formalin per 100 mg of tissue.

- ii. Due to the slow rate of diffusion of formalin (0.5 mm hr), cut the sections of tissue into 3 mm slices before transfer into formalin. This will ensure the best possible preservation of tissue and offers rapid uniform penetration and fixation of tissue within 3 hours.
- iii. Tissue should be fixed for a minimum 48 hours at room temperature.
- iv. After 48 hours of fixation, move tissue into 50% ethanol (for 30 min) and then to 70% ethanol for long term storage. Keep the fixing conditions uniform for a particular study.

The technique of getting fixed tissue into paraffin is called tissue processing or tissue embedding. Once fixed, tissue is processed as follows by gently transferring from one solution to the other:

- a) Fresh 70% ethanol: 1 hour;
- b) 95% ethanol: 1 hour;
- c) First time absolute ethanol: 1 hour;
- d) Second time absolute ethanol: 1½ hours;
- e) Third time absolute ethanol: 1½ hours;
- f) Fourth time absolute ethanol: 2 hour;
- g) First time clearing agent (Xylene or substitute*): 1 hour;
- h) Second time clearing agent (Xylene or substitute*): 1 hour;
- i) First time wax (Paraplast X-tra) at 58 °C: 1 hour in incubator;
- j) Second time wax (Paraplast X-tra) at 58 °C: 1 hour.

* *Toluene, chloroform, methyl and alicylate may also be used as substitute clearing agent.*



Fig. 1: Paraffin block preparation

4.2. Preparation of the Paraffin Block of Tissue for Sectioning:

- i. Set up the paraplast block (the block must be completely dry).
- ii. Pour the molten wax gently without bubbles into the block.
- iii. Gently drop the tissue into the molten wax. Using a hot needle orient the tissue.
- iv. Take care not to allow the tissue to be cooled before dropping it into the wax block.
- v. Press the paraplast block into the molten Paraplast. Allow the block to set and harden by cooling it thoroughly in cold water.
- vi. Embedded tissues may be stored indefinitely in Paraplast blocks

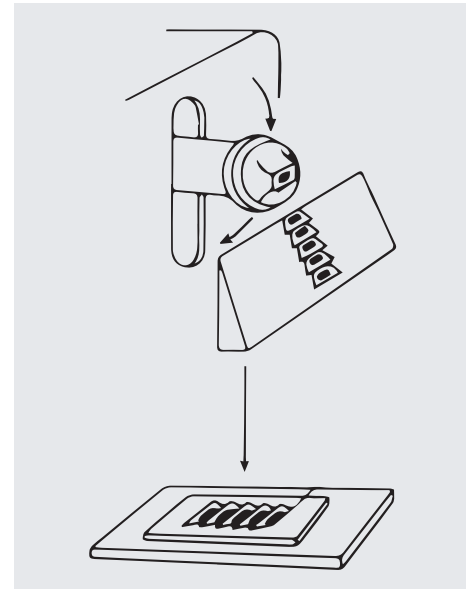


Fig. 2: Sectioning of paraffin embedded tissue using Microtome

Steps for sectioning the tissue embedded in paraffin wax using a microtome are as follows:

- i. Take the hardened paraplast block and remove excess paraplast with a razor blade from the stainless-steel mould so that the tissue surrounded by paraplast, stands out from the block.
- ii. Trim the face of the block to a size such that the tissue is surrounded by about a 1 mm frame of paraffin.
- iii. Trim the upper and lower edges of the block to make them parallel.
- iv. Trim the lateral surfaces so that the resultant face resembles a trapezoid

4.3. Preparation of the Microtome for Sectioning:

- i. Place the blocks face down on an ice block or heat sink for 10 minutes.
- ii. Clean and oil the microtome. Chuck mount should be cranked back.
- iii. Place a fresh blade on the microtome.
- iv. Set thickness of sections at 10 microns. However, with practice, thinner sections upto 5 microns may be prepared, which will allow a more detailed study of the tissue.
- v. Set the angle of the blade at 5°.
- vi. Blades may be used to section up to 10 blocks, but can be replaced whenever sections cease to be made properly.
- vii. Clamp the block into the microtome chuck so that the upper and lower surfaces are parallel with the knife edge. Align the broad end of the trapezium close to knife edge.
- viii. Do not change the angle of the blade. Move the blade toward the block so that it just clears the block and lock the blade so it does not move.

4.4. Sectioning:

- i. Turn on the water bath and check that the temp is 37-40 °C.
- ii. Use fresh deionized water. Face the block by cutting it down to the desired tissue plane and discard the paraffin ribbon.
- iii. As the paraplast is cut, the block face will gradually be cut completely and the sections will appear in trapezoid sections.
- iv. The sections should form ribbons as they are cut.

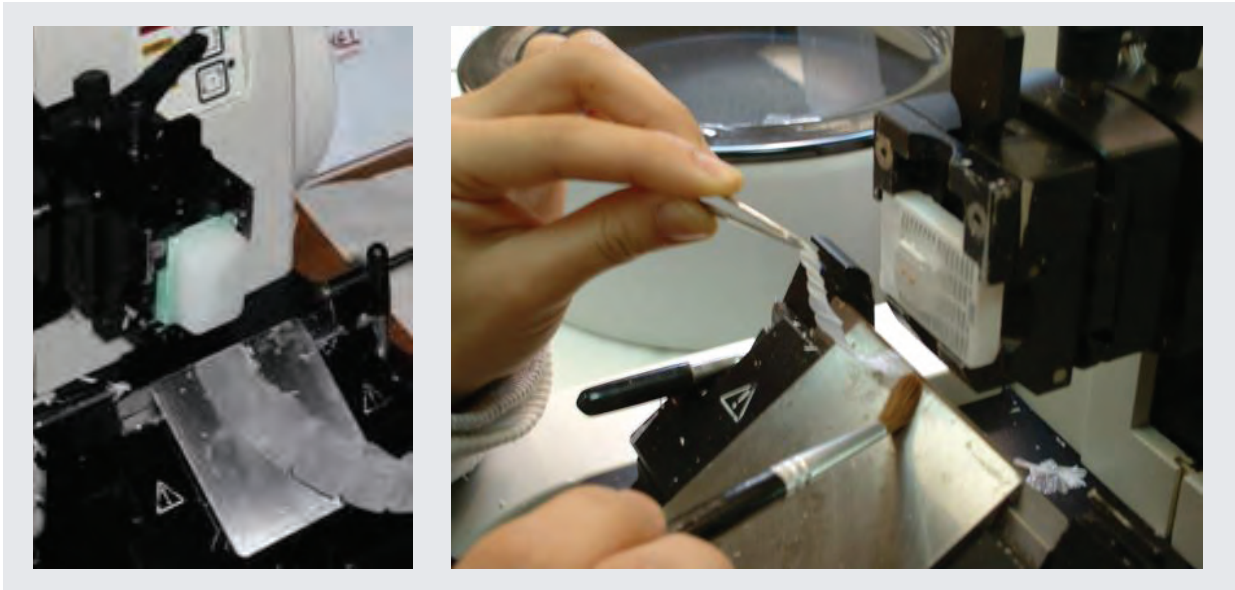


Fig. 3: Sectioning of tissue using a microtome forming paraffin ribbons

- v. If the block is ribboning well then cut another four sections and pick them up with forceps or a fine paint brush and float them on the surface of the 40 °C water bath.

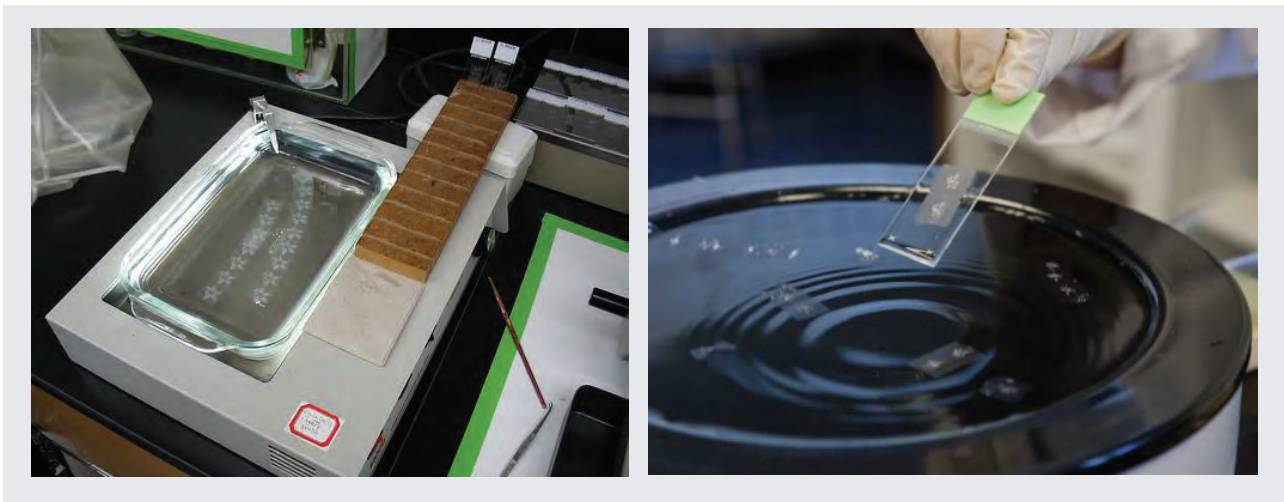


Fig. 4: Paraffin ribbons straightened in a water bath and then mounted on slides.

- vi. The paraffin sections will start straightening and the folds will disappear. Float the sections onto the surface of clean glass slides. Do not use more than 4 sections because paraffin expands upto 20%.
- vii. Place the slides with paraffin sections on the warming block at 55 °C (about 4 -5 °C lower than the melting point of was used) for few minutes. The wax around the tissue section, just starts to melt and become translucent, but the shape of the section is not changed.

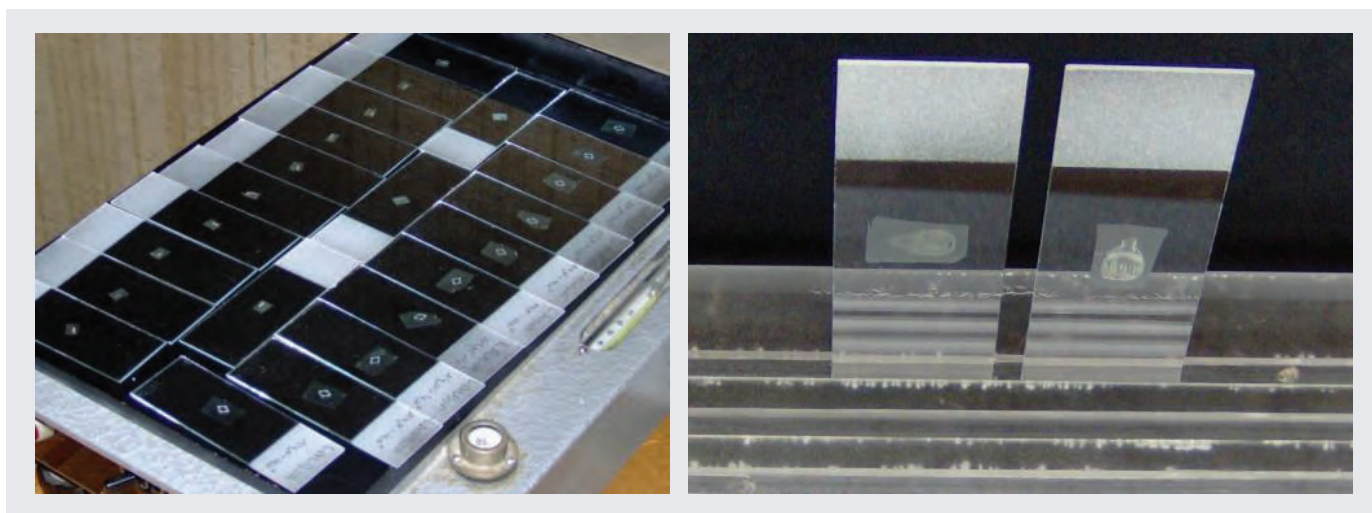


Fig. 5: Slides with sections kept on hot plate for fixing sections to glass slide and perfectly stretched and fixed sections on glass slide. (Note that the paraffin is not melted, but is translucent)

- viii. The paraffin sections will now get fixed to the glass and the sections will look translucent.
- ix. Label the slides by fixing labels on the surface of the side where the sections are fixed (Labelling can also be done using a diamond glass marking pen).
- x. Store the slides overnight at room temperature.

4.5. Staining of paraffin sections is as follows:

- i. Use Coplin jars to stain slides.
- ii. Before staining the tissue, remove the paraffin by immersing the slide in xylene twice. The sections will not fall off, if they are fixed properly to the glass surface.
- iii. Hydrate the sections using series of hydration and dehydrate the sections using dehydration steps of various alcohol grades as given in the (Fig. 3).
- iv. Water soluble staining is done after hydration and alcohol soluble staining is done after dehydration, just prior to clearing step.



Fig. 6: Staining of dewaxed sections in Coplin jars.

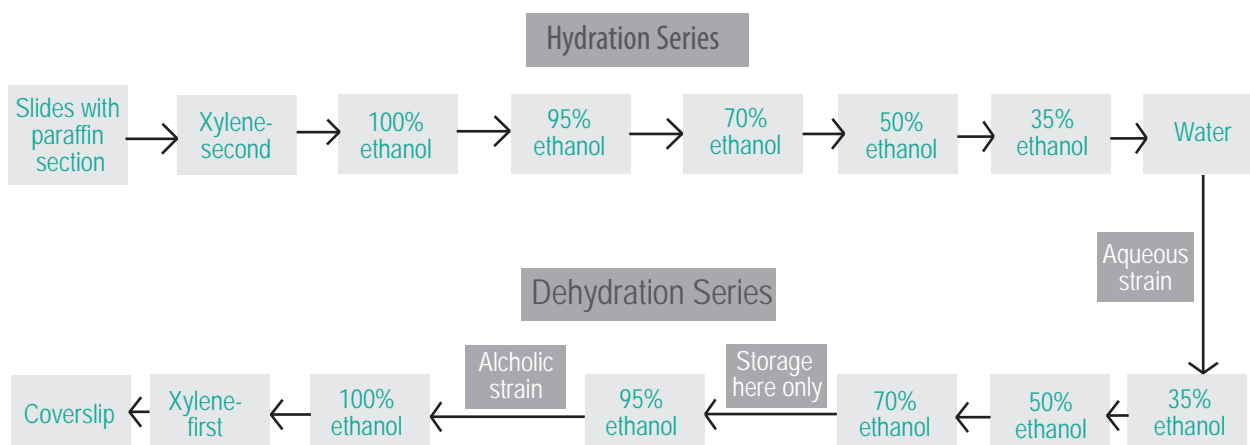


Fig. 7: Stained slide preparation for Hematoxylin and Eosin

- a) Xylene (labelled Xylene I): 5 minutes.
- b) Xylene (labelled Xylene II): 2 minutes.
- c) 100% ethanol: 3 minutes.
- d) 95% ethanol: 3 minutes.
- e) 70% ethanol: 3 minutes.
- f) 50 % ethanol: 3 minutes.
- g) 35 % ethanol: 3 minutes.
- h) Water: 3 minutes.
- i) Delafield's hematoxylin: about 5 minutes.
- j) Rinse off the stain in running tap water and check the slide on a protective glass-plate under the microscope (only for a minute). Nuclei should be dark blue, Cytoplasm grey to very pale blue.
- k) 35% ethanol: 2 minutes.
- l) 50 % ethanol: 2 minutes.
- m) If the cytoplasm is blue, the sections are over stained, add acid alcohol (two drops of concentrated hydrochloric acid in a Coplin jar of 70 % ethanol) until the sections are red-purple. Be careful not to remove too much stain.
- n) 70 % ethanol, 1 minute.
- o) 70% ethanol saturated with lithium carbonate, 5 minutes or until all the cells show a blue color. If the cells are now understained, hydrate back down the alcohol series and restain in haematoxylin.
- p) 95% ethanol 2 minutes
- q) Eosin Y solution, 2 to 5 minutes.
- r) 95% Ethanol, 2 or 3 minutes to rinse off the eosin stain. If the eosin is too dark, leave the slide in 95% ethanol to destain slowly.
- s) 100% ethanol I, 2 minutes.
- t) 100% ethanol II, 2 minutes.
- u) Xylene I, 5 minutes.
- v) Xylene II, 5 minutes.
- w) Drain off the xylene.
- x) Place a small drop of resin on the sections.
- y) Quickly, cover the sections carefully with a coverslip, taking care not to allow the slide to dry.
- z) Place the slide at room temperature overnight.

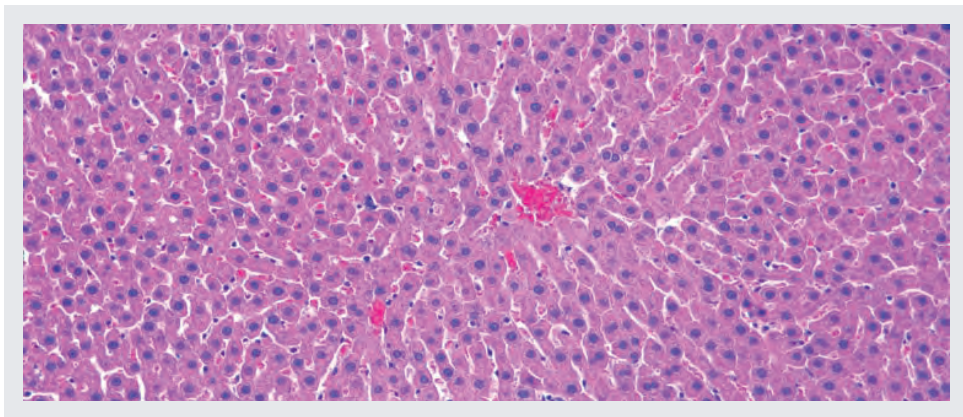


Fig. 8: Liver tissue section stained with haematoxylin and Eosin (under 10X)

5. Precautions

- i. Tissues that are soft (testis, lung etc.) will need to be kept in fixative for 15 minutes and then should be slit to allow the fixative to enter inside the tissue. Such partially fixed tissue should be sliced after about 30 – 45 minutes for proper fixation.
- ii. Embedding should be done in quick and smooth action to avoid formation of wax crystals due to drastic change in temperature between the tissue and the molten wax in the block.
- iii. The slides should be transferred quickly from one solution to the other during staining to avoid fogging of sections due to changes in humidity.
- iv. While staining, it is preferable to tap the bottom edge of the slide on a filter paper before introducing it in the next solution. This allows the draining of the excess solution from the slide.
- v. Use good quality absolute alcohol.
- vi. In monsoon and in high humidity areas, dehydration may be slow and therefore increase the time of immersion or give more changes in 70%, 90%, 100% alcohol and xylene.

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MICROBIOLOGY

Separator Photo Credit

(Lactobacillus)

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BASIC MICROBIOLOGY LABORATORY PRACTICES



1. Aim

Introduction to the basic microbiology laboratory practices and equipment.

2. Introduction

A micro-organism is any small organism that cannot be clearly seen without the help of a microscope. The microorganisms that is used to carry out the study of microbiological techniques include bacteria, fungi and viruses. While working with living microorganisms we must use good laboratory practices. These practices are important for several reasons:

- i. To ensure that we do not contaminate self or others in the laboratory, or contaminate the laboratory itself.
- ii. To avoid contaminating the cultures with which you are working.
- iii. To prevent accidentally taking microorganisms out of the laboratory.

Aseptic technique is the name given to all the procedures that are used when working with micro-organisms to prevent contamination. The most commonly used technique to create aseptic conditions is Sterilization.

Sterilization can be defined as any process that effectively kills or eliminates transmissible agents (such as fungi, bacteria, viruses and prions) from a surface, equipment, foods, medications, or biological culture medium. In practice sterility is achieved by exposure of the object to be sterilized to chemical or physical agents for a specified time.

2.1. Cleaning of glassware:

All glassware used for microbiological work should be perfectly cleaned. Used and soiled glassware should be thoroughly washed to remove all dirt and solid material adhering to the surface and dried before subjecting the material for sterilization.

a. Cleaning of new glassware:

Place the articles, test tubes, petriplates, and conical flask in a suitable container, preferably stainless steel and cover them with 1% solution of trisodium phosphate and heat to boiling. Remove all the glassware from solution, rinse with tap water and immerse in 1% HCl to neutralize the alkalinity. Rinse again in tap water and lastly in distilled water. The glassware is allowed to dry and then sterilization is done.

b. Cleaning of used glassware:

Empty the contents of glassware, rinse in cold water, then in warm water (50-55 °C). Then washing can be done either with 1% detergent solution or 1% trisodium phosphate solution. Wash with tap water, rinse with distilled water. Dry and sterilize.

c. Cleaning of pipettes and microscopic slides:

Used pipettes should be immersed in chromic acid solution in a tall jar made of glass or some non-reactive material; preferably they should be kept overnight before cleaning. This helps in removing the solid film formed over the glass surface. Special care is required in cleaning microscopic slides. They must be perfectly clean.

d. Preparation of chromic acid:

Dissolve 80 g of potassium dichromate in 300 mL of water. Add 460 mL of concentrated H_2SO_4 with constant stirring. Resulting chromic acid can be stored in glass bottles as it is reactive in nature. Instead of chromic acid, 1% KMnO_4 solution can be used for primary cleaning. They can be rinsed with detergent solution also.

Washed glassware/pipettes are allowed to dry completely first in the hot air oven at $100\text{ }^\circ\text{C}$, then plugged with cotton. They are sterilized at a temperature of $160\text{-}180\text{ }^\circ\text{C}$ for 1–2 hrs in the hot air oven.

2.2. Sterilization:

Aseptic technique is the name given to all the procedures that are used when working with micro-organisms to prevent contamination. The most commonly used technique to create aseptic conditions is Sterilization.

Sterilization can be defined as any process that effectively kills or eliminates transmissible agents (such as fungi, bacteria, viruses and prions) from a surface, equipment, foods, medications, or biological culture medium. In practice sterility is achieved by exposure of the object to be sterilized to chemical or physical agents for a specified time.

Methods of Sterilization:

The various methods of sterilization are:

i. Physical Method:

- a. Thermal (Heat) methods
- b. Radiation method
- c. Filtration method

ii. Chemical Method:

- a. Gaseous method

a. Heat Sterilization:

Heat sterilization is the most widely used and reliable method of sterilization, involving destruction of enzymes and other essential cell constituents. The process is more effective in hydrated state where under conditions of high humidity, hydrolysis and denaturation occur, thus lower heat input is required. Under dry state, oxidative changes take place, and higher heat input is required.

This method of sterilization can be applied only to thermostable products. It can be used for moisture-sensitive materials for which dry heat ($160\text{-}180\text{ }^\circ\text{C}$) sterilization is done and for moisture-resistant materials moist heat $121\text{ }^\circ\text{C}$ sterilization is done.

Comparison of time and temperature to achieve sterilization with moist and dry air		
	Temperature	Time to sterilize
MOIST HEAT	121 °C	15 min
	125 °C	10 min
	135 °C	3 min
DRY HEAT	160 °C	120 min
	170 °C	60 min
	180 °C	30 min

i. Dry Heat Sterilization: Examples of Dry heat sterilization are:

- a. Incineration
- b. Red heat
- c. Flaming
- d. Hot air oven

It employs higher temperatures in the range of 160-180 °C and requires exposures time up to 2 hours, depending upon the temperature employed. The benefit of dry heat includes good penetrability and non-corrosive nature which makes it applicable for sterilizing glassware and metallic instruments. Dry heat destroys bacterial endo toxins (or pyrogens) which are difficult to eliminate by other means.

Hot-air oven

Dry heat sterilization is usually carried out in a hot air oven.

ii. Moist Heat Sterilization: Moist heat may be used in three forms to achieve microbial inactivation.

- a. Saturated steam – Autoclaving
- b. Boiling water/ steam at atmospheric pressure
- c. Hot water below boiling point

Moist heat sterilization involves the use of steam in the range of 121 °C. Steam under pressure is used to generate high temperature needed for sterilization. Saturated steam (steam in thermal equilibrium with water from which it is derived) acts as an effective sterilizing agent.

This method of sterilization is used for all ordinary media and fluid material, used and discarded culture media and glassware and other apparatus like bacterial filters and rubber caps using an **autoclave**. The lid is tightened and is heated till the desired pressure of 15 psi (lb per square inch) is obtained (121 °C). The time for sterilization is 15-20 min. Next, the contents are removed and stored until further used.

NOTE: Before closing the main knob of the autoclave, the air content inside must be displaced for effective sterilization.

Autoclaves use pressurized steam to destroy microorganisms, and are the most dependable systems available for the decontamination of laboratory waste and the sterilization of laboratory glassware, media, and reagents.

The stages of operation of autoclaves include air removal, steam admission and sterilization cycle (includes heating up, holding/exposure, and cooling stages).

This method of sterilization can be applied only to thermostable products. It can be used for moisture-sensitive materials for which dry heat (160-180 °C) sterilization is done and for moisture-resistant materials moist heat 121 °C sterilization is done.

b. Gaseous Sterilization:

The chemically reactive gases such as formaldehyde, (methanol, HCHO) and ethylene oxide (CH₂)₂O possess biocidal activity. Ethylene oxide is a colorless, odorless, and flammable gas. Both of these gases being alkylating agents are potentially mutagenic and carcinogenic. They also produce acute toxicity including irritation of the skin, conjunctiva and nasal mucosa.

c. Radiation Sterilization:

Many types of radiation are used for sterilization like electromagnetic radiation (e.g. gamma rays and UV light), particulate radiation (e.g. accelerated electrons). The major target for these radiations is microbial DNA. Gamma rays and electrons cause ionization and free radical production while UV light causes excitation. Radiation sterilization is generally applied to articles in the dry state. UV light, with its much lower energy, and poor penetrability finds uses in the sterilization of air, for surface sterilization of aseptic work areas, for treatment of manufacturing grade water. The optimum wavelength for UV sterilization is 260 nm. A mercury lamp giving peak emission at 254 nm is a suitable source of UV light in this region of the spectrum.

d. Filtration Sterilization:

Filtration process does not destroy but removes microorganisms. It is used for both the clarification and sterilization of liquids and gases as it is capable of preventing the passage of both viable and non-viable particles.

Application of filtration for sterilization of gases:

HEPA (High efficiency particulate air) filters can remove up to 99.97% of particles that are >0.3 micrometer in diameter. Air is first passed through prefilters to remove larger particles and then passed through HEPA filters.

There are two types of filters used in filtration sterilization;

(a) Depth filters: Consist of fibrous or granular materials so packed as to form twisted channels of minute dimensions. They are made of diatomaceous earth, unglazed porcelain filter, sintered glass or asbestos.

(b) Membrane filters: These are porous membrane about 0.1 mm thick, made of cellulose acetate, cellulose nitrate, polycarbonate, and polyvinylidene fluoride, or some other synthetic material. The membranes are supported on a frame and held in special holders. Fluids are made to traverse membranes by positive or negative pressure or by centrifugation.

Application of filtration for sterilization of liquids:

Membrane filters of 0.22 micrometer nominal pore diameter are generally used, but sintered filters are used for corrosive liquids, viscous fluids and organic solvents

The merits, demerits and applications of different methods of sterilization are as follows:

Method	Mechanism	Merits	Demerits	Applications
Heat sterilization	Destroys bacterial endotoxins	Most widely used and reliable method of sterilization, involving destruction of enzymes and other essential cell constituents.	Can be applied only to thermo-stable products	Dry heat is applicable for sterilizing glassware and moist heat is the most dependable method for decontamination of laboratory waste and the sterilization of laboratory glassware, media, and reagents.
Gaseous sterilization	Alkylation	Penetrating ability of gases .	Gases being alkylating agents are potentially mutagenic and carcinogenic	Ethylene oxide gas has been used widely to process heat-sensitive devices.
Radiation sterilization	Ionization of nucleic acids	It is a useful method for the industrial sterilization of heat sensitive products. .	Undesirable changes occur in irradiated products, an example is aqueous solution where radiolysis of water occurs.	Radiation sterilization is generally applied to articles in the dry state
Filtration sterilization	Does not destroy but removes the microorganisms	It is used for both the clarification and sterilization of liquids and gases as it is capable of preventing the passage of both viable and non viable particles	Does not differentiate between viable and non viable particles	In this method sterilizing grade filters are used in the treatment of air and other gases for supply to aseptic areas.

3. Good Laboratory Practices

Most microbiological laboratory procedures require the use of living organisms, thus use of aseptic techniques is an integral part of all laboratory sessions.

The following rules should be followed at all times to reduce ubiquitous microflora and the laboratory accidents.

1. Always wear a laboratory coat or an apron before entering a laboratory to protect clothing from contamination or accidental discoloration by staining solutions.
2. At the beginning and termination of each laboratory session, wipe bench tops with a disinfectant like Lysol (1:500), phenol (1:100), spirit or 90% ethanol.
3. Keep your laboratory bench clear of everything except your laboratory equipment and notebook.
4. Don't smoke, eat or drink in the laboratory.
5. Never remove media, equipment and especially bacterial cultures from the laboratory.
6. Don't place contaminated instruments such as inoculating loops, needles and pipettes on the bench tops.
7. Loops and needles should be sterilized by incineration
8. Pipettes and cultures should be disposed off in designated receptacles.

9. All microbial cultures should be handled as being potential pathogens.
10. Wash your hands with liquid detergent/soap upon entering and prior to leaving the laboratory.
11. Long hair should be tied back to minimize contamination of cultures and fire hazards.
12. Carry cultures in a test tube rack when moving around and stack in a test tube stand or basket on the laboratory bench.
13. Immediately cover spilled cultures or broken culture tubes with filter paper and saturate with disinfectant and after 15 minutes, clean the area.
14. Report accidental cuts or burns to the instructor immediately.
15. Never pipette by mouth any broth cultures or chemical reagents. Pipetting is to be carried out with the aid of a mechanical pipetting device.
16. Aseptic techniques must be rigorously observed at all times.
17. Familiarize yourself in advance with the exercise to be performed.
18. Label all the plates, tubes, cultures properly before starting an exercise.
19. Don't lick labels. Use only self-stick labels for the experimental purposes.

4. Basic Requirements In A Microbiological Laboratory

The following are needed for various microbiological laboratory exercises.

Equipments	Glassware and Plastic ware	Chemicals	Miscellaneous
<ul style="list-style-type: none"> • Laminar air flow chamber • Microscopes and their accessories • Micrometer (ocular and stage) • Water bath • Hot air oven • Autoclave or pressure cooker • Incubators • Refrigerators • Centrifuge • Spectrophotometer • Quebec colony counter • Balances • Homogenizer • pH meter • Hot plate 	<ul style="list-style-type: none"> • Petriplates • Conical flasks • Culture tubes without screw caps • Screw cap tubes • Durham fermentation tubes • Beakers • Funnel • Graduated cylinders • Bacteriological pipettes • Breed's pipettes • Reagent bottles • Microscopic slides • Coverslips • Cavity slid • Glass dropperse 	<ul style="list-style-type: none"> • Culture Media • Disinfectant • Immersion oil • Stains • Spirit • Ethyl Alcohol 	<ul style="list-style-type: none"> • Test tube stands • Baskets • Pipette box • Pipette stand • Cotton • Petriplate box • Labels and tapes • Markers or glass marking pen • Filter paper • Pestle mortar • Lens paper • Trays • Corks (plastic) • Inoculating loops • Transfer needle • Burette stand

<ul style="list-style-type: none"> • Bunsen burner or spirit lamp • Vortex shaker • Magnetic stirrer • Membrane filter assembly • Water distillation assembly • Lab Blender • Stomacher 	<ul style="list-style-type: none"> • Water storage bottle 	<ul style="list-style-type: none"> • Thermometer • Forceps • Scissors • Dropper bottles for staining
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5. Understanding the Functioning and Use of Common Microbiological Equipments

The application of heat is a common means of destroying micro-organisms. Both dry and moist heat is effective. Moist heat is more effective as it has a greater penetration power and causes protein coagulation leading to cell death at a lower temperature than dry heat sterilization. The destruction of all forms of life is accomplished in 15 minutes at 121 °C with moist heat and dry heat requires a temperature of 160-180 °C for 2- 4 hours. For sterilization purposes, autoclave and hot air oven are used.

I. HOT AIR OVEN:

Hot air ovens are commonly used for sterilizing glassware (like petriplates, test tubes, pipettes, etc.), oils, powders and waxes. An oven is based on the principle of achieving sterilization by dry heat or hot air. Sterilization is usually accomplished by exposure of items to 160-180 °C for 2-4 hours.

An oven consists of an insulated cabinet which is held at a constant temperature by means of an electric heating mechanism and a thermostat. An electric fan is provided for the circulation of hot air.

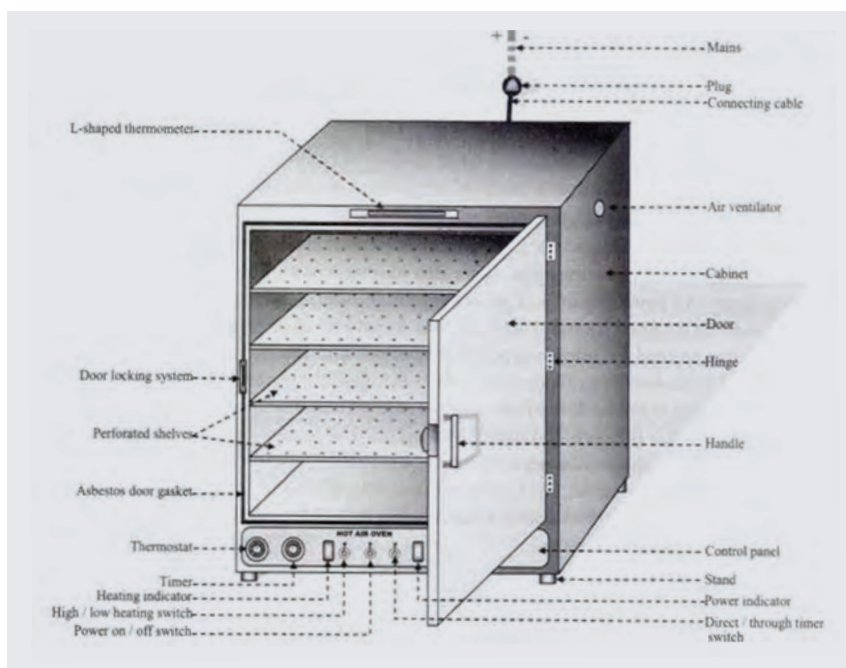


Fig. 1: A conventional hot air oven

II. AUTOCLAVE:

It is the most efficient instrument used for sterilizing solid and liquid media for microbial cultures, heat stable liquids such as the common media ingredients, heat resistant instruments as well as contaminated material, glassware and plastic / rubber products. Autoclaving is not recommended for oils, powders, heat sensitive fluids and plastics that melt.

An autoclave is a double walled cylindrical metallic vessel usually made of thick stainless steel having a pressure gauge timer, steam exhaust valve, controls for adjusting the pressure and temperature. The articles to be sterilized are kept loosely in a basket provided with holes for the free circulation of steam.

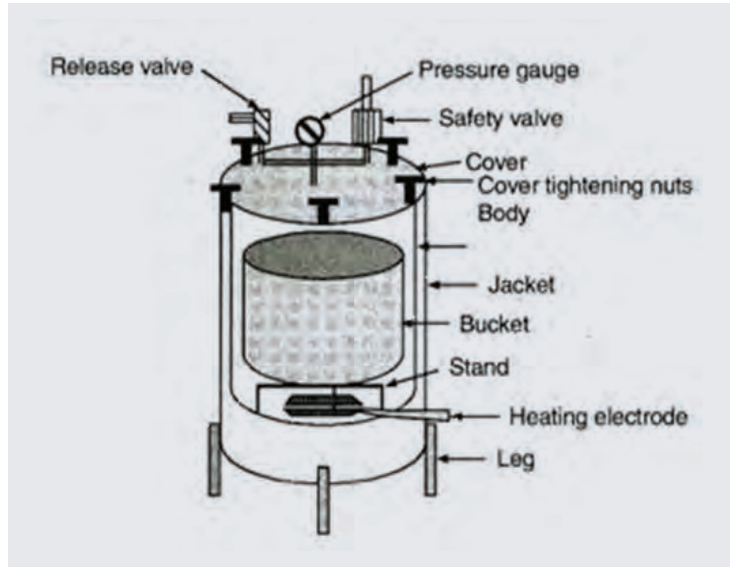


Fig. 2: A schematic diagram of laboratory autoclave

It works on the principle of sterilization using saturated steam under pressure. The increased pressure increases the boiling point of water and produces steam with high temperature. A pressure of 15 psi achieves a temperature of 121 °C and sterilizes in 15 minutes.

(A pressure cooker can also be used instead of autoclave, as the working principle is same for both).

III. INCUBATOR:

An incubator is used for the incubation of the micro-organisms i.e. providing them with a constant temperature for their optimum growth. The temperature usually ranges from 37-50 °C. The B.O.D incubators have a temperature range of 25-27 °C. Both the temperature and humidity can be controlled. An incubator is very similar to an oven in construction but the temperature range is different.

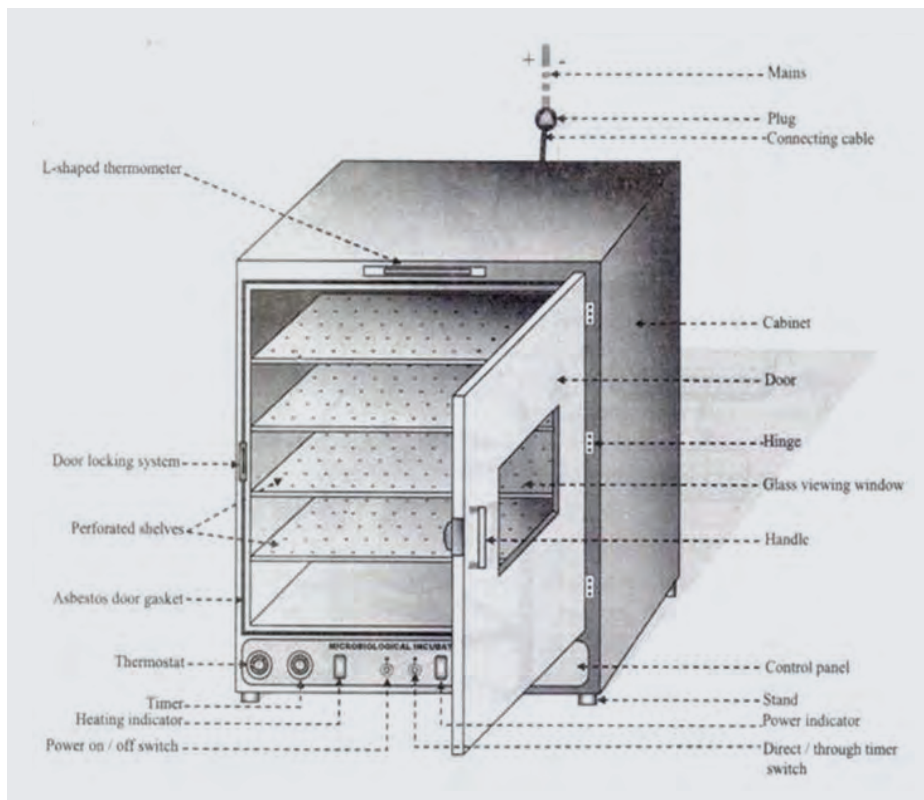


Fig. 3: A conventional microbiological Incubator

IV. LAMINAR AIR FLOW CHAMBER / LAMINAR HOOD:

It is a clean air bench where germ free pure air is introduced on the working bench via a combination of air purifying filters (HEPA filters i.e. High Efficiency Particulate Air filters). It has separate switches / controls for air, UV light and fluorescent light.

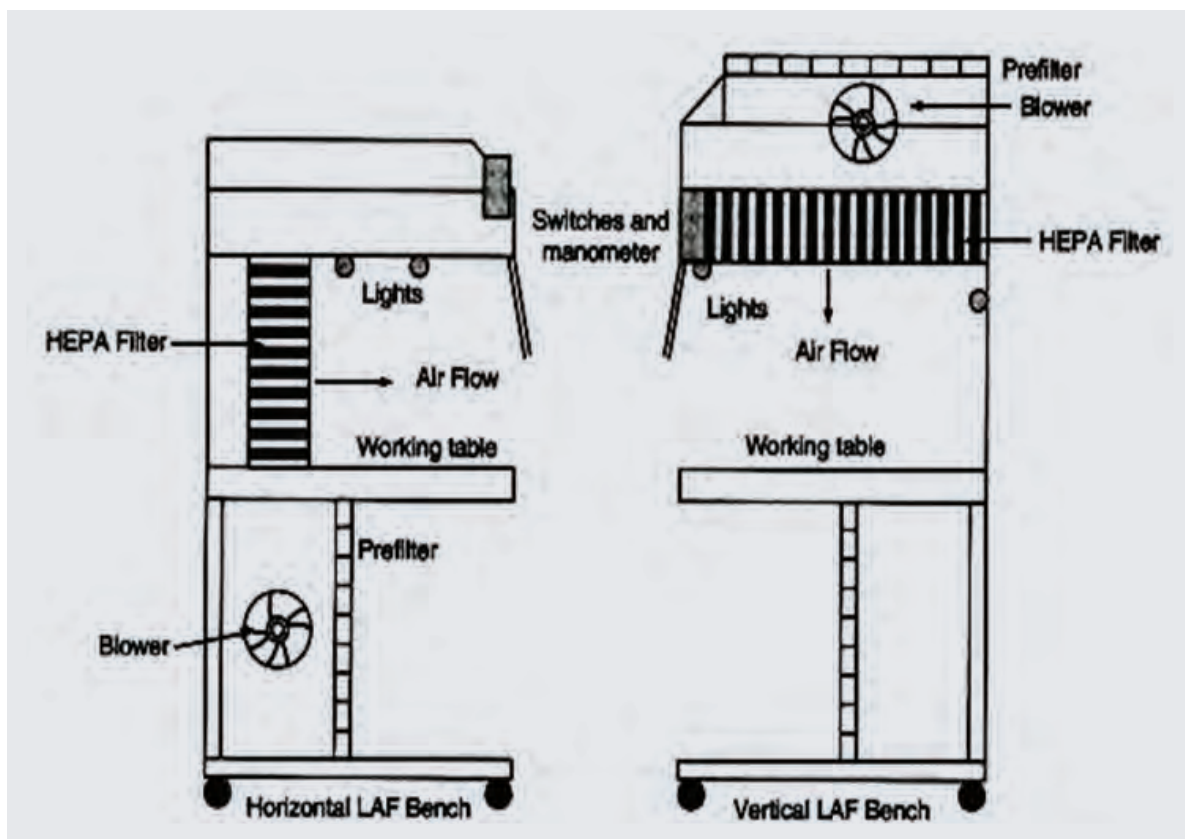


Fig. 4: Labeled diagram of laminar air flow

Apart from the above equipment a microbiology laboratory requires the following:

S.No	EQUIPMENTS	USE
1.	Quebec colony counter	Counting of total number of microbial colonies growing on a petriplate.
2.	Water bath	Maintenance of constant temperature for various microbiological and enzymatic activities.
3.	Shaking water bath	Cultivation of micro-organisms in a broth medium.
4.	Bunsen burner	Used for sterilization of inoculating loops / needles and flaming the mouth of test tubes, conical flasks and other glassware to prevent contamination.
5.	Refrigerator	Used for storing stock cultures of micro-organisms as well as thermo labile solutions, sera, antibiotics and biochemical reagents.
6.	Centrifuge	Used for separation of particles dispersed in a suspended matter by use of centrifugal force. (Usually concentrating micro-organisms for various assays).

7.	Spectrophotometer	Used for determination of bacterial population based on the turbidity measurements.
8.	pH meter	Used to determine the pH (acidity or alkalinity of a solution).
9.	Vortex shaker	Used to mix the contents.
10.	Auto sterilizer	Used for sterilization of loops and inoculating needles.

6. Precautions

- i. Final rinse of the glassware should be done by distilled water.
- ii. Use non-absorbent cotton for making plugs.
- iii. Preparation of chromic acid should be done carefully and preferably in a sink full of water.
- iv. Use of distilled water should be done for the process of autoclaving. It should be regularly changed.
- v. The steam valve should be closed when the water begins to undergo condensation.
- vi. The time for autoclaving should be 15 minutes after 15lbs pressure is attained.

Suggested Reading(s)

- i. Cappuccino, J., & Sherman, N. (2010). *Microbiology: A Laboratory Manual*. (9th ed.). Pearson Education Limited.
- ii. Wiley, J. M., Sherwood, L. M., & Woolverton, C. J. (2013). *Prescott's Microbiology*. (9th ed.). McGraw Hill International.
- iii. Goldman, E., & Green, L. H. (2015). *Practical Handbook of Microbiology*. (3th ed.). CRC Press.

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STRUCTURE AND FUNCTION OF COMPOUND MICROSCOPE



1. Aim

To study the structure and functioning of a compound microscope.

2. Introduction

The bright field compound microscope is a type of microscope, which is most commonly used for general laboratory observations. It consists of a series of two lens system between the eye and the object and a direct light source (sun and mirror or lamp) where light shines directly on the specimen, and dark objects in a bright field are observed.

A typical compound microscope consists of four basic parts:

- i. **Base:** It is usually a horse shoe shaped structure and provides firm support and stability to the microscope.
- ii. **Arm:** It is usually curved and used for handling the microscope.
- iii. **Stage:** It is usually a rectangular flat plate (platform) attached to the lower end of arm and is used to hold the slide or magnifying object i.e. object to be magnified.
- iv. **Body Tube:** It is a tubular hollow part attached to the upper part of the arm. It can be moved up and down with the help of screws.

The other mechanical parts are:

a) Condenser:

Attached to the stage is a sub stage consisting of a condenser, an iris diaphragm and a mirror. The condenser consists of several lenses that concentrate light on the slide. The iris diaphragm controls the angle and amount of light used. The mirror reflects light from the source of illumination via the condenser.

b) Coarse adjustment screw and Fine adjustment screw:

The coarse and fine adjustment knobs are used for focusing the image of the specimen. The coarse adjustment which raises or lowers the body tube is used for focusing the low power objective and the fine adjustment is used for focusing high power and oil immersion objectives. The area seen through a microscope is known as the field of vision.

The optical system consists of two series of lenses,

- i. Objective lens
- ii. Ocular lens (eye piece)

The upper end of the body tube holds the ocular lens (10X /15X) through which the image is viewed. The lower end of body tube which is lodged close to the objective being examined is fitted with a rotating nose piece holding generally three objective lens, which provide magnification powers of 10X , 40X and 100X.

(A microscope possessing a single ocular lens is called a monocular microscope and that possessing two ocular lenses is called a binocular microscope.)

The magnification of microscope depends on the following factors: a) focal tube length, b) the focal length of the objective and c) magnification of eye piece.

The primary image produced by the objective is real inverted image. The eye piece used in the compound microscope is a simple magnifier. It magnifies the image produced by the objective. The image produced by the eyepiece is the virtual, inverted, magnified image. An important property of a microscope is its resolving power. It is defined as its ability to distinguish two points that are close together as distinct and separate. Resolving power depends on the wavelength of the light and optical property i.e. numerical aperture (NA) of the lens used.

$$\text{Resolving power} = \lambda/2NA$$

$$\text{Numerical aperture} = n \sin\theta$$

Where, n is the refractive index and θ is half of the aperture angle.

The n value is 1 when medium is air whose refractive index is 1. However, n is 1.56 if oil immersion objective is used since the refractive index of oil is 1.56. If 58° is half of aperture angle, then,

$$NA = n \sin\theta = 1.56 \times \sin 58^\circ = 1.33$$

3. Working of Microscope

- i. Adjust the mirror so that sufficient amount of light enters into the microscope by looking through the eye piece.
- ii. Keep a clean prepared slide in the centre of the stage. Use clips to fix the slide in the centre of the stage.
- iii. Move the coarse adjustment screw to bring the slide in focus. Use the fine adjustment screw for sharp focusing.
- iv. To use high magnification, first adjust at low magnification and then change to 40X objective lens to a desired position with the help of nose piece.
- v. Focus the area to be enlarged, then shift 40X lens away, add a drop of immersion oil and adjust 100X objective to observe the magnified view.

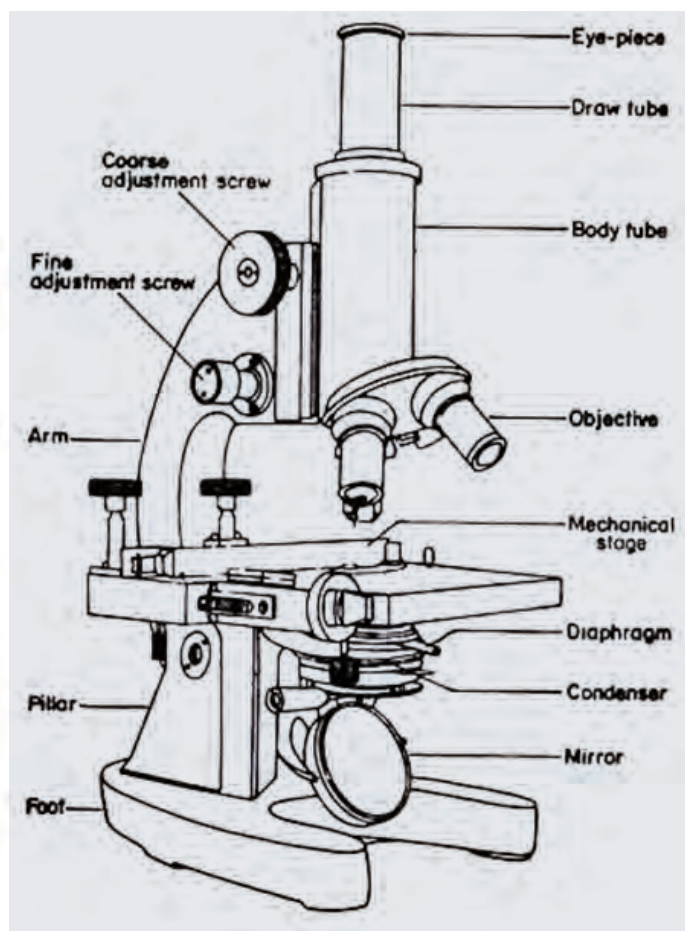


Fig. 1: Compound microscope

Source: <http://cdn.biologydiscussion.com>

4. Precautions

- i. Always focus your slide with low power objective first.
- ii. Keep the microscope and its parts (e.g. lens, stage, and mirror) clean and handle all parts with care.
- iii. Always keep the microscope covered when not in use to keep out dust.
- iv. Never use a slide without cover slip while examining an object under high magnification power of the microscope; otherwise the chemicals will damage the objective.

Suggested Reading(s)

- i. Wiley, J. M., Sherwood, L. M., & Woolverton, C. J. (2013). *Prescott's Microbiology*. (9th ed.). McGraw Hill International.
- ii. Pelczar, M. J., Chan, E. C. S., & Krieg, N. R. (1993). *Microbiology*. (5th ed.). McGraw Hill Book Company
- iii. Goldman, E., & Green, L. H. (2015). *Practical Handbook of Microbiology*. (3th ed.) CRC Press.

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MEDIA PREPARATION FOR BACTERIAL SPECIES



1. Aim

To prepare general purpose media for cultivation of different bacterial species.

2. Introduction

A culture medium is a solid or liquid preparation containing all the nutrients required by microorganisms for growth. It is used to grow, transport and store microorganisms. Liquid culture medium is called broth. It can be solidified by adding solidifying agent agar-agar at a concentration of 1.5-2.0% for complete solid agar and less than 1% for semi solid medium. Solid media provides a hardened surface on which microorganisms can be grown in the form of discrete colonies to get pure cultures.

Depending upon the purpose, media can be categorized as –

General Purpose Media – These support the growth of most microorganisms. Example – nutrient agar, trypticase soy agar etc.

Specialized media – These are used for isolation of specific bacteria from a mixed microbial population, for differentiation among closely related bacterial groups, enumeration of bacteria etc. These media can be grouped as-

- i. Selective media
- ii. Enrichment media
- iii. Differential media
- iv. Selective and differential media
- v. Enriched media

3. Materials Required

3.1. Biological Materials: *E. coli*, *Bacillus*, *Pseudomonas* and *Staphylococcus*.

3.2. Chemicals/Reagents: Peptone, beef extract, agar, distilled water.

3.3. Equipment: pH meter, magnetic stirrer, autoclave, hot air oven, bunsen burner.

3.4. Glassware/Plastic ware: Conical flask, measuring cylinder, petri plates and test tubes.

4. Procedure

4.1. Nutrient Agar and Nutrient Broth:

Nutrient agar and nutrient broth is a general-purpose medium of complex nature. It is widely used in laboratories for cultivation and maintenance of bacteria. It contains peptone and beef extracts, which are the source of various amino acids and vitamins etc.

Composition of Nutrient broth:-

Peptone – 10.0 gm

Beef extract – 10.0 gm

Sodium chloride – 5.0 gm

Distilled water – 1000 mL

pH – 7.3

NOTE: For making nutrient agar, add 15 gm (3%) of agar into nutrient broth

- i. Weigh and dissolve each ingredient except agar in 500 mL of distilled water.
- ii. Make the volume to 1000 mL with distilled water. Set the pH to 7.0. This is nutrient broth.
- iii. Autoclave the nutrient broth at 15 psi for 15- 20 minutes and dispense in sterile test tubes aseptically.
- iv. For nutrient agar, add 15 gm of agar in 1000 mL of nutrient broth. Heat the mixture to dissolve agar.
- v. Dispense the mixture into flasks and autoclave.
- vi. After autoclaving, cool the nutrient agar medium to 45 °C and dispense it aseptically into sterile petri plates and tubes to make agar plates, slants and deep tubes.
- vii. Allow to solidify. For making slants, keep the tubes in inclined position for agar solidification.
- viii. Inoculate nutrient agar plates, nutrient agar tubes and nutrient broth with bacterial culture and incubate for 24 hours at 37 °C.
- ix. Note the observation and result.

5. Observations

Method of cultivation	Colony Characteristics (Colonies/Streak/Turbidity)
Nutrient Agar Plates Nutrient Agar Tubes Nutrient Broth	

6. Result

On the inoculated nutrient agar plate and tubes bacterial colonies will be obtained. The nutrient broth will appear turbid.

7. Precautions

- i. Dissolve ingredients one by one in distilled water.
- ii. Check and set the medium to appropriate pH.
- iii. Autoclave the medium properly.
- iv. Dispensing of the medium should be done aseptically.

Suggested Reading(s)

- i. Cappuccino, J., & Sherman, N. (2010). *Microbiology: A Laboratory Manual*. (9th ed.). Pearson Education Limited.
- ii. Wiley, J. M., Sherwood, L. M., & Woolverton, C. J. (2013). *Prescott's Microbiology*. (9th ed.). McGraw Hill International.
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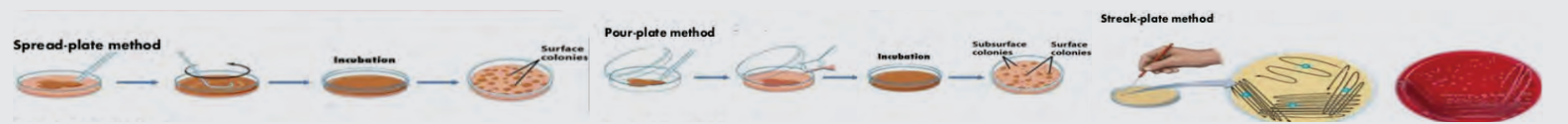
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CULTIVATION AND SUB CULTURING OF MICROBIAL CULTURES



1. Aim

To study the cultivation and sub culturing of microbial cultures.

2. Introduction

Bacteria and fungi are grown on or in microbiological media of various types. The medium that is used to culture the microorganism depends on the microorganism that one is trying to isolate or identify. Microorganisms need nutrients, a source of energy and certain environmental conditions in order to grow and reproduce.

The mixture of necessary nutrients can be used as a liquid medium known as broth or a solidifying agent can be added. "Agar agar" is a natural polysaccharide produced by marine algae and is the most commonly used solidifying agent added to media (end concentration usually 1.5 % w/v). This medium is known as agar.

Finally, in working with microorganisms, we must have a method of transferring growing organisms (called the inoculum) from a pure culture to a sterile medium without introducing any unwanted contaminants. This method of preventing unwanted microorganisms from gaining access is termed aseptic technique.

3. Materials Required

3.1. Biological Materials: 18-24 hour old nutrient agar slant or nutrient broth cultures of *E. coli*.

3.2. Chemicals/Reagents: Sterile nutrient broth and nutrient agar, sterile nutrient agar slants and stabs.

3.3. Glassware/Plastic ware: Petri plates, sugar tubes (10 mL), test tubes.

3.3. Equipment: Laminar air flow chamber, incubator.

3.4. Miscellaneous: Inoculating loop and needles, bunsen burner.

4. Procedure

4.1. Sterilize the inoculating loop:

- i. Sterilize the inoculation loop by passing it at an angle through the flame of a gas burner until the entire length of the wire becomes orange from the heat. In this way all contaminants on the wire are incinerated

4.2. Remove the inoculum:

a. **Removing inoculum from a broth culture** (organisms growing in a liquid medium):

- i. Hold the culture tube in one hand and in your other hand, hold the sterilized inoculating loop as if it were a pencil.
- ii. Remove the cap of the pure culture tube with the little finger of your loop hand.

- iii. Very briefly flame the lip of the culture tube. This creates a convection current which forces air out of the tube and preventing airborne contaminants from entering the tube. The heat of the gas burner also causes the air around work area to rise, and this also reduces the chance of airborne microorganisms contaminating cultures.
- iv. Keeping the culture tube at an angle, insert the inoculating loop and remove a loopful of inoculum.
- v. Again flame the lip of the culture tube.
- vi. Replace the cap.

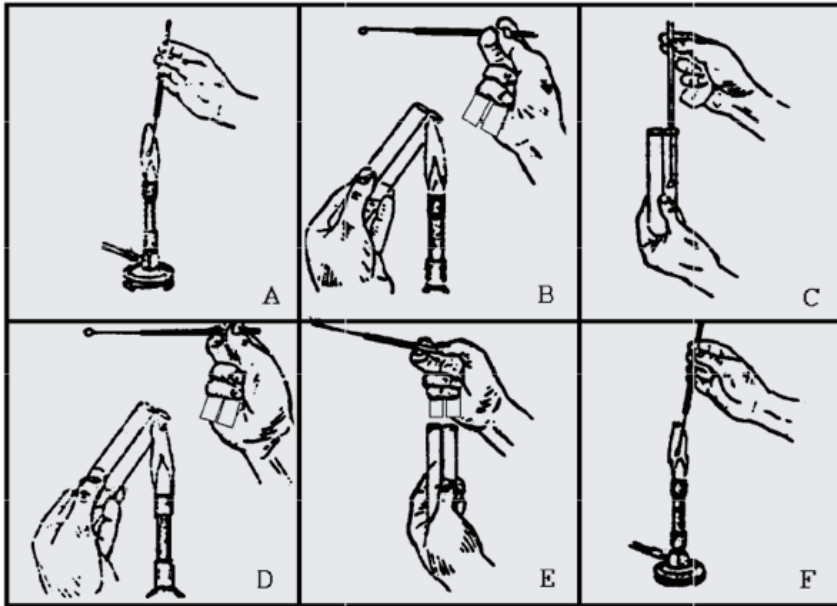


Fig. 1: Showing tube to tube transfer

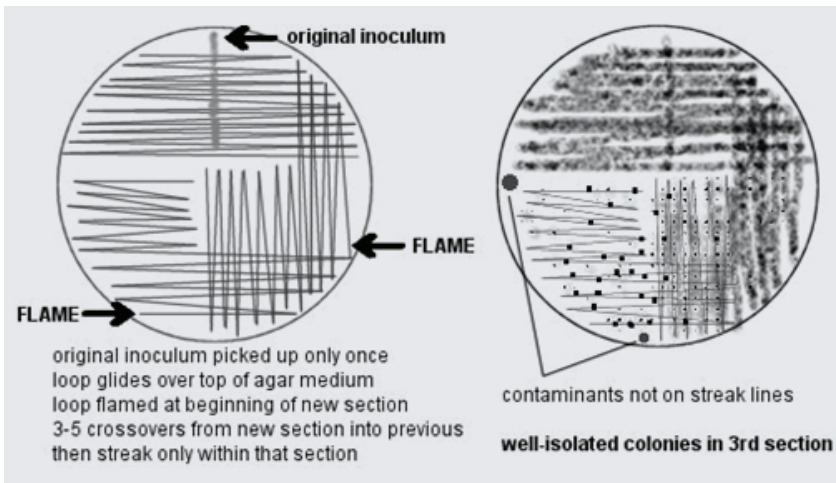


Fig. 2: Streaking on agar plate

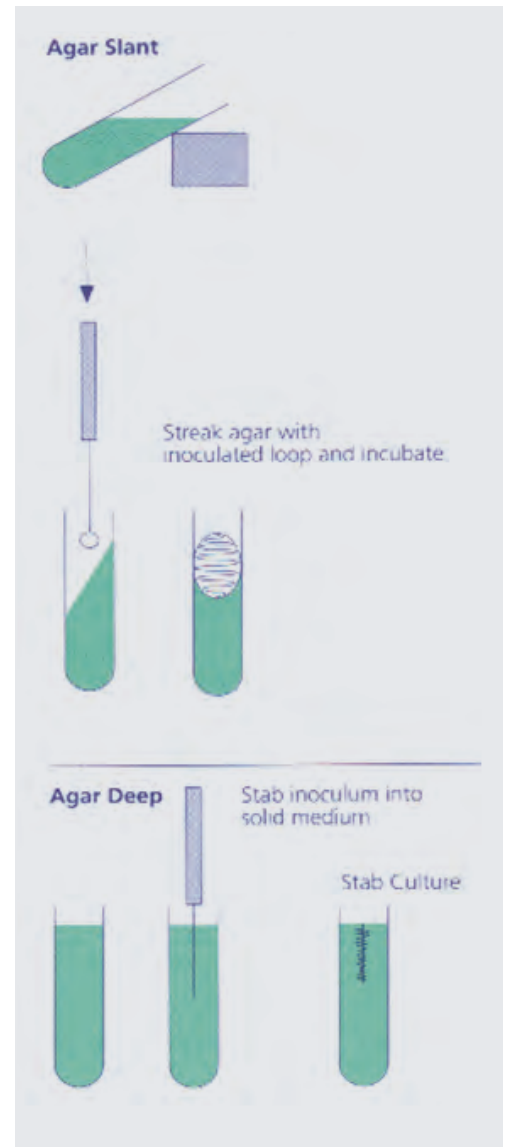


Fig. 3: Sub culturing into agar slant and agar stab

b. Removing inoculum from a plate culture (organisms growing on an agar surface in a petri plate)/agar slant or stab:

- i. Sterilize the inoculating loop in the flame of a gas burner.
- ii. Lift the lid of the culture plate slightly and stab the loop into the agar away from any growth to cool the loop. In case of slant or stab, remove cap near the flame.
- iii. Scrape off a small amount of the organisms and close the lid or cap.

4.3. Transferring the Inoculum to the Sterile Medium:

a. Transferring the inoculum into a broth tube:

- i. Pick up the sterile broth tube and remove the cap with the little finger of your loop hand.
- ii. Briefly flame the lip of the broth tube.
- iii. Place the loopful of inoculum into the broth, and withdraw the loop. Do not lay the loop down.
- iv. Again flame the lip of the tube.
- v. Replace the cap.
- vi. Resterilize the loop by placing it in the flame until it is orange. Now you may lay the loop down until it is needed again.

b. Transferring the inoculum into a petri plate:

- i. Lift the edge of the lid just enough to insert the loop.
- ii. Streak the loop across the surface of the agar medium using either the pattern shown or the pattern shown. These streaking patterns allow you to obtain single isolated bacterial colonies originating from a single bacterium or arrangement of bacteria.
- iii. In order to avoid digging into the agar as you streak the loop over the top of the agar you must **keep the loop parallel to the agar surface**. Always start streaking at the "12:00 position" of the plate and streak side-to-side as you pull the loop toward you. Each time you flame and cool the loop between sectors, rotate the plate counter clockwise so you are **always working in the "12:00 position" of the plate**. This keeps the inoculating loop parallel with the agar surface and helps prevent the loop from digging into the agar.
- iv. Remove the loop and close the lid.
- v. Resterilize the inoculating loop.

(Add the inoculum then pour the sterile molten and cooled agar for preparation of the **pour plates** whereas for a **spread plate**, the inoculum is spread on the solidified agar surface of the plate with an L-shaped spreader.)

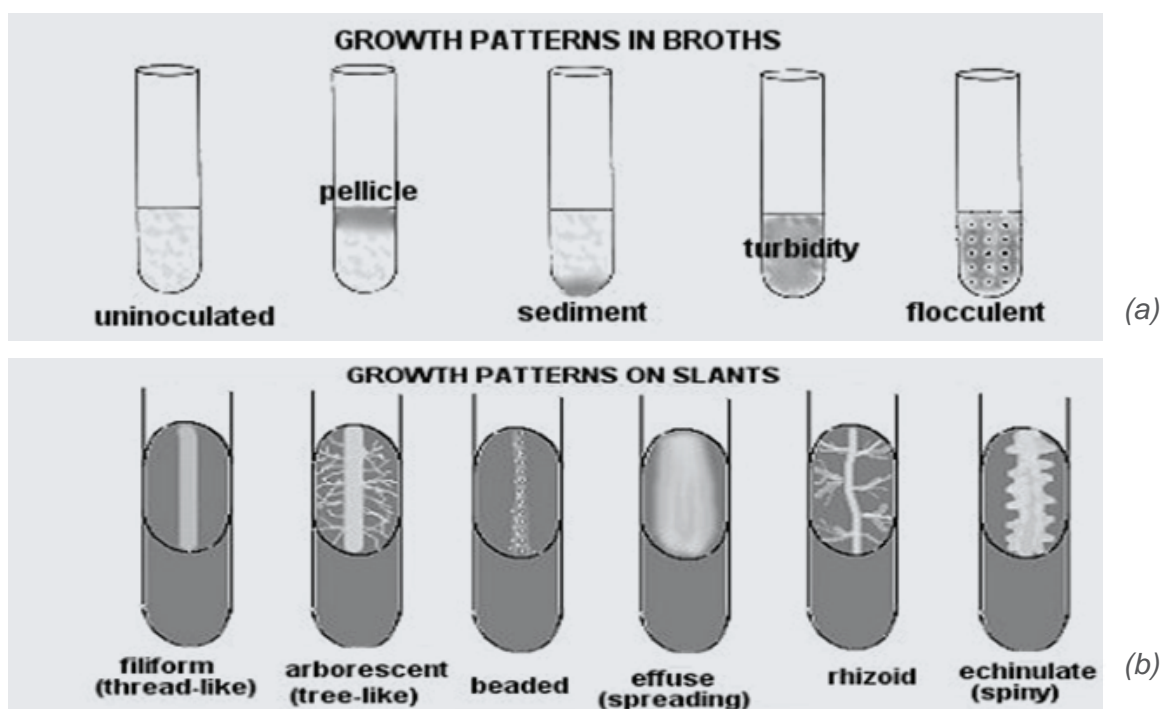


Fig. 4: Growth patterns (a) In broth, (b) On slants

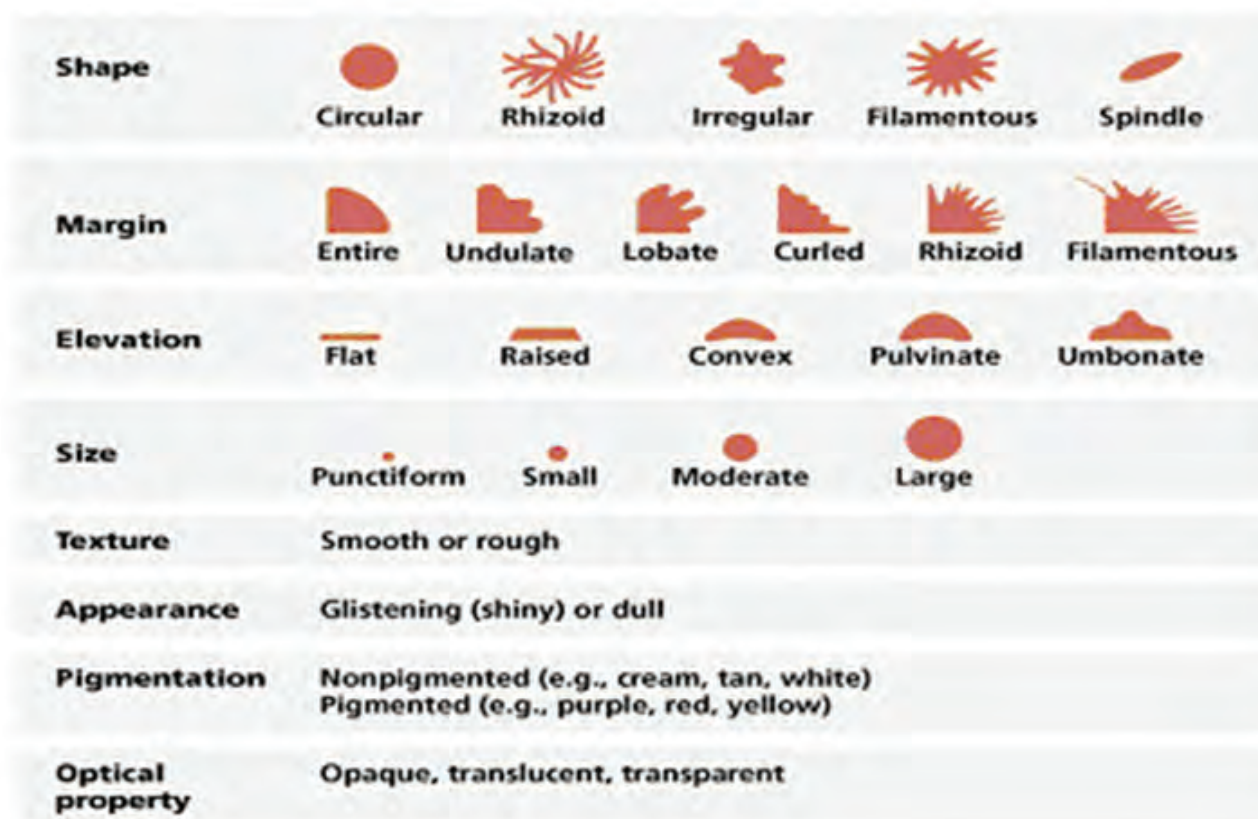


Fig. 5: Colony characteristics on agar slant, and agar plate

c. Transferring the inoculum into an agar slant or agar stab:

- i. Remove the cap or plug of the tube still holding within the fingers of the hand.
- ii. Insert the loop carrying the scraped inoculum into the tube.
- iii. Streak along the surface of the slant with the loop. In case of agar stab, stab the agar with the needle as shown.

4.4. Incubate the inoculated tube, agar slant, agar deep tube or agar plates at the optimum growth temperature of the inoculated cultures.

4.5. Observe the various growth patterns.

5. Observations

After incubation, check the following growth patterns of all tubes and plates. Note down the observations in the given table.

Colony Characteristics	A	B
Form of colony		
Elevation		
Margin (edge)		
Surface		
Optical characteristics		
Pigmentation		

6. Result

Isolated colony can be purified by streak plate method and characterized on the agar plate. In case of broth, depending upon the type of microorganism after inoculation and incubation, the turbidity increases and the growth can be seen in the form of pellicle, sediment or a flocculent mass.

7. Precautions

- i. Glassware should be properly clean and sterilized.
- ii. Weighing should be done accurately.
- iii. pH adjustment should be done carefully.
- iv. Filtering should be proper.
- v. Cotton plugs should be tight enough.
- vi. Never lay the cap down or it may become contaminated.

Suggested Reading(s)

- i. Cappuccino, J., & Sherman, N. (2010). *Microbiology: A laboratory manual*. (9th ed.). Pearson Education Limited.
- ii. Wiley, J. M., Sherwood, L. M., & Woolverton, C. J. (2013). *Prescott's Microbiology*. (9th ed.). McGraw Hill International.
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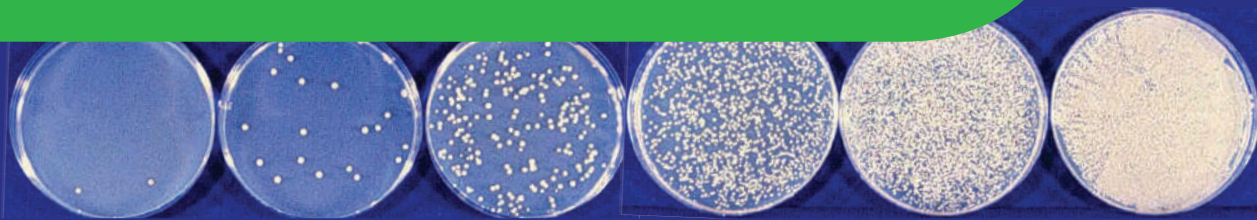
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ISOLATION AND ENUMERATION OF BACTERIA



1. Aim

To isolate *Escherichia coli* culture and assess its viability using the viable count method.

2. Introduction

The study of any microbe requires its isolation from a mixed natural population and growing it as a pure culture in a suitable medium. Once a pure culture is developed, microbiologists have to routinely maintain such cultures to ensure long-term viability and genetic stability, and to protect them from contamination during storage or transfers.

The most common method to maintain a bacterial culture and estimate the viability is the plate count method. This method is based on the fact that, on a solid nutrient medium, the viable cells grow to form colonies that are visible to the naked eye. The resultant colonies are visible to the naked eye making visualization, selection and counting simpler. Plates suitable for counting must contain between 30-300 colonies. To arrive at a suitable dilution that results in this number, serial dilution method is used. The method involves making a tenfold dilution at a time by taking a small amount of the original sample (For eg. 1 mL) and making up the volume (to say 10 mL) by adding an appropriate buffer or normal saline. For every dilution, a specific volume is plated on a suitable medium.

Plate count method can be of two types:

Pour plate method: It allows the growth of anaerobes (beneath the surface) as well as aerobes (on the surface of the plate). Refer to Fig. 1 and the procedure.

Spread plate method: A known volume of the dilution is plated on the nutrient agar plate. The colonies obtained can be selected and sub cultured easily for further use.

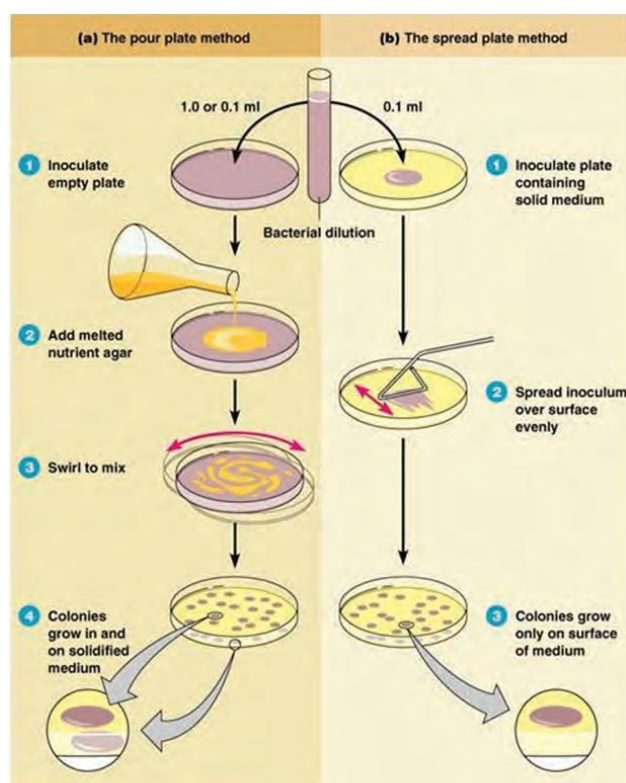
Refer to Figure 1 and the procedure

The bacterial count in the original sample is estimated as CFU/mL (Colony Forming units/mL).

$$\text{CFU/mL} = \text{CFU/volume of dilution plated} \times \text{Dilution factor}$$

3. Materials Required

- 3.1. Biological Materials:** *E. coli* culture.
- 3.2. Chemicals / Reagents:** Peptone, yeast extract, sodium chloride, agar agar, ethanol.
- 3.3. Equipment:** Autoclave, shaker incubator, laminar flow cabinet, incubator, water bath set at 48-50 °C.



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Fig. 1: Pour plate and spread plate method for enumeration of the bacterial count of a sample.

3.4. Glassware/Plastic ware: Erlenmeyer flasks (1 liter; 100 mL), Culture tubes, Petri -plates, spreader, Beakers (250 mL), Pipette.

3.5. Miscellaneous: Cotton plugs (non-absorbent), Micro tips. Micropipettes, test tube stand, inoculation loop.

4. Procedure

4.1. Culturing of *E. coli* in liquid broth:

- Take a measured volume of nutrient broth medium in a culture tube (5 mL) or in a flask (20 mL in a 50 mL flask).
- Aseptically transfer 1% v/v of inoculum to the culture tube. Alternatively, using an inoculation loop, pick up a colony from a plate and inoculate it into the broth medium.
- Incubate the tubes overnight at 37 °C in a shaker incubator (set at 200 rpm).
- Observe the liquid broth for growth. The medium will be turbid if the organism has grown.

4.2. Streak-plate method:

- Sterilize an inoculation loop by heating it until red hot in a flame. Allow it to cool. Touch the loop onto the agar surface to check if the loop is cool enough.
- Pick up a loopful of liquid inoculum or bacterial growth from the surface of an agar plate and streak back and forth with the loop, making close parallel streaks. A number of variations of this technique are practiced such as the four-way (quadrant) streak-plate method.
- It is important to realize that in this technique we gradually thin out the number of bacteria in each successive streak/ quadrant, with the goal of obtaining isolated colonies.
- Invert the plate and incubate the plates overnight at 37 °C
- Observe the plates for isolated colonies and the quality of streaking.

4.3. Serial dilution:

- Place the bacterial culture, nutrient broth, nutrient agar plates, test tubes, measuring cylinder, micro pipette, micro-tips and glass rod in a laminar air flow chamber.
- Dispense 9 mL of normal saline/nutrient broth in each test tube
- Add 1mL of bacterial culture to test tube number 1. Mix the contents well by shaking. Transfer 1 mL from this tube to next tube.
- Repeat the process till the last dilution (refer diagram).

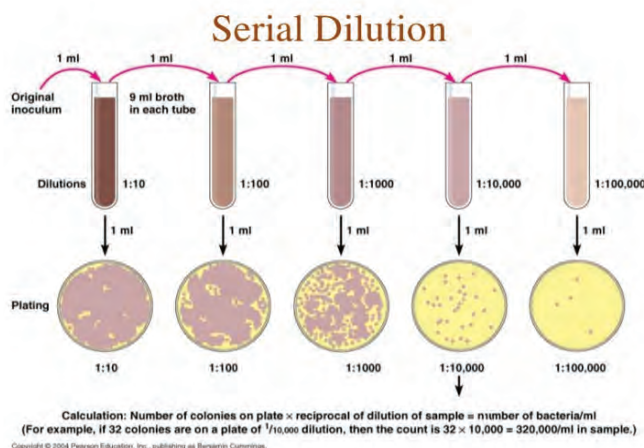


Fig. 2: Preparing serial dilutions of a bacterial culture.

4.4. Pour plate method

- i. Add 1 mL of the bacterial cultures in the centre of a Petri plate.
- ii. Pour 15-20 mL of autoclaved cooled, molten agar (at a temperature of about 45 °C) in the Petri plate. Swirl the plate gently 3-4 times clockwise and anti-clockwise.
- iii. Repeat the above steps for all dilutions to be plated.
- iv. Leave the plate undisturbed for 15-20 minutes in the laminar flow hood to allow the medium to solidify.
- v. Incubate the plates overnight in an inverted position at 37 °C.
- vi. Count the surface as well as subsurface colonies and calculate cfu/mL.

Note: Remember that the embedded colonies will be much smaller than those that grow on the surface.

4.5. Spread-plate method

- i. Pour autoclaved molten nutrient agar medium in sterile Petri plates and allow to fully solidify.
- ii. Add 100 µL of different dilutions on the surface of the petri plate.
- iii. Sterilize the spreader in alcohol (95%) and pass it through the burner flame. Alcohol will burn up. Now allow the spreader to cool. Hold the spreader briefly on the surface of agar to finish cooling.
- iv. Gently spread the sample over the agar surface using the sterilized spreader while rotating the plates by hand or using a turn table.
- v. Leave the plates undisturbed for 15-20 minutes to allow the sample to be absorbed.
- vi. Incubate the plates overnight in an inverted position at 37 °C.
- vii. Count the colonies and calculate cfu/mL.

5. Observations

Dilution	Volume of inoculum in ml*	No. of colonies	Cfu/mL of diluted culture

(*If 100 µL of inoculum is used for plating then cfu/mL is given by = no. of colonies/100 × 10³ . Using a smaller volume of inoculum would produce countable colonies at a lower dilution)

6. Result

The bacterial count of the given sample was calculated to be CFU/mL.

7. Precautions

- i. Aseptic conditions should be maintained at all steps. Disinfect the laminar flow surface before and after the experiment with alcohol. Always wash hands prior to and after the completion of the experiment and remove protective clothing after leaving the laboratory.
- ii. After pouring the medium, incubate all plates and culture tubes overnight to ensure sterility. There should be no turbidity in the broth and no colonies on the plates.

- iii. Incubate petri plates in an inverted position to prevent water droplets that are formed on the cover during solidification from dropping down on the surface of agar. This can lead to spreading of surface colonies.
- iv. Open sterile glassware and medium only inside the laminar flow cabinet, close to the flame for a minimum period of time to transfer.
- v. Do not do mouth pipetting. Use mechanical pipetting devices to transfer medium or inoculum. Auto pipettes with sterile disposable tips can also be used.
- vi. The platinum loop should not dig into the solid medium while streaking.
- vii. Keep the beaker containing alcohol away from the flame.

Suggested Reading(s)

- i. Cappuccino, J., & Sherman, N. (2010). *Microbiology: A Laboratory Manual*. (9th ed.). Pearson Education Limited.
- ii. Wiley, J. M., Sherwood, L. M., & Woolverton, C. J. (2013) *Prescott' Microbiology*. (9th ed.). McGraw Hill International.
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STAINING OF BACTERIA



1. Aim

To perform differential staining of bacteria.

2. Introduction

Differential staining is a staining process which uses more than one chemical stain. One commonly recognizable use of differential staining is the Gram stain. Bacteria can be quickly divided into two distinct morphological and functional groups on the basis of the Gram stain. By this technique, Gram positive bacteria stain purple and gram negative stain pink. The bacteria are first stained with crystal violet followed by a brief treatment with Gram's iodine. The iodine functions as a mordant to help the crystal violet bind more firmly. The bacteria are then rinsed with ethanol. Gram positive bacteria, which have multiple layers of peptidoglycan, retain the crystal violet while it is quickly rinsed out of Gram negative bacteria because their peptidoglycan is a single layer thick. The bacteria are stained a second time (counter stained) with the dye safranin which will not show up on the already purple Gram positive but will stain the decolonized Gram negative bacteria pink.

3. Materials Required

3.1. Biological Materials: Log phase (overnight grown) cultures of bacteria or bacterial colonies on solid medium.

3.2. Chemicals/Reagents: NaCl, Nutrient broth / nutrient agar plates, crystal violet, absolute alcohol, ethyl alcohol, potassium iodide, iodine, safranin.

3.3. Equipment: Incubator, analytical/electronic balance, Bunsen burner, inoculating needle, microscopes, laminar air flow.

3.4 Glassware/Plastic ware: Sterile petri plates, glass spreader, pipettes, staining tray, glass slides.

4. Procedure

4.1. Reagents and media preparation:

- i. Normal saline solution (0.9% w/v NaCl in distilled water)
- ii. Preparation of nutrient agar:

Peptone	-	5 g
Beef extract	-	3 g
NaCl	-	5 g
Agar	-	20 g

Dissolve in 1 liter of distilled water, set the pH to 7 before adding agar and autoclave it.

- iii. Crystal violet stain (2 g crystal violet dissolved in 20 mL of absolute alcohol and volume made up to 1 liter using distilled water).
- iv. Gram's iodine (dissolve 2 g potassium iodide in 25 mL distilled water, then add 1g iodine and make the volume up to 1 liter with distilled water). Alternatively this can be obtained commercially.
- v. Counter stain Safranin (dissolve 0.1g of safranin into 95% ethyl alcohol and add 400 mL distilled water).

4.2. Gram Staining:

- i. Choose a well separated colony, of bacteria, suspend it in 0.2 mL of normal saline, take a loopful from the suspension and prepare a smear by spreading it on clean grease-free glass slide or alternatively take a loopful of overnight grown culture of bacteria and prepare the smear.
- ii. Heat fixes the smear by heating gently by passing the slide over the flame of the spirit lamp few times.
- iii. Pour a few drops of crystal violet on the heat fixed smear.
- iv. Wait for 1 minute and wash with tap water.
- v. Now flood the slide with Gram's iodine for 1 minute and again wash with tap water.
- vi. Decolourize the stain with 95% ethanol for 30 seconds – 1 minute by pouring the 95% ethanol on the slide. Wash the slide with running tap water again.
- vii. Counter stain the slide with Safranin for 40 seconds.
- viii. Dry the slide and observe under oil immersion (100X) objective lens of the

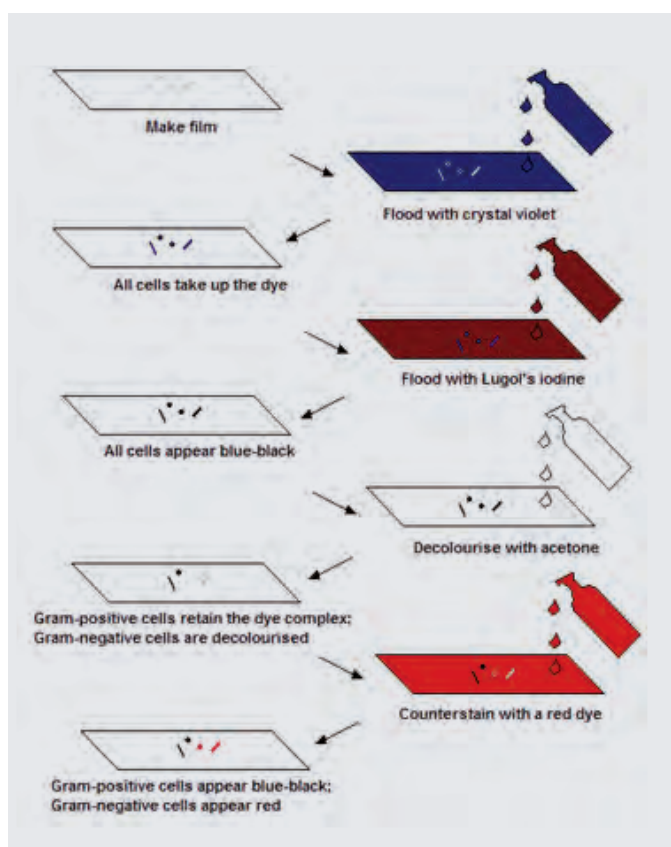


Fig. 1: Gram staining procedure

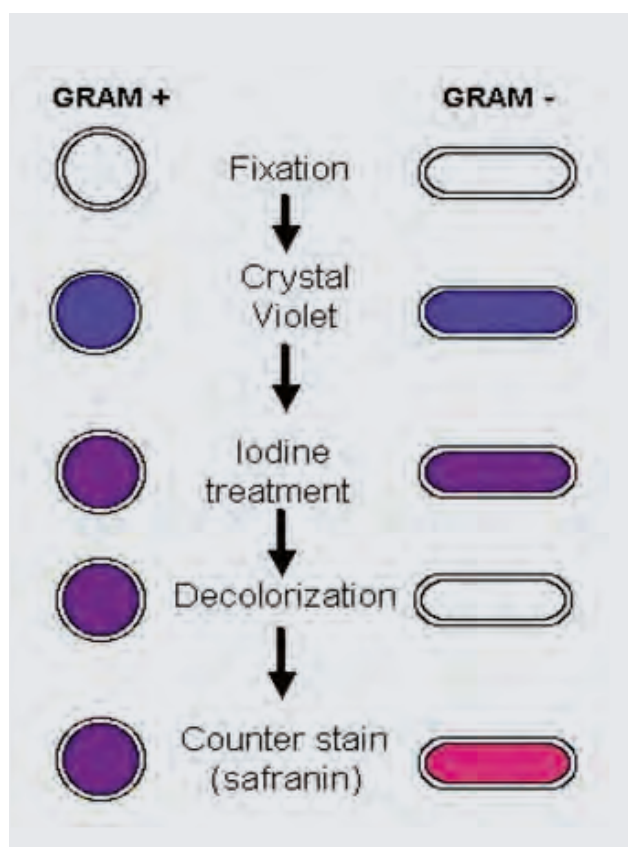


Fig. 2: Gram positive cocci and Gram Negative bacilli

6. Observation and Result

The bacteria that take up stain, appears purple or Blue-black are Gram positive bacteria while those which do not take up the stain and appear pink due to the counter stain Safranin, are Gram negative bacteria. (In case of curd sample, you will find both Gram positive bacilli and cocci under the microscope).

7. Precautions

- i. Only sterile glassware should be used.
- ii. For staining do not take too many cells.
- iii. Do not over heat the smear or else the cells will clump.
- iv. Smear should be thin and uniform.
- v. Over decolourization of the cells should be avoided.

Suggested Reading(s)

- i. Cappuccino, J., & Sherman, N. (2010). *Microbiology: A Laboratory Manual*. (9th ed.). Pearson Education Limited.
- ii. Wiley, J. M., Sherwood, L. M., & Woolverton, C. J. (2013) *Prescott' Microbiology*. (9th ed.). McGraw Hill International.
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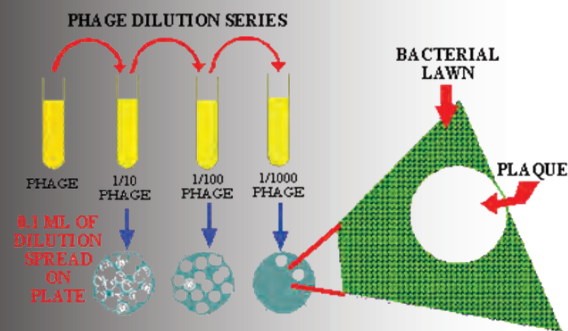
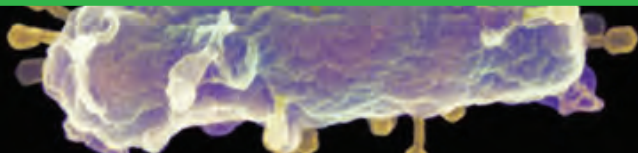
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GROWTH CURVE OF BACTERIA



1. Aim

To study various phases of the growth curve of bacteria by Turbidometric method.

2. Introduction

Bacteria are generally found out in nature as mixtures but are studied as pure culture. These are most conveniently isolated organism on solid media, because each colony can be derived from single aggregate of identical cells with those species whose cells do not separate regularly after cell division. Growth in liquid medium is used for many kinds of physiological studies and large scale cultivation. Bacteria can be quantitated in terms of either of mass for biochemical studies or of cell numbers for studies of genetics.

Bacterial population growth studies require inoculation of viable cells into a sterile broth medium and incubation of the culture under optimum temperature, pH, and gaseous conditions. Under these conditions, the cells will reproduce rapidly and the dynamics of the microbial growth can be charted by means of a population growth curve, which is constructed by plotting the increase in cell numbers versus time of incubation and can be used to delineate stages of the growth cycle. It also facilitates measurement of cell numbers and the rate of growth of a particular organism under standardized conditions as expressed by its generation time, the time required for a microbial population to double.

Cell mass can be measured in terms of dry weight or some chemical component (nitrogen, DNA etc.). The most practical index is turbidity, which can be conveniently followed in growing culture; by photoelectric measurement of decrease in transmission (increase in optical density is O.D.). This decrease is attributable mostly to light scattering by highly retractile bacteria. It measures nonliving cells also. As the O.D. is greater the lower the wavelength, but it is generally measured above 490 nm in order to avoid light absorption by the yellow products of autoclaving of the medium.

When bacteria are inoculated in to fresh media, the growth curve usually exhibits three phases, Lag, Exponential (log), and Stationary. The cessation of growth may be secondary to exhaustion of required nutrient or of the oxygen or to accumulation of an inhibitory product. In the logarithmic phase of growth the rate of increase of bacterial mass at any time is proportional to the mass present. Hence in this phase a plot of the logarithm of number of bacteria against time gives a straight line, it is convenient to express bacterial growth rate in term of doubling time or mean generation time.

3. Materials Required

3.1. Biological Materials: Actively growing culture of *E. coli*.

3.2 Chemicals/Reagents: Nutrient broth

3.3 Equipment: Shaking Incubator, bunsen burner, inoculating needle, laminar air flow, colorimeter.

3.4 Glassware/Plastic ware: Sterile petri plates, inoculation loop, pipettes.

4. Procedure

- Dispense 100 mL nutrient medium in 250ml Erlenmeyer flask (Side-arm flask) and autoclave at 121 °C for 30 minutes.
- Inoculate 5-10% of inoculums in the sterile medium with actively growing *E.coli* culture under aseptic conditions.
- Shake well the flask and note down initial O.D. of zero hour reading (O.D. 600 nm in colorimeter) and place the flask in shaker adjusted at 150 rpm at 37 °C for 24-48 hrs.
- Read O.D. of growth medium at the interval of half an hour and record in record book. Un-inoculated growth medium is treated as blank.
- Prepare growth curve by plotting graph in terms of absorbance against time (hours).

5. Observations

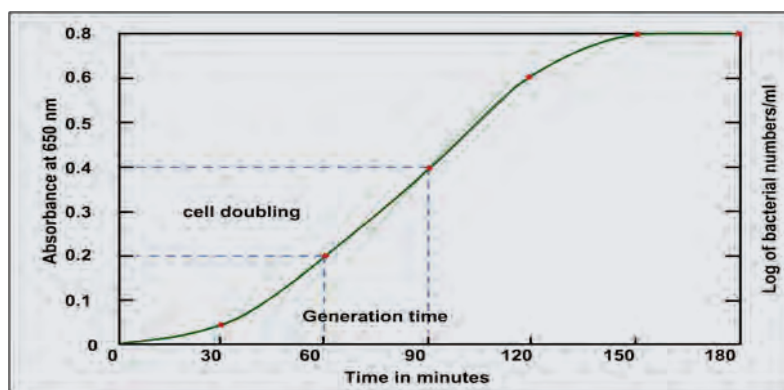


Fig. 1 : Calculation of Generation Time

Take the O.D. at regular time interval as shown in Table 1

Table1: Observation table for Growth Curve.

S. No.	Time in Minutes	Optical Density (O.D.) at 600 nm
1	00	
2	30	
3	60	
4	90	
5	120	
6	150	
7	180	
8	210	
9	240	
10	270	
Read O.D. till Absorbance becomes static		

6. Result

Lag, log and stationary phase can be observed and generation time can be calculated with growth curve.

7. Precautions

- i. Perform all steps under aseptic conditions.
- ii. There should not be contamination during removal of media.

Suggested Reading(s)

- i. Cappuccino, J., & Sherman, N. (2010). Microbiology: A Laboratory Manual. (9th ed.). Pearson Education Limited.
- ii. Atlas, R. M. (1997). Principles of Microbiology. (2nd ed.) WM.T.Brown Publishers.

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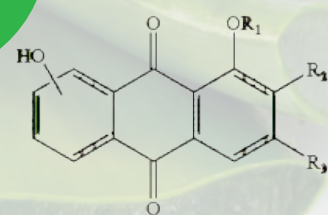
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ANTI-BACTERIAL POTENTIAL OF NATURAL PRODUCTS



1. Aim

To perform the screening of antibacterial potential of selected natural products.

2. Introduction

Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Infections caused by bacteria can be prevented, managed and treated through anti-bacterial group of compounds known as antibiotics. Antibiotics are natural, semi-synthetic or synthetic compounds that kill or inhibit the growth of bacteria. Bacteria, however, have evolved numerous defenses against anti-microbial agents, and synthetic antibiotics have their own sets of side effects. In view of this, the use of natural products like plant extracts can be of great significance. The success of effective screening of antimicrobial activity depends on the type of screening method adapted. Among the vast number of methodologies used, the most popular ones have been the disc/well diffusion method and the broth dilution method. The effectiveness of a given extract/compound is then expressed in terms of the Minimal Inhibitory Concentration (MIC) which is defined as the lowest concentration (highest dilution) of the test compound required to inhibit any visible growth on agar plates or in broths

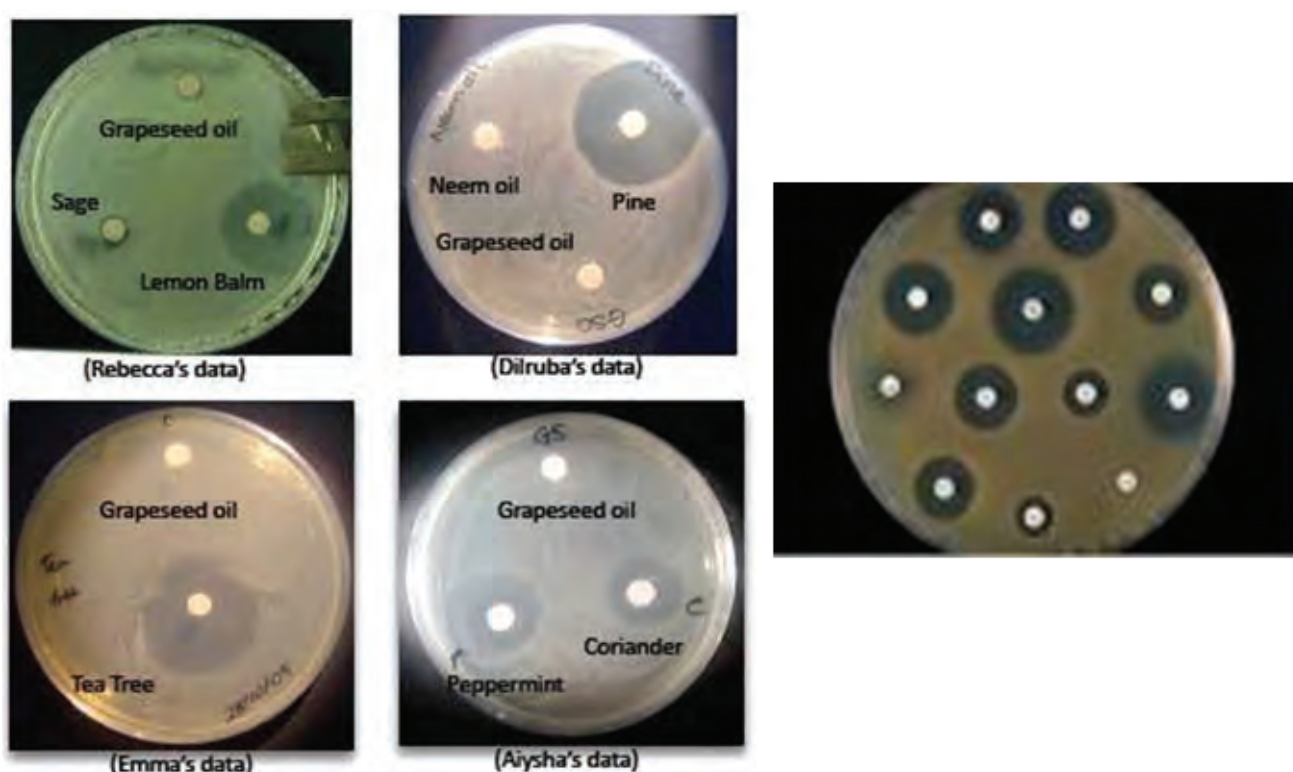


Fig. 1: Agar well diffusion assay of some essential oils by Agar Disc Diffusion assay

Plant	Antimicrobial compound
1. <i>Aloe vera</i>	Phenolic acids/polyphenols, (specially the non-flavonoid polyphenols) phytosterols, fatty acids, indoles, alkanes, pyrimidines, alkaloids, organic acids, aldehydes, dicarboxylic acids, ketones and alcohols
2. <i>Curcumin longa</i> (Turmeric)	polyphenolic compound curcumin
3. <i>Syzygium aromaticum</i> (Cloves)	Eugenol comprises 72-90% of the essential oil extracted from cloves. Other important essential oil constituents of clove oil include acetyleneugenol, beta-caryophyllene and vanillin, categolic acid, tannins such as bicornin, gallotannic acid, methyl salicylate (painkiller), the flavonoids eugenin, kaempferol, rhamnetin, and eugenitin, triterpenoids such as oleanolic acid, stigmasterol, and campesterol, and several sesquiterpenes

3. Materials Required

- 3.1. Biological Materials:** Test organisms: Overnight grown bacterial cultures of *Escherichia coli*, *Klebsiella sp.* and *Staphylococcus aureus*. Test Samples: Extracts of *Aloe vera*, *Curcuma longa*, *Syzygium aromaticum*.
- 3.2. Chemicals/Reagents:** Nutrient broth, Luria Bertani (LB) broth, agar powder, ethanol, methanol, acetone, ethyl acetate, known/available antibiotic, Tween 80.
- 3.3. Equipment:** Laminar air flow, incubator, weighing balance, pH meter, autoclave, magnetic stirrer, Bunsen burner.
- 3.4. Glassware/Plastic ware:** Erlenmeyer flasks, test tubes, spreader, inoculating loop, petri plates

4. Reagents and Media Preparation

- 4.1.** Nutrient broth (0.5% peptone, 0.3% beef extract/yeast extract, 0.5% NaCl, distilled water pH adjusted to 6.8 at 25 °C).
- 4.2.** Luria Bertani (LB) broth (1% casein hydrolysate, 0.5% yeast extract, 1% NaCl, distilled water, pH adjusted to 7.5 at 25 °C).
- 4.3.** 1.5% Agar powder was used for making solid plates of the respective media.
- 4.4.** Positive controls were prepared by using discs of standard antibiotics of known concentrations.
- 4.5.** Plant extracts preparations:

4.5.1. Preparation of *Curcumin longa* (Turmeric) extract:

Clean and cut the fresh rhizomes of *Curcumin longa* and air dry for 2 days. Dry the air dried samples again in a hot air oven at 50 °C for 24 hrs. Grind the samples into powder and pass through a sieve with nominal mesh size of 2 mm in diameter. Soak 100 g of this powder in 200ml of distilled water and keep at room temperature for 24 hours. Filter through Whatman No. 1 filter paper. Heat the filtrate at 40-50 °C in water bath, till a thick paste forms. The thick paste is considered as 100% concentration of extract. Store the extract at 4°C in refrigerator. Evaluate the antimicrobial activity using different dilutions of this extract.

4.5.2. Preparation of *Aloe vera* extract:

Wash the leaves of the plant with distilled water, cut open and collect the fresh pulp. Dry the gel in an oven at 80 °C for 48 hours and powder it. Prepare an ethanol extract by dissolving 20 grams of the powder in 200 mL of ethanol. Filter the contents using Whatmann filter paper No. 1 and evaporate the filtrate to dryness before being used as the test compound against bacterial growth.

4.5.3. Preparation of *Syzygium aromaticum* (clove) extract:

Dry *Syzygium aromaticum* in an incubator at 37 °C for 3-4 days and grind it into fine powder. Dissolve the powder in ethanol-methanol solution (70% ethanol and 80% methanol) in the ratio 2:15 w/v. Keep the mixture in the dark for 3 days at room temperature in sterilized beakers wrapped with aluminum foil to avoid evaporation and exposure to sunlight. After 3 days, filter the mixture through Whatman No.1 filter paper and keep it in incubator at 37 °C till ethanol or methanol gets completely evaporated. Dissolve the left over sample in TrisHCl buffer, pH 8.0. (volume of buffer should be one tenth of the volume of ethanol-methanol mix used initially to dissolve the clove powder)

5. Procedure

5.1. Agar well diffusion method (for *Curcumin longa* extract)

- i. 100 µL of overnight (12-16 hrs) grown bacterial cultures were spread uniformly over the surface of Nutrient agar (NA) and LB Agar plates.
- ii. Wells (10 mm in diameter and about 2 cm apart) were made in each of these plates using sterile cork borer.
- iii. Stock solution of each plant extract was prepared at a concentration of 1 mg/mL.
- iv. About 100 µL of different concentrations of plant solvent extracts were added with sterile microtips into the wells and allowed to diffuse at room temperature for 2hrs.
- v. Appropriate solvent wells were marked as negative controls, while antibiotic wells were observed marked as positive controls.
- vi. The plates will be incubated at 37 °C for 18-24 hrs and the diameter of the inhibition zone (mm) (at least three values) was measured for each sample.

5.2. Disc diffusion method (for *Aloe vera* extract):

- i. In this method, sterile circular filter papers of the same size as that of the cups impregnated with different dilutions of the test compounds were placed on the surface of the agar plates coated with the respective bacterial cultures.
- ii. Antibacterial activity was assessed through zone of inhibitions in a similar way as that of the agar well diffusion method.

5.3. Broth dilution method (using *Syzygium aromaticum* (clove) extract):

- i. The clove extract (oil) was first emulsified by adding 0.02% tween 80.
- ii. A series of test tubes containing 10 mL nutrient broth were marked with different dilutions of the test compound.

- iii. The emulsified clove oil was then serially diluted in sterile nutrient broths and 100 μL of each of the dilutions was transferred to the broths (10 mL each) in each of the test tubes to achieve the corresponding effective dilutions as marked in the test tube. 100 μL of 0.02% Tween 80 was added in a separate tube in each set to serve as the tween 80 control.
- iv. Subsequently 100 μL of the respective overnight bacterial cultures will be transferred to each test tube of a given set of dilutions (marked for a given test compound).
- v. The tubes will be incubated at 37 $^{\circ}\text{C}$ for 18-24 hrs and growth will be assessed in each tube by determining the absorbance at 600 nm.
- vi. The highest dilution corresponding to which there no visible turbidity in the medium is resulting from bacterial growth was the Minimum Inhibitory Concentration (MIC) of clove oil.

6. Observations

Table 1: Inhibition zone obtained by Agar Well/Disc diffusion method:

Diameter of Zone of Inhibition (mm)		
Plant Extract		
Bacteria	Aloevera	Curcumin longa
<i>Escherichia coli</i>		
<i>Klebsiella sp.</i>		
<i>Staphylococcus aureus</i>		

Table 2: Turbidometric analysis of growth by broth dilution method:

Bacteria	Absorbance (OD_{600})					
	Original extract	2X diluted	4X diluted	8X diluted	16X diluted	32X diluted
<i>Escherichia coli</i>	+/-	+/-	+/-	+/-	+/-	+/-
<i>Klebsiella sp.</i>	+/-	+/-	+/-	+/-	+/-	+/-
<i>Staphylococcus aureus</i>	+/-	+/-	+/-	+/-	+/-	+/-

+/- : + indicates visible turbidity (growth), - indicates clear medium (no growth)

7. Result

- i. Inhibition zone obtained by Agar Well Diffusion method: Zone diameter for the four natural extracts to be compared and to be reported.

- ii. Turbidometric analysis of growth by modified broth dilution method: The results obtained in terms of visible growth (or Optical density values) of test bacterial isolates, in tubes containing different dilutions of the original extract is to be compared with control and reported. MIC of test extract for different bacterial isolates should also be reported.

MIC of clove oil: for *E.coli*, *Klebsiella sp.*, and *Staphylococcus aureus* was found to be _____, _____ and _____, respectively.

8. Precautions:

- i. Only sterile glassware should be used.
- ii. Care should be taken during evaporation of filtrate to dryness.
- iii. Each dilution should be thoroughly shaken before removing an aliquot for subsequent dilution.

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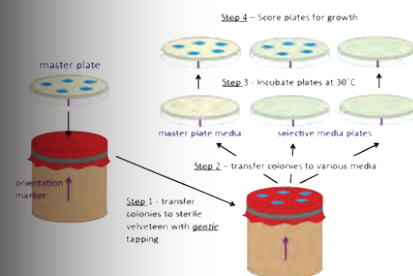
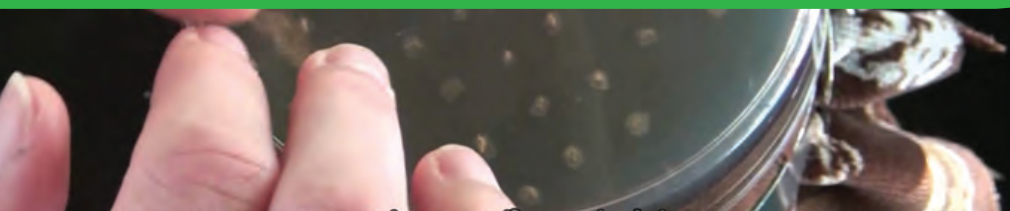
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REPLICA PLATING



1. Aim

To study the technique of replica plating for identification of *E. coli* auxotrophic mutants.

2. Introduction

Replica plating is a technique in which one or more secondary Petri plates containing different solid (agar-based) selective growth media (lacking nutrients or containing chemical growth inhibitors such as antibiotics) are inoculated with the same colonies of microorganisms from a primary plate (or master plate), reproducing the original spatial pattern of colonies. The technique involves pressing a velveteen-covered disk, and then imprinting secondary plates with cells in colonies obtained from the original plate. Generally, large numbers of colonies (roughly 30-300) are replica plated due to the difficulty in streaking each out individually onto a separate plate.

The purpose of replica plating is to be able to compare the master plate and any secondary plates, typically to screen for a desired phenotype. For example, when a colony that was present on the primary plate (or master plate), fails to appear on a secondary plate, it shows that the colony was sensitive to a substance on that particular secondary plate. Common screenable phenotypes include auxotrophic and antibiotic resistance.

Replica plating is especially useful for "negative screening". For example, if one wanted to select colonies that were sensitive to ampicillin, the primary plate could be replica plated on a secondary Amp^r agar plate. The sensitive colonies on the secondary plate would die but the colonies could still be deduced from the primary plate since the two have the same spatial patterns from ampicillin resistant colonies. The sensitive colonies could then be picked off from the primary plate. A non-selective plate should be replica plated after the Amp^r plate to confirm that the absence of growth on the selective plate is due to the selection itself and not because of a problem with transferring cells. If one sees growth on the third (non-selective) plate but not the second one, the selective agent is responsible for the lack of growth. If the non-selective plate shows no growth, one cannot say whether viable cells were transferred at all, and no conclusions can be made about the presence or absence of growth on selective media. This is particularly useful if there are questions about the age or viability of the cells on the original plate.

3. Materials Required

3.1. Biological Material: Primary cultures of *E. coli* XL-1 blue

3.2. Chemicals/Reagents: Luria Broth media (LB), LB-agar media, minimal media (Solution A and Solution B), amino acid solutions (1mg/mL) each of leucine, lysine, tryptophan, histidine; ampicillin solution (50 µg/mL).

3.3. Equipment: Laminar flow hood, spectrophotometer, shaker incubator, static incubator.

3.4. Glassware/Plastic ware: Autoclaved petri-plates, conical flasks, velvet cloth, micropipettes, toothpicks.

4. Procedure

- i. A primary culture of the *E. coli* XL-1 Blue strain was setup by inoculating 1% v/v from an overnight grown *E. coli* culture.
- ii. When the O.D of the culture became 0.6-0.7 at 600 nm, 200 μ L of the culture was taken in 8 centrifuge tubes labeled as A1 to A8 and similarly 200 μ L of culture was taken in 8 centrifuge tubes labeled as B1 to B8.
- iii. The tubes labeled A were exposed to UV radiation for 30mins and the tubes labelled B were exposed to UV for 20 mins.
- iv. Two plates with LB agar were marked A and B respectively and divided into 8 sections A1 to A8, and B1 to B8.
- v. The bacterial culture from each centrifuge tube was streaked on the corresponding section on the plate, using an autoclaved toothpick.
- vi. The plates were left overnight at 37 °C.
- vii. Two plates with LB agar were marked A and B respectively and divided into 5 \times 5 grids with cells labeled A1 to A25.
- viii. Single colonies from A1, A2 etc were picked and placed on the corresponding cell in the grid.
- ix. The same was repeated on the second plate.
- x. Incubate overnight at 37 °C.
- xi. Colonies were obtained on each cell the next day. These plates are called master plates.
- xii. 6 types of minimal media plates were prepared:
 - a. Control: Only minimal media
 - b. Leu⁺: minimal media + leucine
 - c. His⁺: minimal media + histidine
 - d. Lys⁺: minimal media + lysine
 - e. Trp⁺: minimal media + tryptophan
 - f. Amp⁺: minimal media + ampicillin
- xiii. Using an autoclaved velvet cloth wrapped on the base of a conical flask as a stamp, a replica of the master plate was picked up and stamped on all 6 minimal media plates.
- xiv. The plates were incubated overnight at 37 °C.
- xv. The plates were observed for mutants the next day.

5. Observations



Fig. 1: Creating a master plate using a modified metal stamp and a 96 well plate

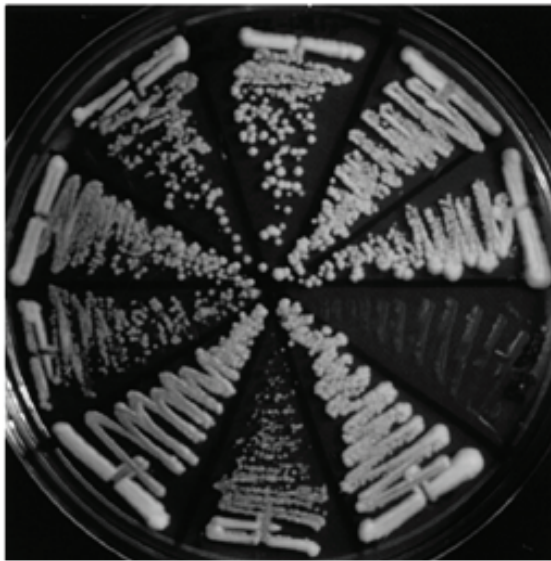


Fig. 2: Colonies grown after UV irradiation (Patch UV irradiated samples on Complete Media)

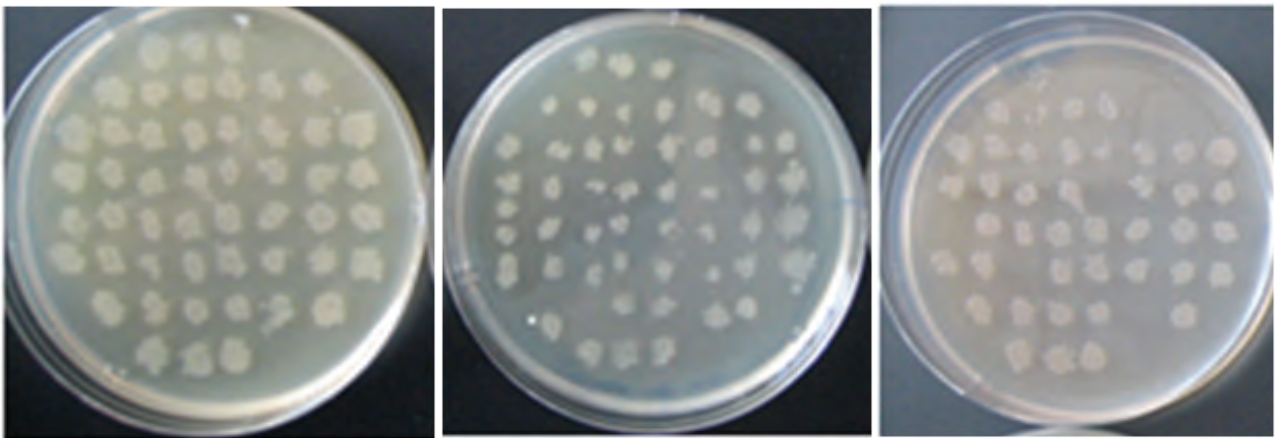


Fig. 3: A) The master plate,

B) Replica on minimal media Leu+

C) Replica on minimal media His+

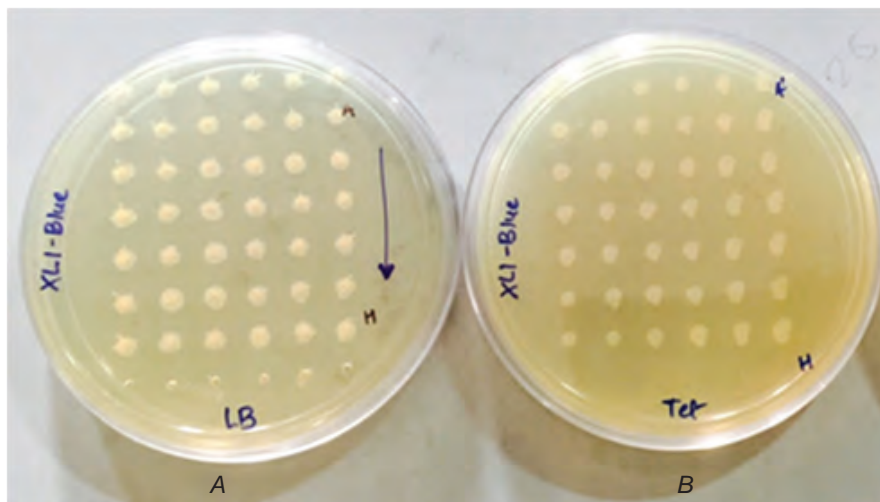


Fig. 4: Colonies of XL-1 Blue on A) Master plate, B) Replica on Tet+

6. Result

The technique of replica plating was performed.

7. Precautions

- i. Perform all steps under aseptic conditions.
- ii. The velvet side of the cloth should be pressed against the master plate as the fibers act as inoculation needle.
- iii. Care must be taken to avoid exposure to UV radiations.

Suggested Reading(s)

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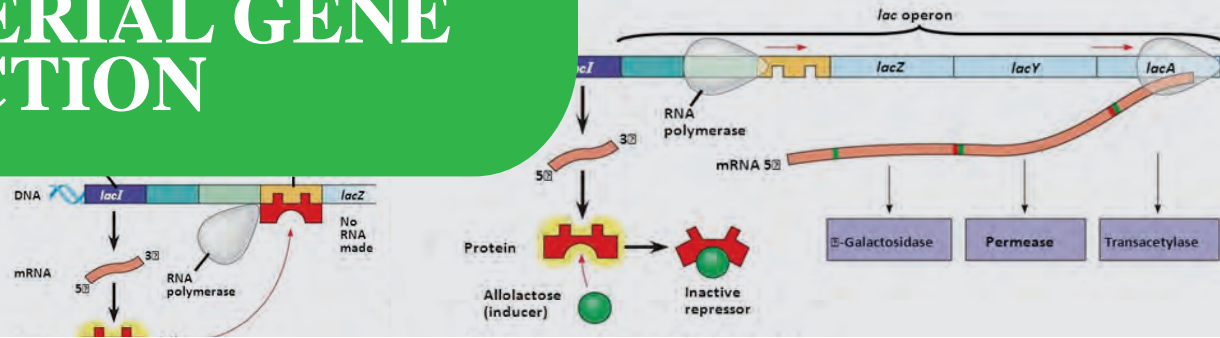
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BACTERIAL GENE INDUCTION



1. Aim

To study bacterial gene induction and comparing the expression of the inducible gene in two strains of *E. coli* (DH5 α and XL-I blue).

2. Introduction

An inducible gene is a gene that is expressed in the presence of a substance (an inducer) in the environment. This substance can control the expression of one or more genes (structural genes) involved in the metabolism of that substance. For example, lactose induces the expression of the lac genes that are involved in lactose metabolism.

Lactose Operon:

The *lactose operon* contains three structural genes that code for enzymes involved in lactose metabolism.

- 1) The *lac z* gene codes for β -galactosidase, an enzyme that breaks down lactose into glucose and galactose.
- 2) The *lac y* gene codes for a permease, which is involved in uptake of lactose.
- 3) The *lac a* gene codes for a galactose transacetylase.

These genes are transcribed from a common promoter into a polycistronic mRNA, which is translated to yield the three enzymes. The expression of structural genes is not only influenced by the presence or absence of the inducer, it is also controlled by a specific regulatory gene which encodes for a repressor protein. The repressor acts by binding to an operator element on DNA which is adjacent to the structural genes being regulated. The structural genes together with the operator region and the promoter are called an Operon.

The binding of the repressor to the operator can be prevented by the inducer, and the inducer can also remove repressor already bound to the operator. Thus, the inducer prevents the repressor from binding the operator, resulting in gene transcription. This kind of control is referred to a negative control since the function of the regulatory gene product (repressor) is to turn off transcription of the structural genes. The inducer for lac operon is lactose or a β -galactoside.

Catabolite repression (Glucose effect):

Many inducible operons are additionally controlled by glucose levels in the environment. The ability of glucose to control the expression of a number of different inducible operons is called Catabolite Repression.

Catabolite repression is generally seen in those operons which are involved in the degradation of compounds that yield energy. Since glucose is the preferred energy source in bacteria, the ability of glucose to regulate the expression of other operons ensures that bacteria will utilize glucose before any other carbon source as a source of energy. This control is edited by cAMP, which is synthesized by the enzyme adenylcyclase when glucose levels are low. In complex with the CAP or CRP, CAMP turns on the transcription of catabolite repression- sensitive operons. Thus maximum expression of the catabolite repression sensitive operon happens when glucose is not available or has been metabolized.

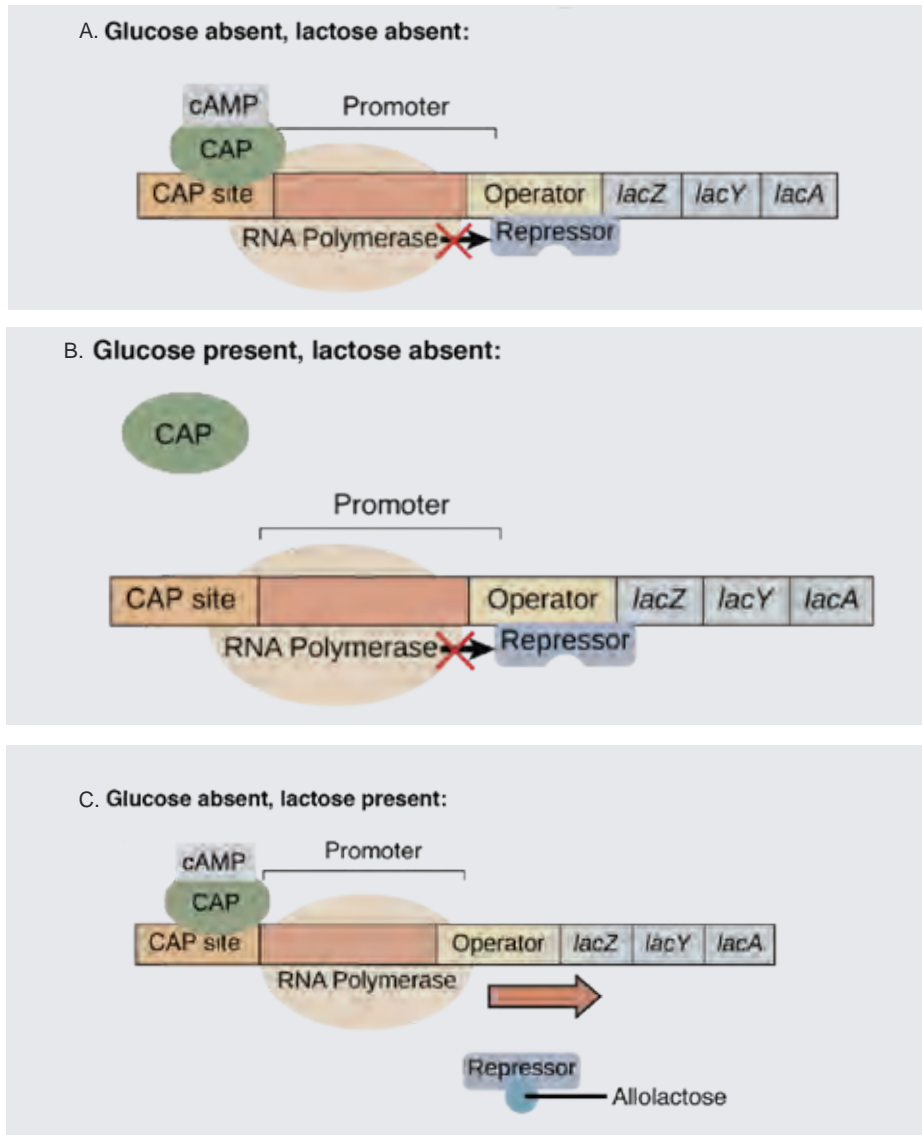


Fig. 1: A,B and C show the three possible regulatory situations of the functional Lac operon

Source: Gene regulation in bacteria: lac operon. Khanacademy.org

3. Materials Required

3.1 Biological Materials: Primary cultures of *E.coli* XL-1 blue, *E.coli* DH5 α (12-18 hrs old)

3.2 Chemicals/Reagents: LB media (1.0% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl in distilled water. The pH was adjusted to 7.0 with a NaOH solution.), Inorganic salt media Ingredients mg /L : Calcium carbonate 3000.0; Calcium chloride, 6H₂O 446.0; Potassium chloride 165.0; Monopotassium phosphate 200.0; Magnesium sulphate. heptahydrate 700.0; Sodium sulphate 200.0; Potassium iodide 0.750; Iron (III) Chloride, 6H₂O 2.50; Boric acid 1.50; Sodium molybdate, 2H₂O 0.250; Manganese sulphate, 4H₂O 6.640; Zinc sulphate, 7H₂O 2.670 ;Copper sulphate, 5H₂O 0.070.), lactose , glucose.

3.3 Equipment: Laminar hood, orbital shaker incubator, spectrophotometer.

3.4 Glassware/Plastic ware: Autoclaved/ sterilized, falcon tubes, pipettes, tips.

4. Procedure

i. Take primary cultures of *E.coli* XL-1 blue and *E.coli* DH5 α grown overnight in LB media and having an OD of 0.8 .

- ii. Add 500 μL of the primary cultures to 8.5 mL inorganic salt media. This was done in 3 falcon tubes (50 mL).
- iii. To 1st tube add 1 mL of 10% glucose stock solution, to the 2nd one, add 1 mL of lactose 10% stock solution and to the 3rd, add 1 mL of distilled water. This ensures a concentration of 1% carbon source in the experimental falcon tubes.
- iv. Incubate all three tubes at 37 °C in an orbital shaker incubator and take the readings after 90 mins, 150 mins, 210 mins; at 600 nm in the spectrophotometer.

5. Observations

After 90 mins incubation:

Media containing	OD _{600nm} (XL-1 Blue)	OD _{600nm} (DH5 α)
Water	0.00	
Glucose	0.276	
Lactose	0.153	

After 150 mins incubation:

Media containing	OD _{600nm} (XL-1 Blue)	OD _{600nm} (DH5 α)
Water	0.00	0.00
Glucose	0.526	0.170
Lactose	0.181	0.010

After 210 mins Incubation:

Media containing	OD _{600nm} (XL-1 Blue)	OD _{600nm} (DH5 α)
Water	0.00	0.00
Glucose	0.603	0.200
Lactose	0.368	0.047

6. Result

Both the cultures grew in media containing Glucose as a carbon source with *E.coli* XL-1 Blue showing a faster growth as compared to *E.coli* DH5 α . The *E.coli* XL-1 Blue culture showed an induction of the lac operon. The *E. coli* DH5 α did not show any significant growth in the presence of lactose even after 210 mins

7. Discussion

It takes time for the lac operon to be induced because glucose is always metabolized first in preference to other sugars. Lactose is not utilized prior to glucose because the cells are unable to transport and cleave the disaccharide. Only when glucose is exhausted, are the enzymes for lactose utilization synthesized when lactose becomes available, it is converted into Allolactose (inducer), hence activating the lac operon. Therefore in the E.coli XL-1 Blue culture which showed an induction of the lac operon, growth was delayed as the primary culture used for inoculation had been grown in LB media and the lac operon would have been repressed and it would take time for the lactose to remove repression and start the transcription of the Lac operon. The E. coli DH5 α has a truncated lac operon in which the lac-Z gene product (B-galactosidase) does not have the Lac Z alpha monomer and hence a functional hetero-tetramer, containing the two monomers - lacZ-alpha, and lacZ-omega is not formed, thus making the bacteria incapable of metabolizing lactose.

8. Precautions

- i. Maintain aseptic conditions throughout the experiment.
- ii. When taking absorbance, wash the cuvettes properly between different samples.

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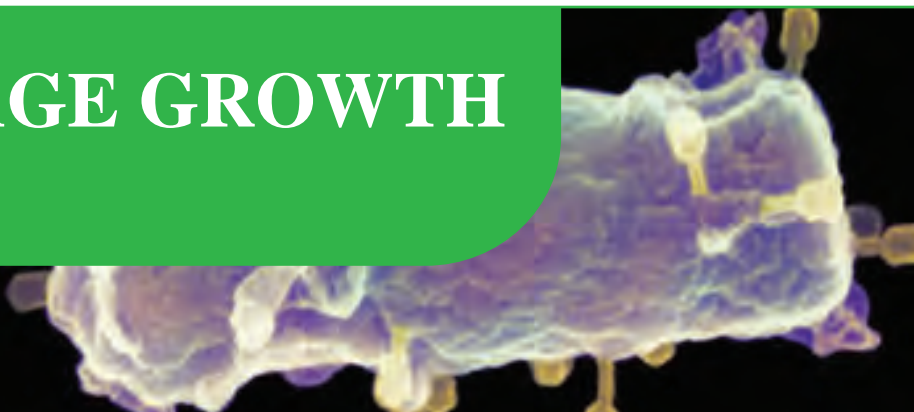
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BACTERIOPHAGE GROWTH ANALYSIS



1. Aim

To study the pattern of bacteriophage growth using plaque technique.

2. Introduction

Viruses that attack bacteria are called bacteriophages or simply phages. Phages, like other viruses, cannot exist without a suitable host. To replicate, a virus should induce its host. To synthesize components that are necessary for the assembly of new virus particles. The virus accomplishes this process by first attaching to the host (adsorption) and then injecting its nucleic acid into the cell (injection or penetration). The viral DNA can stay free in the cell and be replicated as such, or it can be incorporated into the host chromosome and be replicated simultaneously with it. Viral proteins are synthesized with the host's machinery under the direction of viral DNA and the new virus particles are assembled. These particles can find their way out of the cell or lyse the cell and be released into the medium, ready to infect new cells.

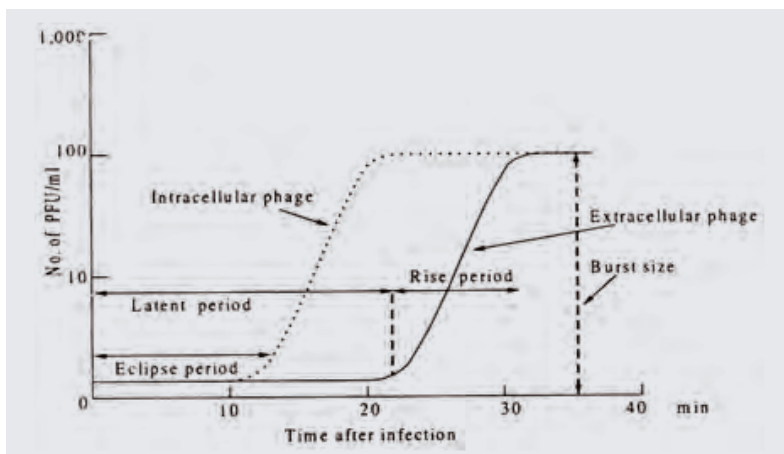
To study the growth of phages, the bacteria are mixed with a phage containing sample and incubated for a short period of time. The mixture is then diluted to drastically reduce the number of bacteria available for phage adsorption. Samples are removed at specified intervals and plated to quantitate the phage present in the culture. The average number of phage released per bacterium is called the burst size and is expressed as PFU (plaque forming units). Plaques are the clear zones formed against a lawn of bacteria because of lysis of all bacteria in a given area. If the number of phage particles was monitored during growth, the growth curve has the following phases:

Eclipse period: It is the period during which viral biosynthesis is on in the host cell. During this period, there are no mature phages in the host cell. By the end of the eclipse period, intracellular phages are present but not in the extracellular medium

Latent period: This marks the period of intracellular accumulation of the phage. It terminates with the lysis of the infected bacterium and release of phages.

Rise period: During this period, there is an increase in the number of phages in the extracellular medium because of increase in the number of bacteria getting lysed.

Once all the infected bacteria are lysed, the number of phages remains constant.



(Source: Article: Infection Cycles of Lytic, Single-Stranded and Temperate Bacteriophages)

3. Materials Required

3.1 Biological Materials: Bacterial culture

3.2 Chemicals/Reagents: Phage lysate, Thin nutrient agar plates (containing 15 mL of medium instead of the usual 25 mL), Phage broth (composition (g/l): Peptone, 100; Yeast extract, 50; NaCl, 25; K₂HPO₄, 80; pH 6.8±0.2)

3.3 Equipment: Water bath 37 °C water bath 50 °C.

3.4 Glassware/Plasticware: Phage soft agar tubes (Nutrient agar with an agar concentration of 0.8%), Sterile 1.0 mL pipettes

4. Procedure

4.1 First Session:

- i. Take bacterial culture with a count of 2×10^8 cfu (colony forming units/mL) and the phage lysate (4×10^6 pfu/mL) in the 37 °C water bath in the lab. Keep them at this temperature all the time. To the "Adsorption Tube" which already contains 0.1 mL of the phage lysate, add 0.9 mL of host culture, mix well, record the exact time and return the tubes to the 37 °C water bath.
- ii. Obtain two test tubes containing 9.9 mL of phage broth and label them 10^{-2} and 10^{-4} and place them at 37 °C.
- iii. At precisely 10 min after starting the incubation of the Adsorption Tube, remove 0.1 mL of adsorption mixture and transfer to the tube marked 10^{-2} using a sterile pipette. Mix thoroughly by vortexing.
- iv. Use another sterile pipette to transfer 0.1 mL from tube 10^{-2} to tube 10^{-4} . Mix thoroughly by vortexing and place at 37 °C.
- v. At exactly 20 minutes into the experiment, add 0.1 mL from the 10^{-4} tube and 0.1 mL of the host culture to a soft agar tube and mix thoroughly by rolling the tube in the palms of your hands.
- vi. Pour the soft agar onto a base plate labeled "20 min" and tilt the plate in all directions to evenly spread the soft agar across the surface of the entire plate. Allow the agar to solidify invert the plate, and incubate at 37 °C overnight.
- vii. Repeat steps v. and vi. at precisely 25, 30, 40, 50, 60, 70 and 80 minutes into the experiment.
- viii. After incubation, store the plates in refrigerator.

4.2 Second Session:

- i. Calculate the multiplicity ratio in the Adsorption Tube. This is done by dividing the number of phage by the number of bacteria in the Adsorption Tube at the start of the experiment. This ratio provides an estimate of the number of phage available to infect each bacterium.
- ii. Count the number of plaques per plate and complete the table in the "Results" section. Plates with a large number of plaques can be divided into 2 or 4 sections and only one section counted and then the result multiplied by 2 or 4 to get the total plaque number per plate.

5. Observations

Calculate the actual counts based for the number of plaques found on your plates multiplied by the dilution factor and the amount of inoculum used. Look at the actual counts and decide whether the very first or the average of the first few counts can be chosen as the base line (eclipse period). Divide all other counts by the base line to obtain the relative titers.

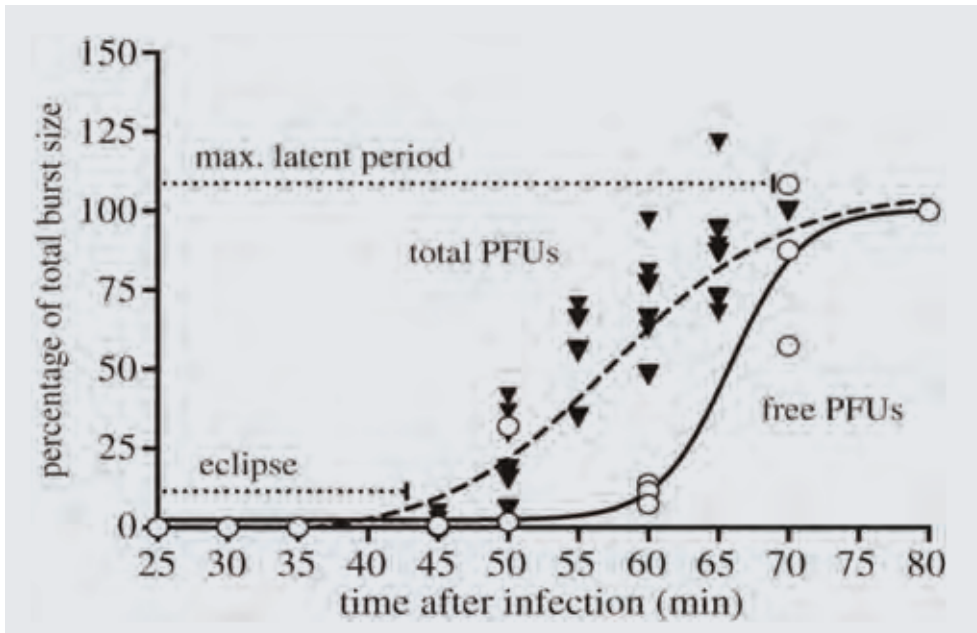


Fig. 1: Growth curve of bacteriophage showing relative titers (y-axis) over time (x-axis).

6. Result

Table 1. Relative titers over time.

Incubation time (min)	No. of plaques observed on plate	Actual counts in Adsorption Tube (pfu/mL)	Relative titers
20			
25			
30			
40			
50			
60			
70			
80			

7. Precautions

- i. Keep soft agar (molten) tubes in the 50 °C water bath. Remove only one tube at a time and use immediately to prevent solidification.
- ii. Tilt the plate gently in all the directions after addition of agar and allow to solidify completely.
- iii. Always incubate the plates in an inverted position.

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- i. Bao, H., Zhang, H., & Wang, R. (2011). *Isolation and characterization of bacteriophages of Salmonella entericserovar Pullorum Poultry Science*, 90(10):2370-2377 doi:103382/ps.2011-01496.
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GENETICS



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(Magnified image of chromosomes)
<https://www.amnh.org>

G & C BANDING OF MAMMALIAN METAPHASE CHROMOSOMES



1. Aim

To stain metaphase spread of mammalian chromosome for G and C banding.

2. Introduction

Chromosomes as seen at metaphase stage appear uniformly stained all through their length. However, they are made up of different structural compartments. These compartments or domains manifest various structural and functional attributes of chromatin. The most obvious distinct domains are Euchromatin and Heterochromatin. At interphase, Euchromatic regions of chromatin decondense while Heterochromatic regions remain condensed. In genetic terms, while Euchromatin comprises potentially active parts of genome, Heterochromatic regions are generally transcriptionally inert. In structural terms, Heterochromatin is generally enriched in highly repeated base sequences. Euchromatin, in contrast, harbors unique sequences of DNA.

The Euchromatin, however, is structurally not uniform. Through its length, different structurally as well as functionally distinct domains are encountered, and these domains are constant for any given species. These domains, though invisible in routinely stained metaphase chromosomes, can be resolved if the chromosomes are treated with certain agents like trypsin. Trypsin treated chromosome preparations elicit transverse bands on chromosomes following staining with Giemsa. They are called "G – bands". Heterochromatin region, on the other hand, can be distinctly visualized by treating chromosome preparations first with a denaturing agent (e.g., an alkali) and then with saline sodium citrate (SSC) solution followed by Giemsa staining. The darkly stained Heterochromatin regions in such preparations are called "C – bands". Besides unraveling certain aspects of chromosome structure and function, these techniques have been particularly useful in clinical cytogenetics and evolutionary studies.

G – banding studies help in identifying homologous regions in chromosomes. It is also useful in evaluating chromosomal abnormalities and understanding gene expression in cancerous cells.

C – banding is useful in paternity and in gene mapping studies. C – banding is also useful in identifying the Y chromosomes in mammalian species.

A. For G – banding of metaphase chromosomes

3. Materials Required

3.1. Biological Materials: Good metaphase chromosome spread from Rat/Mouse (Refer the protocol for preparing metaphase spread using mammalian bone marrow cells).

3.2. Chemicals/Reagents: Trypsin solution (30 mg/mL in 0.9% NaCl, stored at $-20\text{ }^{\circ}\text{C}$), 0.9% NaCl (stored at $4\text{ }^{\circ}\text{C}$), Giemsa stain,

Phosphate buffer (stored at $4\text{ }^{\circ}\text{C}$)

Solution 1: 9.073 g KH_2PO_4 in 1000 mL distilled water

Solution 2: 11.87 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in 1000 mL distilled water

Mix equal parts of solution 1 and solution 2 .

3.3. Equipment: Microscope with camera attachment

3.4. Miscellaneous: Distilled water (deionized), Coplin jars

4. Procedure for G – banding

- i. Thaw 1 mL of trypsin stock solution and dilute up to 50 mL with 0.9% NaCl in a Coplin jar.
- ii. Adjust the pH of the solution with 1 M NaOH to 7.5-7.8 using pH paper (1-2 drops of 1 M NaOH may be required).
- iii. Pour 50 mL phosphate buffer to another Coplin jar.
- iv. Pour 7-10% Giemsa stain in phosphate buffer in another Coplin jar.
- v. Pour distilled water (deionized) in another Coplin jar.
- vi. Take 3 – 4 slides with good spread of metaphase chromosomes.
- vii. Label the slides with a diamond tipped glass marker on the smear surface.
- viii. Dip one slide in trypsin for 5 seconds.
- ix. Rinse the slide (gently dip in and pull out the slide, 4 – 5 times) in phosphate buffer immediately after trypsin exposure.
- x. Tap the narrow edge of the slide on filter paper to remove excess buffer.
- xi. Place the slide in Giemsa Stain for 3 – 5 minutes in Giemsa.
- xii. Rinse in water, and monitor under the microscope.
 - a. If stain is less, put again in Giemsa; if treatment is less, put the slide back again in trypsin for a few seconds and repeat the staining steps until satisfactory bands emerge.
 - b. If the slide gets overtreated (i.e. chromosome will look hollow and chewed up), take a new slide and repeat the process giving shorter trypsin treatment.
- xiii. Air dry the slide, mount in DPX and observe under the microscope.

B. C – banding of metaphase chromosomes

3. Materials Required

3.1. Biological Materials: Good Metaphase Chromosome Spread from Rat/Mouse (Refer the protocol for preparing metaphase spread using mammalian bone marrow cells)

3.2. Chemicals/Reagents: 0.2 N HCl (1 mL Conc. HCl in 54 mL distilled water), Giemsa stain

- a. 5% Barium hydroxide
 - i. Boil 100 mL of distilled water in a conical flask
 - ii. Add 5 g Ba(OH)₂ while the water is steaming.
 - iii. Stir vigorously to get as much Ba(OH)₂ in solution as possible.
 - iv. Filter into a Coplin jar and maintain the solution at 50 °C in a water bath
- b. Saline Sodium Citrate (SSC) Solution : (6X)
 - i. 52.6 g NaCl
 - ii. 26.5 g Sodium Citrate
 - iii. Dissolve in 800 mL deionized water in a 1 L standard flask
 - iv. Adjust pH to 7.0 with 10 M NaOH
 - v. Dilute to 1000 mL mark with more deionized water
- c. 2X SSC (pH 7.2)
 - i. 333 mL SSC Solution (6X)
 - ii. 667 mL Deionized water
 - iii. Mix the two solutions well
 - iv. Giemsa stain

3.3. Glassware/Plastic ware: DPX mountant, 500 mL conical flask, Coplin jars

3.4. Equipment: Microscope with camera attachment, Water bath set at 50 °C (for Ba(OH)₂ solution), Water bath set at 60 °C (for 2X SSC).

4. Procedure for C – banding

- i. Take 3 – 4 slides with good spread of metaphase chromosomes. Keep them aside for a week before further use.
- ii. Label the slides with a diamond tipped glass marker on the smear surface.
- iii. Pour 0.2 N HCl in a Coplin jar.
- iv. Pour distilled water in six different Coplin jars and mark them 1 to 6 serially.
- v. Pour Ba(OH)₂ in a Coplin jar and keep the Coplin jar in water bath at 50 °C for 30 minutes.
- vi. Pour 2X SSC in a Coplin jar and keep the Coplin jar in water bath at 60 °C for 30 minutes.
- vii. Pour 7-10% Giemsa stain in phosphate buffer in another Coplin jar.
- viii. Dip the slides in 0.2 N HCl in a Coplin jar for 30 minutes.
- ix. Transfer slides into Coplin jar No. 1 of distilled water for 5 minutes.
- x. Transfer slides into Coplin jar No. 2 of distilled water for 5 minutes.
- xi. Air dry the slides by keeping them in a dry Coplin jar.
- xii. Transfer the air-dried slides to Ba(OH)₂ in water bath at 50 °C; treat different slides for varying time intervals ranging from 1 min to 5 min (remove the precipitate formed in the solution, before placing slides in the Ba(OH)₂ solution,).
- xiii. Transfer slides into Coplin jar No. 3 of distilled water for 5 minutes.
- xiv. Transfer slides into Coplin jar No. 4 of distilled water for 5 minutes.
- xv. Air dry the slides by keeping them in a dry Coplin jar.

- xvi. Transfer the air-dried slides in 2X SSC in a waterbath maintained at 60 °C for 120 minutes.
- xvii. Transfer slides into Coplin jar No. 5 of distilled water for 5 minutes.
- xviii. Transfer slides into Coplin jar No. 6 of distilled water for 5 minutes.
- xix. Air dry the slides by keeping them in a dry Coplin jar.
- xx. Transfer the slides to Giemsa and stain for 15-20 minutes.
- xxi. After 10 minutes in Giemsa, intermittently take the slide out, tap the narrow end of the slide on a filter paper and check for proper staining under a microscope (Take care not to dry the slide).
- xxii. After appropriate staining, air dry the slide and mount with DPX.

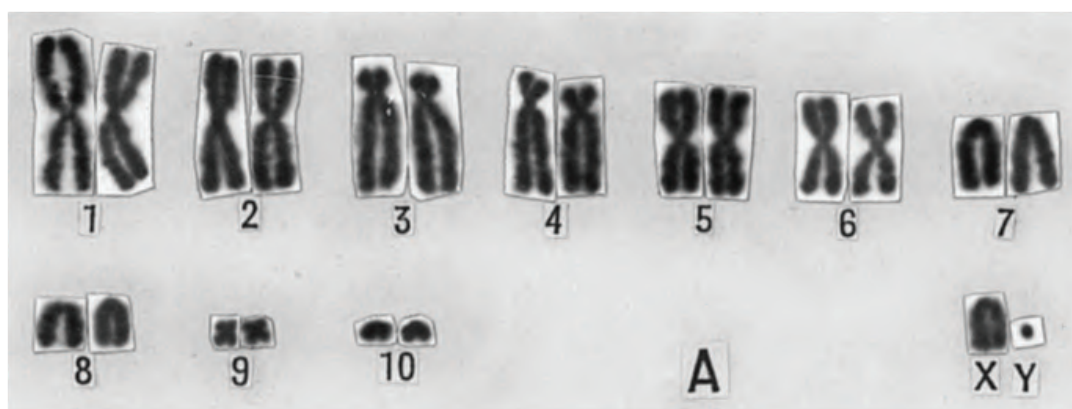
5. Recording Banding Pattern

- i. Focus the area of the chromosome spread under low power and then under high power
- ii. Locate chromosomes which are properly banded.
- iii. Focus them under the 1000X of the microscope.
- iv. Using an image capture software obtain a microphotograph of the banded chromosomes.
- v. Print the microphotograph and obtain cut outs of each individual chromosome.
- vi. Arrange the chromosomes, in descending order of their sizes and stick them on a white paper.
- vii. Count the number of bands on each chromosome.
- viii. Record the number of bands seen on each chromosome in a table.

6. Observation

After G-banding, the long chromosomes show clearly distinct bands while in the short ones bands are not always visible. The homologous chromosomes can be paired easily based on banding patterns. In the male rat, G – banding patterns have been recorded between 189 to 282 in 20 autosomes and two sex chromosomes.

C-bands are present in the centromeric regions of chromosomes and represent constitutive heterochromatin. This heterochromatin is highly condensed A-T or G-C rich, with multiple copies of DNA, with no known genes.



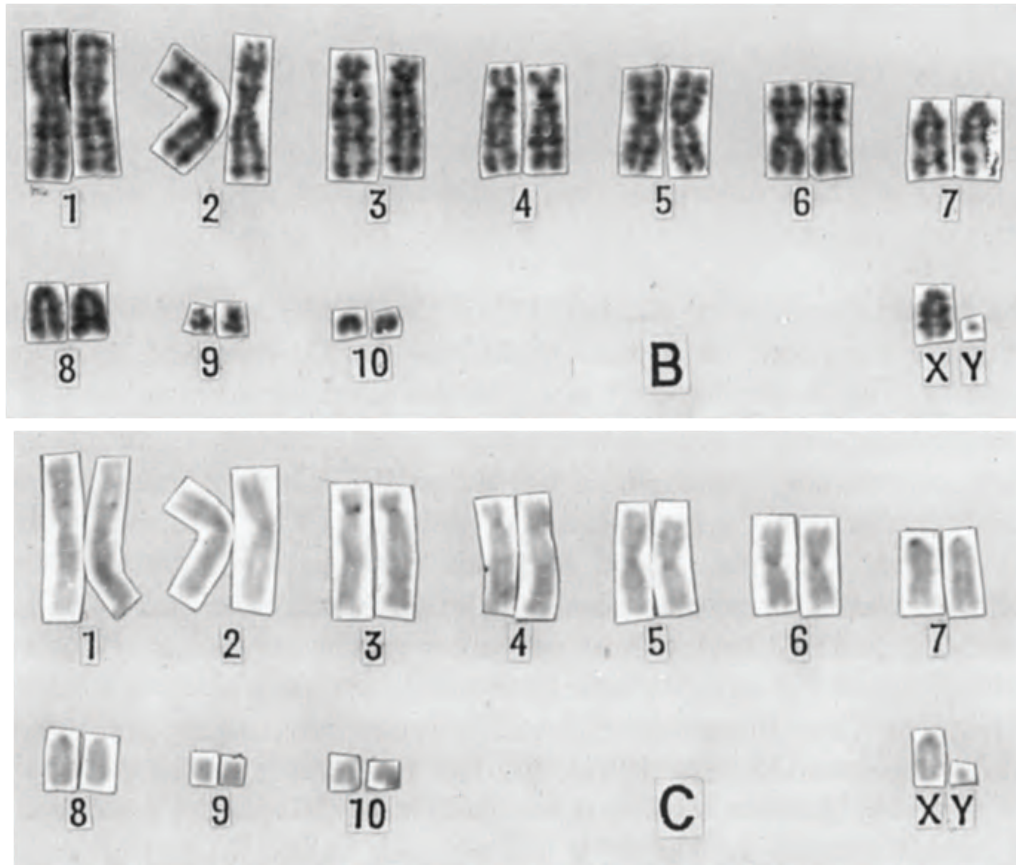


Fig. 1: Images of A). normal karyogram, B). after G – banding, and C). after C – banding in a rat species

(Source: Ping et al, Caryologia, Vol. 48, 1995.)

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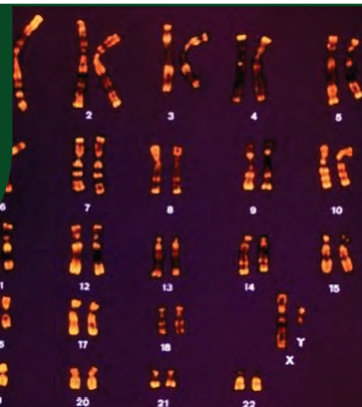
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KARYOTYPE ANALYSIS OF CELLS FROM MAMMALIAN BONE MARROW



1. Aim

To prepare metaphase spread of chromosomes from cells of bone marrow and prepare a karyogram

2. Introduction

The marrow from long bones, like the limb bones, provides convenient material for analysis of karyotype of small animals. In animals like snakes, which do not have limbs, ribs can provide the marrow. Bone marrow also provides useful material in diagnosis of leukemia. The technique of air-dried chromosome preparation can also be used for other soft tissues, viz., spleen, liver, kidney and gills. This is useful in cases where marrow may be difficult to obtain e.g., fish, new born mammals etc. Most importantly, bone marrow cells can be incubated *in vitro* for at least one cell cycle without any exogenous mitogenic stimulation. Thus, they may be used for certain *in vitro* experiments (mutagenesis, cell metabolism, cell cycle) as very accurate representative of *in vitro* conditions. Such preparations can be made even in field, where power supply is not available. A hand centrifuge and a burner can substitute for incubator and power-driven centrifuge.

3. Materials Required

3.1. Biological Materials: Rat or mouse

3.2. Chemicals/Reagents: Colchicine, hypotonic Solution: 0.56% KCl (should be kept pre-warmed to 37 °C before use), fixative: (aceto-methanol: 1:3, mix 25 mL glacial acetic acid with 75 mL methanol), Giemsa stain

3.3. Glassware/Plastic ware: Centrifuge tubes (15 mL graduated), brown cellophane tape, sterile disposable syringe (5 mL) and needles no. 22 and no. 18

3.4. Equipment: Incubator (37-38 °C), instruments for dissection, agitator (Cyclo-Mixer), clinical centrifuge, pasteur pipettes (with fire drawn long narrow tips)

4. Procedure

4.1. Harvesting bone marrow cells:

- i. Inject colchicine (0.2 mg/kg body weight) to the animal 2-3 hrs prior to sacrifice.
- ii. Sacrifice by cervical dislocation and dissect out femur bone (any long bone will do)
- iii. Cut both ends of the bone. Take about 0.5 mL of prewarmed hypotonic solution in a 5 mL syringe (with no. 18 needle for rat and no. 22 for mouse) and by inserting through one of the cut ends of the bone flush the marrow into a centrifuge tube.
- iv. Agitate the marrow with a rubber agitator (Cyclo mixture at low speed) so that it breaks into a uniform cell suspension. Alternatively, use a long capillary tipped pasture pipette and aspirate up and down, the cell pellet, to make a suspension.

- v. Make up the volume with the pre-warmed hypotonic solution to 10 mL and keep in incubator (37 °C).
- vi. Centrifuge the cell suspension (1,000 rpm) for 5 min to obtain a cell pellet at the bottom of the tube.
- vii. Discard the supernatant.
- viii. Fix the cells by adding the fixative drop-by-drop (upto about 0.5 mL) and agitating the tube to make a suspension.
- ix. Make up the volume to 10 mL with the fixative.
- x. Keep aside for 15 min.
- xi. Agitate the cells and centrifuge at 1000 rpm for 5 min.
- xii. Obtain a cell pellet at the bottom of the tube and discard the supernatant.
- xiii. Add a small volume of fixative (0.3 - 0.5 mL) and re-suspend the pellet.
- xiv. Place a clean glass slide in a petridish, slating at an angle, with one end resting on the side of the petridish.
- xv. Use a Pasteur pipette and drop 2-3 drops of the cell suspension one drop at a time, from about a foot height, at the centre of the slide such that each drop spreads after falling on the slide.
- xvi. Heat the slide over a gentle flame, by moving the slide in and out of the flame (spirit lamp/match stick flame) till the cell suspension almost dries. (Do not overheat the slide).
- xvii. Keep the slide aside in a petridish to air dry.
- xviii. To begin with, prepare only one slide and observe under the microscope to judge the density of cell suspension (an unstained slide can be examined for this purpose). If there are too many cells on the slide, dilute the suspension by adding more fixative. If the cells are too few, re-spin the tube and suspend the cells in a smaller volume of fixative.

4.2. Staining with Giemsa Stain:

- i. After proper drying, stain the slide with Giemsa with following steps;
 - a. With a pipette add three to four drops of Giemsa stain (undiluted) such that the stain fully covers the smear area. Do not allow the stain to flow out of the slide.
 - b. Immediately, cover the petri dish and stain for 3 – 4 minutes.
 - c. With a dropper or pipette, gently add few drops of deionized water on the stain, such that the diluted stain spreads till all edges of the slide but does not flow out from the slide.
 - d. Notice a green shiny scum formed over the surface of the diluted stain.
 - e. Gently cover the petri dish and keep aside undisturbed for 15 minutes (differentiation).
 - f. Open the petri dish and lift the slide by one of its narrow ends and drain the diluted stain into the petri dish.
 - g. Place the slide, for two minutes, in a Coplin jar into which a gentle stream of running water is directed. Do not allow the stream of water to fall directly on the smear.
- ii. Place the slide on strip of filter paper such that the smear surface is facing upwards.
- iii. When completely dried, mount with DPX mountant, using a large cover glass.
- iv. Observe the slide first under the low power of the microscope (10X) and then under the high power (40X).
- v. Place a drop of immersion oil and focus the slide under the Oil Immersion Lens.

4.3. Preparation of Karyogram:

- i. Set up a camera lucida and trace the chromosomes on to a graph paper.
- ii. Trace each chromosome separately on the graph paper. Note the magnification.

- iii. Take a photocopy of the same (with enlargement) and cut out each individual chromosome.
- iv. Arrange the chromosomes, in descending order of their sizes and stick them on a white paper.

Alternatively,

- i. Focus the area of the chromosome spread under 1000X of the microscope.
- ii. Using an image capture software obtain a microphotograph of the chromosome spread.
- iii. Print the microphotograph and obtain cut outs of each individual chromosome.
- iv. Arrange the chromosomes, in descending order of their sizes and stick them on a white paper.

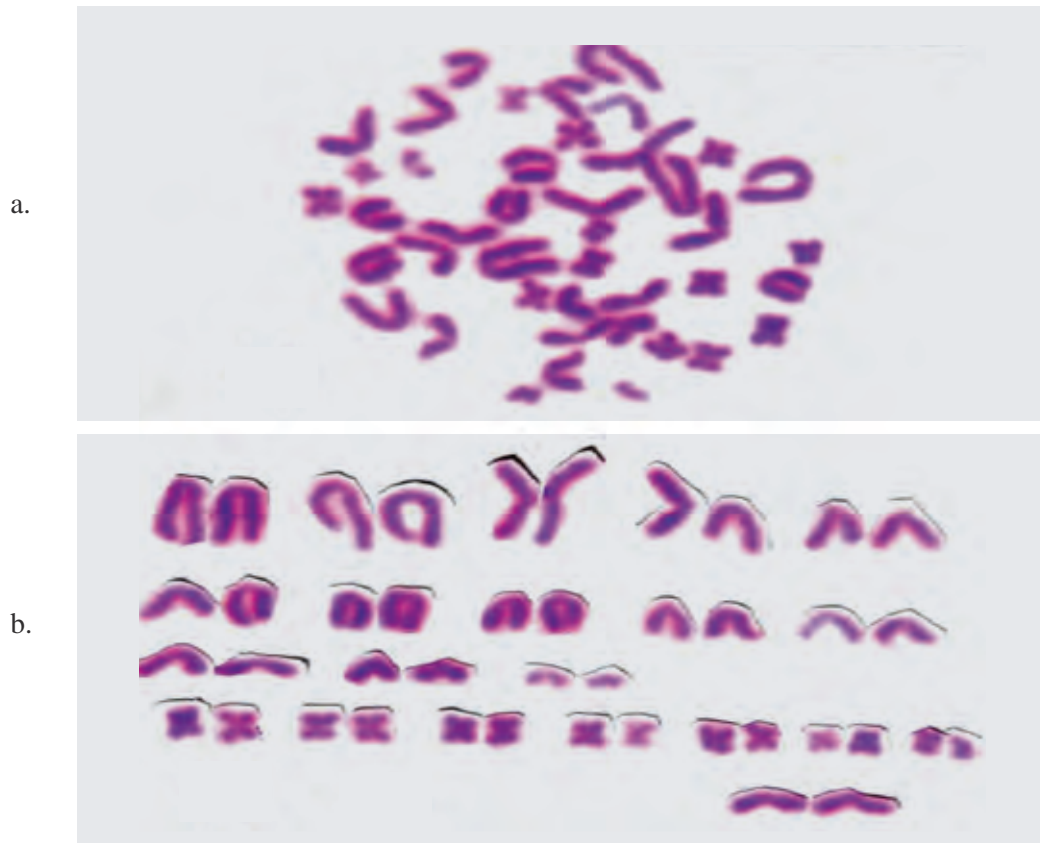


Fig. 1: (a) Metaphase spread and, (b) Karyogram of *Rattus norvegicus*

(Source: Chingangbamet et al., African Journal of Biotechnology, Vol.13(53), 2014)

5. Observations

The rat species (*Rattus rattus*) has diploid number, $2n = 42$. There 14 metacentric and 26 acrocentric chromosomes and two sex chromosomes. The X chromosome is 11th of autosomal chromosome in length and Y chromosome is the shortest in the male genome

6. Extension Activity

- i. Use a good chromosome spread and carry out G-C banding of the chromosomes.

Suggested Reading(s)

- i. Goran, L. (1974). Nomenclature for G-bands in rat chromosomes. *Hereditas*, Vol (77: 37-52).
- ii. Toshihide, H. Y. (1979). Genetic aspects of polymorphic C-bands in the chromosomes of the Black Rat (*Rattusrattus tanezumi*) in Japan. *Cytologia*, vol (44: 265-274), .

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SEX-LINKED INHERITANCE IN *DROSOPHILA MELANOGASTER*



1. Aim

To demonstrate sex-linked inheritance of eye-colour in *Drosophila melanogaster*.

2. Introduction

If a trait is located on “X” chromosome, the trait would get expressed in alternate sex from one generation to the other. The inheritance pattern depends on which parent carries the gene on the X chromosome. The trait or character can be transmitted from father to daughter because X- chromosome of father is always received by daughter. The trait can be transmitted from mother to son or mother to daughter because X- chromosome of mother is distributed between both daughter and son. This is called Cris-Cross inheritance. The recessive trait normally gets expressed in male only since the female carries a double dose of X chromosome, and therefore she remains a carrier.

3. Materials Required

3.1. Biological Materials: *Drosophila melanogaster* cultures (including wild and eye colour mutants).

3.2. Chemicals/Reagents: Ether.

3.3. Equipment: Binocular microscope.

3.3. Miscellaneous: Food culture bottle, fly etherizer, soft hair brush, glassware etc.

4. Procedure

4.1. Collecting Virgin Female flies:

- Remove all flies from the culture vessel a day before the pupae become ready to hatch (Flies normally emerge on 11th or 12th day after eggs are laid).
- Check the surface of food for any remaining flies.
- Collect newly emerged adult virgin flies early in the morning, 7:00 am – 8:00 am (flies emerge in early morning after sunrise and mate within 8 hours).
- Anesthetize the flies and drop them on a filter paper.
- Identify the male flies (darker abdomen) and female flies (lighter abdomen) and isolate the female flies. (Virgin flies are with soft bodies and should be handled with soft hair brush)
- Identify and separate virgin female flies as follows;
 - Virgin female flies have much larger abdomens.
 - Virgin females have a dot on the side of their abdomen.
 - Females with folded wings and wet must have just emerged and will be virgin.

Generation	Date of Mating	No. of Males	No. of Females	No. of Flies (Parents for mating)			
				Males		Females	
				Red Eye	White Eye	Red Eye	White Eye
F1		10	10	00	10	10	00
Results after selfing of F1 flies							
F2	Date of separating Adult F1 flies	Date of hatching for 1 st F2 Batch	Date of hatching for 2 nd F2 Batch	No. of Flies (F2 generation)			
				Males		Females	
				Red Eye	White Eye	Red Eye	White Eye

6. Results

F1 Generation	Red eyed female	White eyed female	Red eyed male	White eyed male
No. of Flies				
Ratio				
F2 Generation	Red eyed female	White eyed female	Red eyed male	White eyed male
No. of Flies				
Ratio				

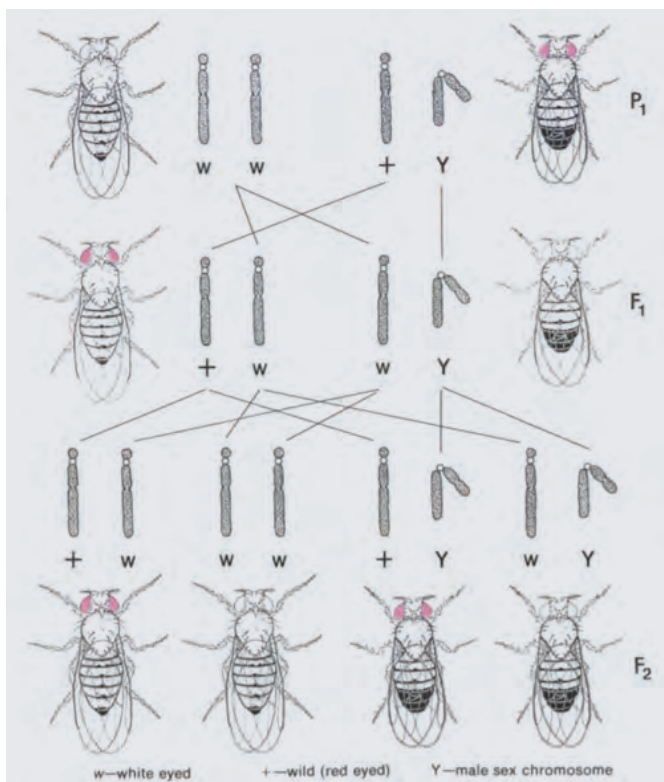


Fig. 1: Sex- linked cross

(Source: Bioreview Sheet 42: 2620, *Drosophila* Genetics, 1. Sex linkage)

6.1. Interpretation:

The results of the experiment can be explained by the presence of white eyed females in F₂ generation but none in F₁ generation. In this F₁ generation, females are heterozygous to red eye while the males are with mutant gene (white eye) on the single X chromosome they carry.

When the cross is made between the F₁ males with F₁ females, then, half of the female flies and half of the male flies are white eyed.

In F₁, the white eye trait appearing in the male are transmitted through their white eyed mother. In F₂, white eyed female passes this trait to their daughters and so white eyed females appear in F₂ generation (Criss–Cross Inheritance) as depicted diagrammatically below.

Suggested Reading(s)

- i. Carolina Drosophila Manual (1988), Carolina biological supply company, USA.,
- ii. Barbara, H. J. (2011). Drosophila – a versatile model in biology & medicine. *Materials Today, Volume 14*(5).
- iii. Genetics on the Fly: A primer on the Drosophila model system by Karen G. H. *et.al*, (2015)

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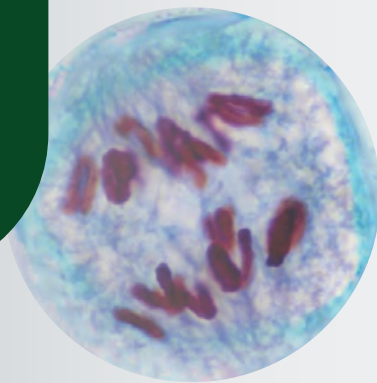
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STUDY OF POLYPLOIDY IN ONION ROOT TIPS



1. Aim

To study polyploidy in onion root tips after treatment with colchicine.

2. Introduction

Colchicine is a poisonous alkaloid that occurs in the liliaceae plant, *Colchicum autumnale* native to Europe. Seeds of this lily are the chief source of colchicine. Colchicine is known to suppress the assembly of the mitotic spindle and thereby inhibit cell division. It is a spindle poison causing depolymerization of mitotic spindles into tubulin subunits. This dissolves the spindle and inhibits the cell from completing its mitotic spindle. The failure of mitotic spindle assembly, results in the chromosomes lagging behind at the center during the metaphase stage. The chromosomes are most condensed at metaphase phase and thus chromosome morphology can be best studied in colchicine treated onion root tips.

Because colchicine can stop plant cells from dividing after the chromatids have separated during anaphase of mitosis, it is a powerful inducer of polyploidy. Seeds and meristematic buds can be treated with colchicine, and the cells inside become polyploid with multiple sets of chromosomes (more than the diploid number). Polyploidy in plants has commercial applications because odd polyploids (such as $3n$ triploids) are sterile and seedless. Polyploid plants (such as $4n$ tetraploids) typically produce larger flowers and fruits. In fact, many of the fruits and vegetables sold at supermarkets are polyploid varieties.

Colchicine has another medical use for human because it reduces the inflammation and pain of gout. It is also used in cancer chemotherapy to stop tumor cells from dividing, thus causing remission of the cancer.

3. Materials Required

3.1. Biological Materials: Onion/garlic/shallot root tips treated with 0.05% colchicine in water.

3.2. Chemicals/Reagents: Carnoy's fixative (acetic acid : ethanol; 1:3), 70% ethanol, 2% aceto-carmin stain, 45% acetic acid.

3.3. Equipment: Microscope

3.4. Glassware/Plastic ware: Coplin jar, test tube, slides and cover glass.

3.5. Miscellaneous: Cavity blocks, forceps, scissor and blade.

4. Procedure

- i. Take medium sized onions/shallots/pods of garlic.
- ii. Remove the dried outer covering and dried roots.

- iii. Place the onions / shallots / pods of garlic over a Copling Jar / test tube such that the base of the bulb is dipped in water.

NOTE: Pierce toothpicks through three sides of shallots and garlic pods to support them, while dipping the bulbs in water.

- iv. Keep the bulbs undisturbed till fresh roots sprout from the base of the bulb.
- v. Transfer the bulbs to Copling Jars/test tubes containing 0.05% Colchicine after the roots are few centimeters long.
- vi. Allow the roots to grow in 0.05% Colchicine for 24 hours.
- vii. Take out the bulbs the next day and cut 1 – 2 cm long the root tips.
- viii. Drop the root tips into a cavity block with Carnoy's fixative and fix them for 2 hours.
- ix. Preserve the root tips in 70% Ethanol till further use.
- x. Remove the tips from alcohol and stain in 1% Aceto-Carmine for 1hr.
- xi. Tease with needles on the slide and prepare a squash of the root tips.
- xii. Observe under the microscope.

5. Observations

- i. Highly condensed chromosomes are randomly dispersed in the cells treated with colchicine.
- ii. Polyploidy can also be observed in the bulbs after 24 hours treatment with colchicine.

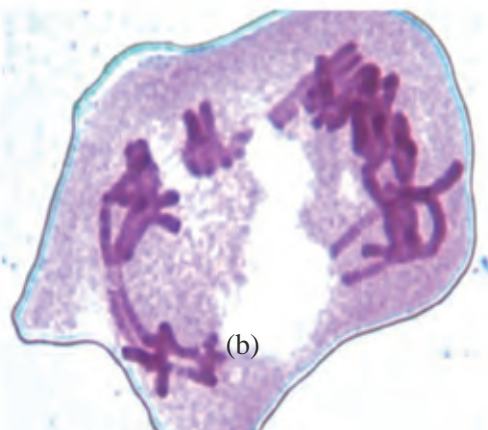
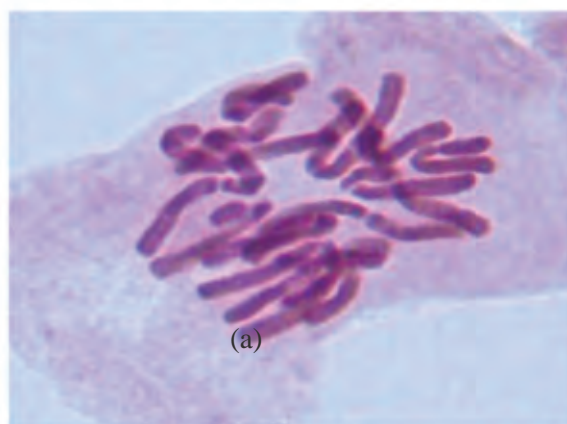


Fig. 1: *Allium sativum*, (a) Diploid; $n = 16$ (left), and

(b) Tetraploid; $n=32$ (right)

(Source: Cheng et al.; Genetics and Molecular Research, July, 2012)

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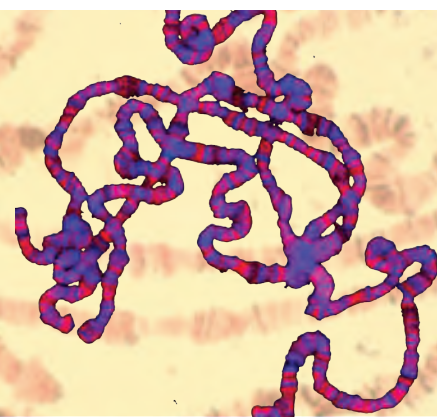
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SQUASH POLYTENE CHROMOSOME PREPARATION



1. Aim

To prepare squash of polytene chromosomes from *Drosophila* larvae.

2. Introduction

Polytene chromosomes are well known for their use in a variety of genetic, cytogenetic and molecular studies. These chromosomes remain in a permanent state of interphase but due to repeated cycles of endoreduplication and tight lateral association of all the chromatids, each chromosome becomes thicker and distinctly visible as a cable-like structure with alternating dark and light regions called the bands and inter-bands, respectively. Polytene chromosomes are most commonly found in dipteran insects, and they are also seen in certain other insects, macronucleus of some ciliates and in certain plant tissues.

Polytene chromosomes or giant chromosomes found in salivary glands of some dipteran larvae like *Chironomus* larvae (of midge fly) and larvae of *Drosophila melanogaster*. These chromosomes are generated by successive replications of a homologous chromosome set without separation of the replicated chromosomes. These chromosomes are exceptionally large and gene expression can be visualized under the microscope.

Polytene chromosomes are seen in the salivary glands which are made up of somatic cells. Polytene chromosomes are formed when DNA replication takes place multiple times (9-10 times) without the daughter chromatids undergoing separation. Thus, about 1024 homologous chromatids lie side by side, attached together without the cell division (endomitosis). The nuclear membrane and nucleolus remain intact throughout the replication of the chromosomes. This results in the formation of giant/polytene chromosome (poly –many, tene- threads). Giant chromosomes were first observed in salivary glands of dipteran flies by Balbiani in 1881.

Giant chromosomes have developmental significance since, these help in gene amplification. More genetic information is made available without much somatic cell growth. The dipteran larvae generally are voracious feeders and grow big. The amplified genes in the secretory glands cater to the increased requirement of genetic information without significant somatic growth of the gland cells.

Drosophila has been very widely used for studies on polytene chromosomes. The salivary glands of late third instar larvae of *Drosophila* provides a cytologically excellent polytene chromosome preparation due to a high level of polyteny achieved by many cells in this tissue. With increasing larval age, the level of polyteny of chromosomes in cells of salivary glands increases. Each salivary gland has about 120 cells. Of these, the more posterior cells endoreplicate more often than the anterior ones and hence posterior cells provide better chromosome preparations. By late 3rd instar larval stage, many of these cells have completed 8 or 9 rounds of replication. All the resulting chromatids (28 or 29 chromatid fibrils) maintain their lateral association in such a way that their differentially coiled regions remain in tight register: this results in the characteristic banding pattern consisting of more dense band regions alternating with light stained inter-bands. The chromatin is more densely packed in bands while it is less coiled in inter-band regions.

Transcriptionally active regions can be easily identified under light microscope as "puffs". The chromatin fibrils are more loosely arranged in a puff and the newly synthesized RNA also accumulates here. These events result in the enlarged diameter and lighter staining of puff regions compared to the transcriptionally inactive regions (bands). The pericentromeric heterochromatic regions of different chromosomes remain in close association with each other and together they form the chromocentre. The euchromatic arms of different chromosomes appear to radiate from this common chromocentre (in some dipterans, like *Chironomus*, chromocentre formation does not occur in polytene cells). A major part of the DNA in chromocentre region does not participate in endoreplication, i.e., it remains under-replicated.

Drosophila melanogaster has an acrocentric X-chromosome, two pairs of metacentric chromosomes (chromosomes 2 and 3) and a pair of very small dot-like 4th chromosome.

Females have two X-chromosomes while males have one X and a large sub-metacentric Y-chromosome. Y-chromosome, like the other heterochromatic regions, remains buried within the chromocentre mass. As in other somatic cells of *Drosophila* and many other diptera, the homologous chromosomes in polytene cells also remain tightly synapsed. As a result, a polytene nucleus of *D. melanogaster* shows a common chromocentre (formed by centromeric and pericentromeric heterochromatic regions of all chromosomes) from which 5 long and a very short euchromatic banded chromosome arms radiate out. The 5 long arms represent the X-chromosome, left and right arms of chromosomes 2 and 3, respectively while the very short arm is formed by the 4th chromosome. Each chromosome arm has a characteristic banding pattern due to which each region of every chromosome can be very easily distinguished and identified. Every band is given a specific number identity: for *D. melanogaster*, the polytene chromosome maps prepared by C. B. Bridges and P. N. Bridges in 1930s and 1940s are followed to identify each of the approximate 5000 bands seen in a salivary gland polytene nucleus.

As the salivary glands are histolysed after pupation, polytene chromosome preparations can be prepared from salivary glands of larvae only. Salivary glands of adult flies do not contain polytene cells. For cytological studies, polytene chromosome preparations are made by the classical squashing technique following a brief fixation and staining. These preparations could be temporary or permanent, depending upon how they are made.

3. Materials Required

3.1. Biological material: Healthy late 3rd instar larvae (*Drosophila* larvae or *Chironomous* larvae)

3.2. Chemicals/ Reagents: KCl, NaCl, NaHPO₄.2H₂O, CaCl₂.2H₂O, MgSO₄.7H₂O, KHCO₃, acetic acid, methanol, Orcein, lactic acid, absolute alcohol, DPX (mixture of distyrene, a plasticizer and xylene).

3.3. Equipment: 37 °C incubator, stereo binocular microscope, light binocular, microscope.

3.4. Glassware/plastic ware: Cavity slides, clean glass slides, cover-glasses, dipping jars, slide tray.

3.5. Miscellaneous: Blotting paper, droppers, dissecting needles and fine forceps, cloth for cleaning slides, diamond marker, marker-pen, slide box, razor-blade.

4. Preparation of Reagents

4.1. Poels' Salt Solution (pH 6.8):

- i. NaCl - 86 mg
- ii. KCl - 313 mg
- iii. CaCl₂.2H₂O - 116 mg

- iv. $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ - 88 mg
- v. KHCO_3 - 18 mg
- vi. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 513 mg
- vii. Distilled H_2O - 100 mL.
- viii. Adjust pH to 7.0 with 1 M NaOH and filter.

4.2 Aceto-Methanol:

- i. Aceto: Methanol- 1:3
- ii. 50% acetic acid
- iii. Methanol - freshly prepared

4.3 Aceto-Orcein (2%):

- i. Dissolve 2 g Orcein in 50% Acetic acid by boiling for 30 min under a reflux condenser.
- ii. Filter when cool.

[Note: it is strongly desirable to filter the stain every time before use.]

4.4 Aceto-Carmine (2%):

- i. Dissolve 2 g Orcein in 50% Acetic acid by boiling for 2 hours under a reflux condenser
- ii. Filter when cool.

[Note: it is strongly desirable to filter the stain every time before use.]

4.5 Lacto-Aceto-Orcein (2%):

- i. Dissolve 2 g Orcein in a solution containing 51 mL Glacial Acetic Acid, 34 mL distilled water and 15 mL of 85% lactic acid by boiling for 2 hours on a very low flame in a flask fitted with a reflux condenser.
- ii. Filter the stain when cooled to room temperature.

[Note: it is strongly desirable to filter the stain every time before use.]

4.6 DPX mountant:

- i. Ethanol grades 70%, 90%, absolute alcohol.

5. Procedure

5.1. Method I (Temporary preparations):

- i. Take late 3rd instar larvae (about 5 day old if grown at 24 °C) from a healthy culture (late 3rd instar larvae crawl out of the food medium and move actively on food-free surface), wash them free of adhering food particles etc with water and transfer to a cavity slide containing a small amount of Poels' salt solution (simple insect saline/Ringer's solution can also be used).

- ii. Using fine forceps and/or dissecting needles, pull forward the mouth parts of larvae to rupture larval skin. It forces out internal organs. Salivary glands are seen as a pair of whitish translucent elongated structures connected at their anterior ends with a common salivary duct. Remove fat bodies adhering to glands.
- iii. Using tips of the dissecting needles, transfer the cleaned salivary glands to a drop of Poels' salt solution on a clean slide. Drain out the salt solution (do not let the glands dry). Keeping the slide in a slanting position, add drops (drop-by-drop) of freshly prepared fixative. Wipe out excess fixative with a piece of filter paper (the total duration of fixation should not exceed 1 min since longer fixation makes chromosomes brittle and difficult to spread). Add a few drops of aceto-orcein stain and leave the slide covered with a watch glass for a few minutes (a better staining is obtained by a mix of Aceto-Orcein and Aceto-Carmine stains: add 2 drops of Aceto-Carmine and 1 drop of Aceto-Orcein and keep covered).
- iv. Drain out the stain and add a few drops of 50% acetic acid to remove excess stain. Finally place a drop of 50% acetic acid, cover with clean cover-glass.
- v. For squashing, put the slide with its cover-glass between folds of a clean filter paper and lightly tap the cover-glass either with the rubber-end of a pencil or with the blunt end of needle-holder or even with the needle (tapping breaks the cell and nuclear membranes and releases chromosomes free in cytoplasm; a very slight movement of cover-glass on the slide may be desirable but too strong a tapping would break chromosomes in pieces). Hold the cover-glass in position with fingers of one hand placed over the filter paper such that they press on two diagonal corners of cover-glass. Apply firm pressure of thumb of the other hand on the cover-glass. This act of squashing spreads the polytene chromosome arms of a nucleus and makes them flat in one plane. Any lateral movement of cover-glass relative to the slide at this stage is likely to cause "rolling" of chromosomes making them totally unsuitable for study. Too strong a thumb pressure may cause the chromosomes unduly stretched ("optimum" thumb-pressure is learnt with experience only).
- vi. After squashing, seal the cover-glass with DPX (to prevent evaporation of acetic acid and drying of the slide) and observe under microscope (these preparations will stay for a few days only; temporary preparations will last longer if the squash preparation is made in Lacto-Aceto-Orcein instead of 50% acetic acid and if the sealed slides are stored in refrigerator at 4 °C).

5.2. Method II (Permanent preparations):

- i. Step i-v same as in Method I. (in certain cases staining with aceto-orcein is not done: the glands are directly transferred to 50% acetic acid after fixation; for certain situations, it may also be desirable that squashes are made on slides which are gelatin-subbed using cover glasses which are siliconized. Gelatin subbing makes chromosomes stick better to slide while siliconization prevents chromosomes from sticking to cover-glasses).
- ii. Transfer the slides with cover-glass to a slide box which has been pre-chilled and immediately store the slide box at -70 °C. Keep at -70 °C for a few hours or over-night.
- iii. After the desired period of freezing, quickly flip off the cover-glass using a sharp razorblade and immediately transfer the slide to a 1:1 solution of 50% acetic acid and 50% ethanol (slides are taken out one by one from the freezing chamber to ensure their frozen state). Pass the slides through 70%, 90%, absolute alcohol and air-dry. Alternatively, after chilling at -70 °C for 15-20 min, briefly dip the slides in liquid nitrogen followed by quick flipping off of the cover-glass with a blade. Immediately transfer the slide to a 1:1 solution of 50% acetic acid and 50% ethanol (slides are taken out one by one from the freezing chamber to ensure their frozen state). Pass the slides through 70%, 90%, absolute alcohol and air-dry.

- iv. Observe the slides under phase-contrast microscope (bright-field microscope can also be used if the light is dimmed and substage diaphragm is closed).
- v. Slides can be stained with Giemsa and mounted with DPX (sometimes, most of the spread chromosomes may stick to the cover-glass and when the cover-glass is flipped off, little material is seen on the slide; in such cases, the cover glass may be mounted with DPX or other mounting medium on a clean slide in such a way that the chromosomes remain on upper exposed surface of the cover-glass; after the mounting medium has dried, these can be processed further like other slides).

6. Observations

A good squash preparation reveals many polytene nuclei with well spread polytene chromosome arms connected to a common chromocentre. The chromocentre is an irregular mass of densely stained chromatin giving a granular appearance. This granular and irregularly arranged chromatin is termed the β -heterochromatin. A small very densely stained compact region, the α -heterochromatin can often be seen within this mass. Five long (corresponding to the X, left and right arms of chromosomes 2 and 3 (2L, 2R, 3L and 3R) respectively and one short (chromosome 4) chromosome arms radiate from the chromocentre. Each chromosome arm displays a typical pattern of dark stained bands and light interbands: this banding pattern allows identification of not only each chromosome arm but also specific chromosome region since each band has been assigned a specific number. Certain specific regions, the puffs, appear swollen (greater diameter) and light stained. Specific regions that are puffed and the size of each puff (the puffing pattern) are characteristic of the developmental stage of the larva.

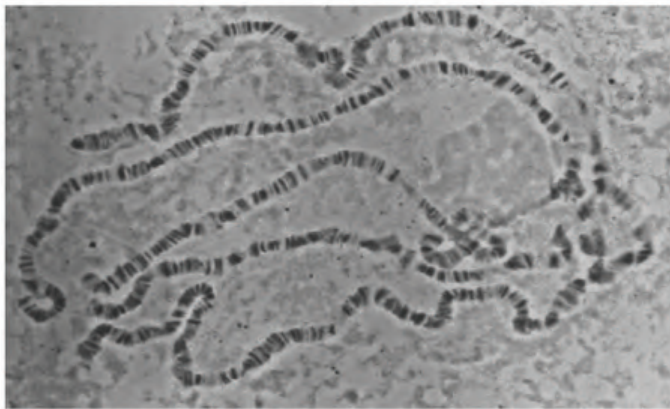


Fig. 1: A polytene nucleus from orcein stained squash preparation of salivary glands of late third instar larva of *Drosophila melanogaster* - note the five long chromosome arms (X, 2L, 2R, 3L, 3R) and the short chromosome 4 connected to a common chromocentre (phase-contrast optics, x 550 approx.)

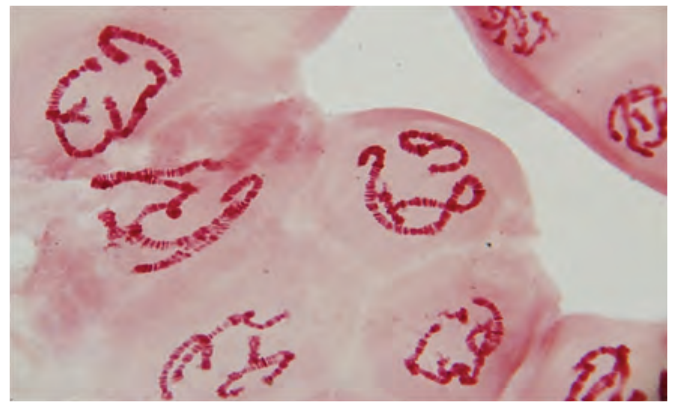


Fig. 2: Giant Chromosomes under microscope at 200X

(Source: Motilal & Banerjee, 2016.)

Under the microscope, polytene chromosomes are seen with bands and puffs (Balbiani Rings). Giant chromosomes show characteristic light and dark banding patterns. Dark bands correspond to Heterochromatin (condensed and inactive chromatin), while light bands (inter-bands) correspond to euchromatin (chromatin in dispersed form and active). Pattern of bands and inter-bands is specific not only to a specific chromosome but also specific at different stages of development. Chromosome puffs (Balbiani ring) are uncoiled regions of the polytene chromosome that are sites of intense transcription.

7. Precautions

Getting good “squash preparations” is an art and requires some practice and care. Following are some of the commonly encountered problems.

- i. Cleaning of slides and cover-glasses: To obtain good squash preparations, it is absolutely essential that the slides and cover-glasses are totally free of any dust-particles, fibers and greasy material. A simple way to achieve this is to store the fresh (or soap-cleaned, if desired) slides and cover-glasses in 90% ethanol in suitable containers and wipe them dry, immediately before use, with a clean soft silken cloth. These are stored covered till use.
- ii. Excessive tapping may lead to breaking of individual chromosome arm into pieces; likewise, excessive pressure during squashing may cause over-stretching of some chromosome regions. Tapping and the thumb pressure have to be "optimal". In a well-spread preparation, all chromosome regions remain in focus at one plane. Any lateral movement of the cover glass relative to the slide during squashing results in chromosomes being “rolled” and fragmented: such chromosomes appear as small “rolls” of homogeneously stained material.
- iii. Imbalanced salt concentrations in the Ringers' or the saline solution in which the glands are dissected or incubated may cause poor morphology of the chromosomes (the bands do not appear "crisp") or the nuclei may not open up at all (most of the polytene nuclei remain rounded up with their nuclear membrane remaining intact).
- iv. Salivary glands from larvae that have not reached the late third instar larval stage or that are weak due to over-crowding or growth at higher temperature, do not provide well spread, thick and distinctly banded chromosomes due to their reduced levels of polyteny.
- v. Any trace of grease on slides or cover glasses hampers good spreading of chromosomes. The slides and cover glasses must be kept in 95% alcohol for some time and should be wiped dry just before use with fresh clean and soft cloth - examination of the slide and cover glasses under reflected light (from a lamp) will reveal if traces of oiliness or fibers etc are present on their surface. Any pieces of tracheae or fibers (e.g., from filter-paper) or stain particles or any cuticular structures (e.g., mouth parts) of larvae left on the slide will not permit good squashing. All these must be carefully removed before applying the cover glass.

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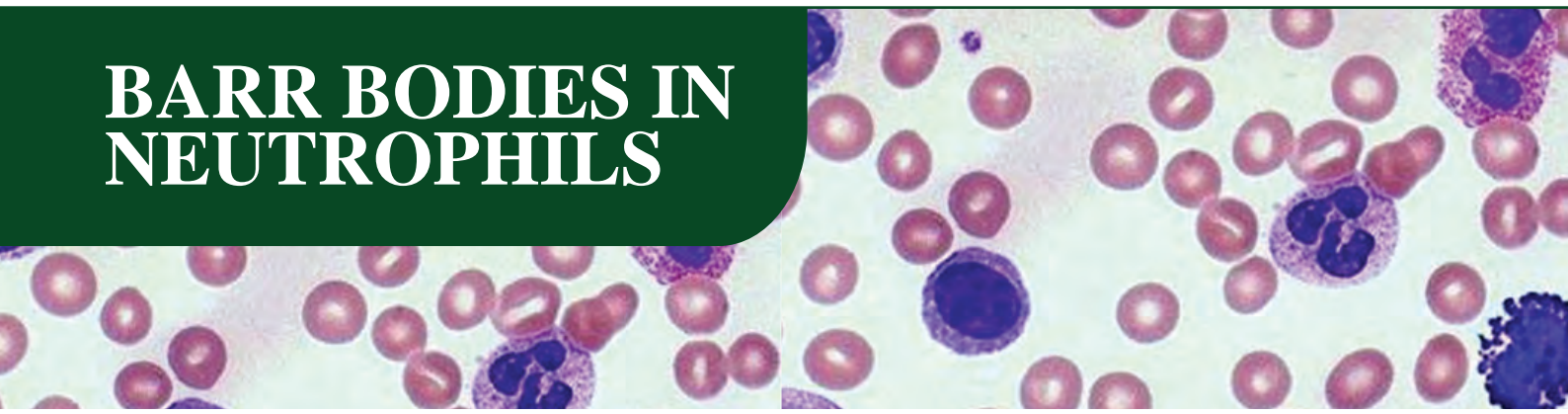
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BARR BODIES IN NEUTROPHILS



1. Aim

To demonstrate Barr body in neutrophils by staining human blood smear.

2. Introduction

Murray. L. Barr and E. G. Bertram observed a darkly stained body in interphase nerve cells of female cats that was absent in similar cells of male cats. This darkly stained body was named as Barr body after the name of its discoverer. It is also called as sex chromatin in humans as it helps in determining the sex of an individual. Barr body can be easily observed as rounded body attached to the nucleus of neutrophils present in the blood of female cells.

In humans, sex chromosomes of female are XX and that of male are XY. The Y chromosome contains the TDF (testis determining factor) or SRY (sex determining region of Y chromosome) which has the genes that lead to the development of male characteristics. Absence of Y chromosome in turn leads to the development of female characteristics.

Mary F. Lyon worked on Tortoise shell cats and proposed the Lyon's hypothesis to explain the presence of Barr body. According to Lyon's hypothesis, in females, only one X chromosome remains active while other X chromosome gets heterochromatinized or condensed during early development (100 cell stage in humans) and this is maintained in all daughter cells. The condensed chromosome becomes genetically inactive and this X inactivation balances the dosages of genetic information between females (XX) and males (XY). This genetically inactive X chromosome is present all cells of the female, but it can be observed easily as a condensed, darkly staining body in the nucleus of squamous epithelium (cheek cells) or in the WBC cells (neutrophils). The number of Barr bodies is always one less than the total number of X chromosomes in the individual. One Barr body means the individual has two X chromosomes, two Barr bodies means the individual has three X chromosomes, etc. A normal male has only one X chromosome so there is no Barr body. Number of Barr bodies = $N - 1$, where 'N' is the total number of X chromosomes present.

Barr body evaluation can be used to determine the genetic sex of an individual especially, in genetic abnormalities related to sex chromosomes;

Sex Chromosomes	Phenotypic condition	No. of Barr bodies or sex chromatin	Remarks
XX	Normal female	1	Normal female
XY	Normal male	Nil	Normal male
X0	Turner Syndrome	Nil	Abnormal female
XXX	Triple X	2	Abnormal female
XXY	Klinefelter Syndrome	1	Abnormal male
XXXY	Klinefelter Syndrome	2	Abnormal male

3. Materials Required

3.1. Biological Materials: Blood sample.

3.2. Chemicals/Reagents: Methanol, Giemsa stain.

3.3. Equipment: Diamond tipped glass marker, compound microscope.

3.4. Glassware/ Plastic ware: Fresh and clean microscope slides, cover glass, petridish.

3.5. Miscellaneous: Sterile pricking needles /lancets, distilled water (deionized).

4. Procedure

4.1. Collecting Blood:

- i. Clean the tip of finger with an alcohol swab.
- ii. Allow the alcohol to dry.
- iii. Hold the finger firmly and gently apply pressure around the fingertip so that the fingertip gorges with blood.
- iv. Use a sterile lancet or a sterile pricking needle to make a wound at the fingertip.
- v. Hold the finger upright and allow the blood to form a drop at the fingertip.
- vi. Take a fresh, clean glass slide and touch it to the blood drop such the blood is transferred to the slide as a drop.
- vii. Place the slide on a flat surface with the blood drop facing upwards.
- viii. Similarly transfer blood drop on to two or three slides.
- ix. Press an alcohol swab at the wound site to stop the bleeding.

4.2. Making blood smear:

- i. Take another clean glass slide with clean smooth edges at the narrow end (“Spreader slide”).
- ii. Touch the narrow end of the “Spreader slide” at the center of the slide with the blood drop (“Blood slide”).
- iii. Hold the “Spreader slide” at an angle such that it makes 40° angle with the “Blood slide”.
- iv. Gently slide the “Spreader slide” towards the blood drop till the narrow end (edge) of the “Spreader slide” just touches the edge of the blood drop.
- v. Allow the blood to spread along the edge of the “Spreader slide” till it covers almost $3/4^{\text{th}}$ the edge of the “Spreader slide”.
- vi. Slide the “Spreader slide” away from the blood drop in a single smooth action till the “Spreader slide” slides out of the “Blood slide” such that the blood is “dragged” to the other end away from the drop.
- vii. Keep the “Blood slide” in a petridish such that the end of the slide with the tail of the smear rests on the edge of the petridish. This will avoid any excess blood from the drop from flowing into the smear area.
- viii. Allow the blood smear to air dry fully.
- ix. The blood smear will be thick near the blood drop (Head of the smear) and very thin towards the other end (Tail of the smear). The Head of the smear should be almost as wide as the breadth of the slide and should not have any bands.
- x. Use the glass marker and label the “Blood Slide” at the Head end of the blood smear.

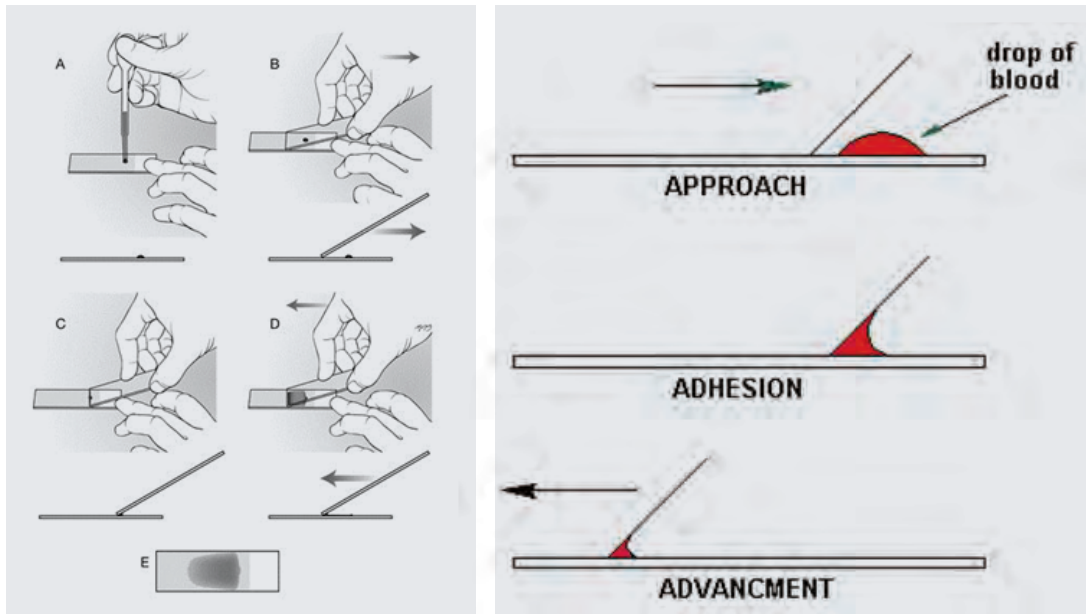


Fig. 1: Preparation of Blood Smear

(Source: *Medicine, Science and More*, April 2013 & *Animal and Plant Health Agency, UK*)

4.3. Staining blood smear:

- i. Place the marked “Blood slide” in a petridish with the smear side upwards.
- ii. With a pipette cover the dried smear with two to three drops of methanol.
- iii. Air dry the smear for 5 minutes till the methanol is evaporated and the cells are fixed to the glass slide.
- iv. With a pipette add three to four drops of Giemsa stain (undiluted) such that the stain fully covers the smear area. Do not allow the stain to flow out of the slide.
- v. Immediately, cover the petri dish and stain for 2 minutes.
- vi. With a dropper or pipette, gently add few drops of deionized water on the stain, such that the diluted stain spreads till all edges of the slide but does not flow out from the slide.
- vii. Notice a green shiny scum formed over the surface of the diluted stain.
- viii. Gently cover the petri dish and keep aside undisturbed for 15 minutes (differentiation).
- ix. Open the petri dish and lift the slide by one of its narrow ends and drain the diluted stain into the petri dish.
- x. Place the slide, for two minutes, in a Coplin jar into which a gentle stream of running water is directed. Do not allow the stream of water to fall directly on the smear.
- xi. Place the slide on strip of filter paper such that the smear surface is facing upwards.
- xii. Observe the slide first under the low power of the microscope (10X) and then under the high power (40X).
- xiii. Place a drop of immersion oil and focus the slide under the Oil Immersion Lens.
- xiv. Locate the neutrophil where the Barr body drum stick is visible as a protrusion of the nucleus.

5. Result and Discussion:

The neutrophils are the more common leukocytes. Their nucleus is divided into 2 - 5 lobes connected by a fine nuclear strand or filament. In the nucleus of the neutrophil, of cells from females, there is an appendage like a little drumstick (Barr body). It is the second X chromosome, which is being inactivated or heterochromatized. Though the drumstick is present in all neutrophils, it is clearly seen only in some neutrophils depending on the angle of vision while observing under the microscope. Barr body helps in sex determination and in diagnosis of abnormal sex aneuploids. The presence or absence of a Barr body in cells is used in medical and criminal forensics to determine and legally define the genetic sex of an individual.

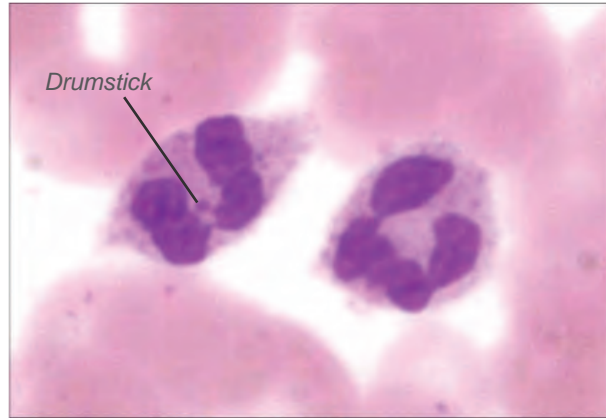


Fig. 2: Drumstick in human neutrophil (1000X)

(Source: Kulkarni M. K et al; Journal of oral and maxillofacial pathology, Vol. 20, 2016.)

6. Precautions

- i. Care should be taken while pricking the finger.
- ii. Use sterile lancets / pricking needles while collecting blood.
- iii. Blood smear should have proper head and a tail.
- iv. Inadequate spreading or uneven sliding will result in “banded” smear.
- v. Observe the slide near the tail of smear.

7. Extension Activity

- i. Use the same smear to carry out differential count of the WBCs in the sample.
- ii. Alternatively collect cheek smear and observe Barr body in the squamous epithelial cells.



Fig. 3: Barr Body in squamous cells of cheek smear from Human female

(Source: Teresa. A. et al. Biology: Life on Earth.; Prentice Hall, 1996.)

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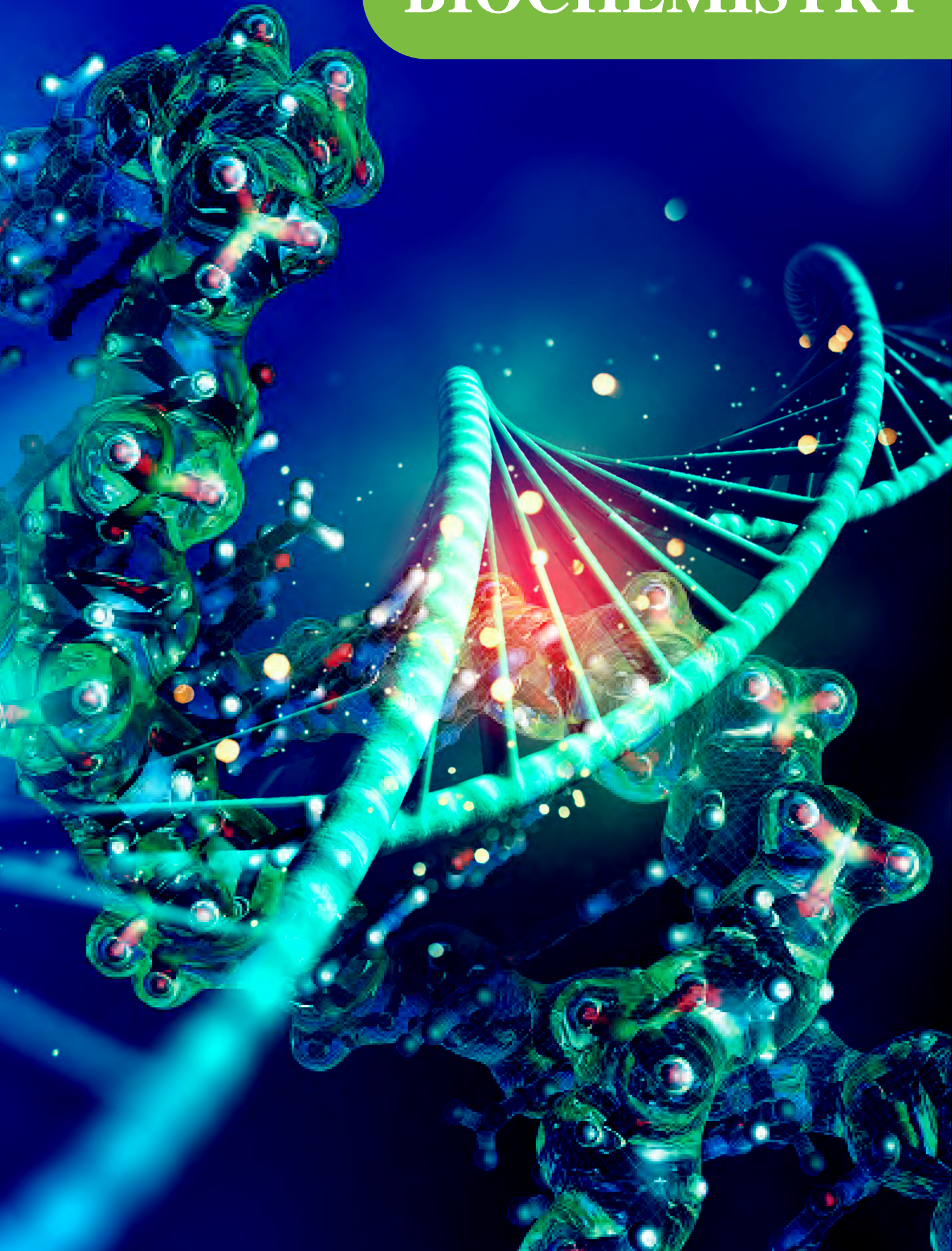
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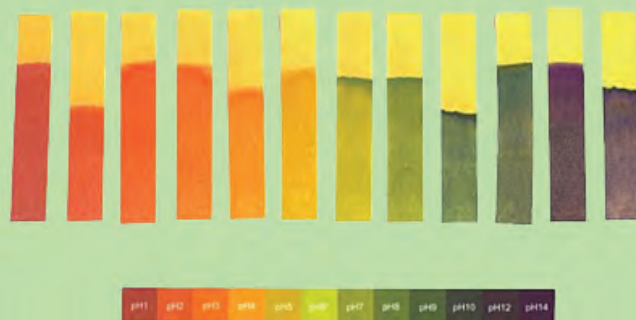
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PREPARATION OF BUFFERS



1. Aim

To prepare buffer solutions of different molarity and to check their pH using a pH meter.

2. Introduction

2.1. Working of pH meter

pH is the unit to measure the degree of acidity or alkalinity of a solution. It is defined as the negative logarithm of hydrogen ion concentration i.e.

$$\text{pH} = -\log [\text{H}^+]$$

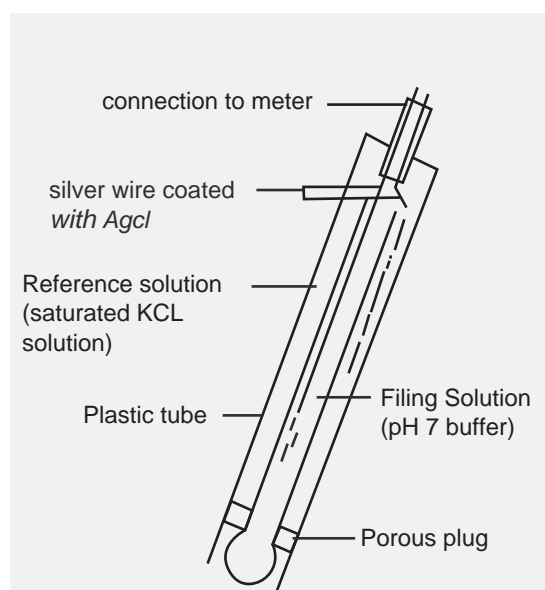
Water molecules dissociate into H^+ and OH^- ions. In the state of equilibrium, the molar concentration of H^+ and OH^- ions is 10^{-7} M each, at 25°C . i.e. $[\text{H}^+][\text{OH}^-] = 10^{-14}$ at 25°C .

The pH value is therefore, measured on a scale of 0 to 14, where pH 7.0 is neutral, $\text{pH} < 7.0$ is acidic and $\text{pH} > 7.0$ is basic. An acidic solution has H^+ concentration higher than OH^- ions whereas a basic solution has higher OH^- concentration than H^+ ions. Since pH scale is a logarithmic scale, an increase in 1 pH unit corresponds to a reduction of $\text{H}^+ : \text{OH}^-$ ratio by a factor of 10.

A standard pH meter consists of an electrode for measuring pH. [Fig. 1(A), (B)]. This electrode has a glass membrane which is sensitive to H^+ ion concentration. Saturated KCl solution is used as the reference solution as its output does not vary with H^+ ion activity. Also, a high input meter measures the difference between the voltages of the two electrodes which then translates the voltage difference into pH and displays it on the screen.



(A) Standard pH meter;



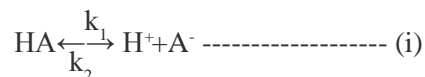
(B) pH measuring probe

Fig. 1: (A) Components of a pH meter;

2.2. Preparation of buffers

Buffer ions are used to maintain solutions at constant pH values. The selection of a buffer for use in the investigation of a biochemical process is of critical importance.

Weak acids and bases do not completely dissociate in solution but exist as equilibrium mixtures [equation (i)]



HA represents a weak acid and A⁻ represents its conjugate bases; k₁ represents the rate constant for dissociation of the acid and k₂ the rate constant for association of the conjugate base and hydrogen ion. The equilibrium constant, K_a, for the weak acid HA is defined by equation (ii)

$$K_a = \frac{k_1}{k_2} = \frac{[\text{H}^+][\text{A}^-]}{[\text{HA}]} \text{ ----- (ii)}$$

which can be rearranged to define [H⁺] [equation (iii)]

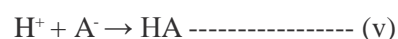
$$[\text{H}^+] = \frac{K_a [\text{HA}]}{[\text{A}^-]} \text{ ----- (iii)}$$

The [H⁺] is often reported as pH, which is -log [H⁺]. In a similar fashion, -log K_a is represented by pK_a. Equation (iii) can be converted to the -log form by substituting pH and pK_a:

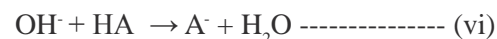
$$\text{pH} = \text{pK}_a + \log \frac{[\text{A}^-]}{[\text{HA}]} \text{ ----- (iv)}$$

Equation (iv) is known as Henderson-Hasselbalch equation, which defines the relationship between pH and the ratio of conjugate acid and conjugate base concentrations. The Henderson- Hasselbalch equation is of great value in buffer chemistry because it can be used to calculate pH of a solution if the molar ratio of buffer ion ([A⁻]/[HA]) and the pK_a of HA are known. Also, the molar ratio of [HA] to [A⁻] that is necessary to prepare a buffer solution at a specific pH can be calculated if the pK_a is known.

A solution containing both HA and A⁻ has the capacity to resist changes in pH; i.e. it acts as a buffer. If acid (H⁺) were added to the buffer solution, it would be neutralized by A⁻ in solution:



Base (OH⁻) added to the buffer solution would be neutralized by reaction with HA:



The most effective buffering system contains equal concentration of the acid, HA, and the conjugate base, A⁻. According to the Henderson-Hasselbalch equation (iv), when [A⁻] is equal to [HA], pH equals pK_a. Therefore, the pK_a of a weak acid-base system represents the center of the buffering region. The effective range of a buffer system is the center of the buffering region. The effective range of a buffer system is generally centered at the pK_a value equation (vii)

Effective pH range of a buffer = pK_a ± 1 ----- (vii)

2.3. Selection of a Biochemical Buffer:

Virtually, all biochemical investigations must be carried out in buffered aqueous solutions. The natural environment of biomolecules and cellular organelles is under strict pH control. When these components are extracted from cell, they are most stable if maintained in their normal pH range of 6.0 to 8.0. An artificial buffer system is found to be the best substitute for the natural cell milieu. It should also be recognized that many biochemical processes (especially some enzyme processes) produce or consume hydrogen ions. The buffer system neutralizes these solutions and maintains a constant chemical environment.

Although most biochemical solutions require buffer system effective in the pH 6.0 to 8.0, there is occasionally a need for buffering over the pH range 2 to 12. Obviously, no single acid-conjugate base pair will be effective over this entire range, but several buffer systems are available that may be used in a discrete pH range. Some buffers (phosphate, succinate and citrate) have more than one pK_a value, so they may be used in different pH regions. Many buffer systems are effective in the biological pH range (6.0 to 8.0); however there may be major problems in their use. Several characteristics of a buffer must be considered before a final selection is made.

Phosphate Buffers:

The phosphates are among the most widely used buffers. These solutions have high buffering capacity and are very useful in the pH range 6.5 to 7.5. Because phosphate is a natural constituent of cells and biological fluids, its presence affords a more “natural” environment than many buffers. Sodium or potassium phosphate solutions of all concentrations are easy to prepare. The major disadvantages of phosphate solutions are

- i. Precipitation or binding of common biological cations (Ca^{2+} and Mg^{2+}) and
- ii. Inhibition of some biological processes, including some enzymes.

Tris Buffer:

The use of the synthetic buffer Tris [tris(hydroxymethyl)aminomethane] is now probably greater than that of phosphate. It is useful in the pH range 7.3 to 9.3. Tris is available in a basic form as highly purified crystals, which makes buffer preparation especially convenient. Although Tris is a primary amine; it causes minimal interference with biochemical processes and does not precipitate calcium ions. However, Tris has several disadvantages, including,

- i. pH dependence on concentration, since the pH decreases 0.1 pH unit for each 10 fold dilution:
- ii. Interference with some pH electrodes.
- iii. A large $\Delta pK_a / ^\circ C$ dependence of pH on temperature.

Most of these drawbacks can be minimized by:

- i. Adjusting the pH after dilution to the appropriate concentration,
- ii. Purchasing electrodes that are compatible with Tris,
- iii. Preparing the buffer at the temperature at which it will be used.

Carboxylic acid Buffers:

The most widely used buffers in this category are acetate, formate, citrate and succinate. This group is useful in the pH range 3 to 6, a region that offers few other buffer choices. All of these acids are natural metabolites, so they may interfere with the biological processes under investigation. Also, citrate and succinate may interfere by binding metal ions (Fe^{3+} , Zn^{2+} , Mg^{2+} etc.).

Borate Buffers:

Buffers of boric acid are useful in the pH range 8.5 to 10.0. Borate has the major disadvantage of complex formation with many metabolites, especially carbohydrates.

Amino acid Buffers:

The most commonly used amino acid buffers are glycine (pH 2 to 3 and 9.5 to 10.5), histidine (pH 5.5 to 6.5), glycine amide (pH 7.8 to 8.8), and glycyglycine (pH 8 to 9). These provide a more “natural” environment to cellular components and extracts; however, they may interfere with some biological processes, as do the carboxylic acid and phosphate buffers.

3. Materials Required

3.1. Chemicals/Reagents: Standard buffer solutions for calibration (pH 4.0, pH 7.0 and pH 9.4), double distilled water (DDW), 0.1 M acetic acid (CH_3COOH), sodium acetate (CH_3COONa), $\text{Na}_2\text{HPO}_4 + \text{NaH}_2\text{PO}_4$ for phosphate buffer.

3.2. Equipment: A standard pH meter, magnetic stirrer.

3.3. Glassware/Plastic ware: Beakers, test tubes, bottles to store buffer.

4. Procedure**4.1. Calibration of pH meter:**

- i. Before measuring the pH of the test solution, calibrate the pH meter using the standard buffer solutions.
- ii. Rinse the probe thoroughly with double distilled water. Immerse the probe in standard buffer solution pH 7.0, allow the display to stabilize and set the display to read 7.0 by adjusting calibration button.
- iii. Rinse the probe with DDW and follow step ii. for standard buffer solutions pH 4.0 or 9.4.
- iv. To measure the pH of the test solution, rinse the probe and gently blot it using a tissue paper. Place the probe into the test solution, the corresponding pH is displayed on the screen.
- v. Rinse the probe and store in 4.0 M KCl solution.

4.2. Preparation of buffers:

- i. Preparation of 1 M acetate buffer of pH 4.1 volume 200 mL by using the following equation

a.

$$\text{pH} = \text{p}k_a + \log \frac{[\text{salt}]}{[\text{acid}]}$$

$$4.1 = 4.76 + \log \frac{[x]}{[200 - x]}$$

$$-0.66 = \log \frac{[x]}{[200 - x]}$$

$$0.66 = \log \frac{[200 - x]}{[x]}$$

$$4.571x = 200 - x$$

$$5.571x = 200, \quad x = \frac{200}{5.571} = 35.90$$

So, volume of sodium acetate to be taken is 35.90 mL

Volume of acetic acid = $200 - 35.90 = 164.1$ mL

b. Prepare 0.1 M acetate buffer from 1 M stock solution:

Using $M_1V_1 = M_2V_2$

$1 \times V_1 = 0.1 \times 200$, $V_1 = 20$ mL, so 20 mL 1 M buffer +180 mL distilled water

ii. Preparation of 1 M $\text{Na}_2\text{HPO}_4 + \text{NaH}_2\text{PO}_4$ Phosphate buffer of pH 6.8 volume 200 mL using the following equation:

$$\text{pH} = \text{p}k_a + \log \frac{[\text{salt}]}{[\text{acid}]}$$

$$6.8 = 6.86 + \log \frac{[x]}{[200 - x]}$$

$$-0.06 = \log \frac{[x]}{[200 - x]}$$

$$\text{antilog } 0.06 = \frac{[200 - x]}{[x]}$$

$$x = 93.10 \text{ mL of } \text{NaH}_2\text{PO}_4$$

$$200 - x = 106.9 \text{ mL of } \text{Na}_2\text{HPO}_4$$

iii. Once all the calculations are done, add the required amount of the chemicals to make their respective buffer together in a flask and keep on stirring using a magnetic stirrer.

iv. Add distilled water to make up the volume to 200 mL and check final pH.

5. Precautions

- Apparatus should be cleaned with distilled water before using.
- Calibration of pH meter should be done properly before using.
- Constant stirring is required while adding the acid/base for pH adjustment.
- Do not add too much acid or base at a time it can immediately change the pH. Always add slowly.
- Always start with small volume of distilled water then raise the volume.
- pH electrode is very fragile do not hit it against the wall of the vessel.

Suggested Reading(s)

- Rodney, F. Boyer. *Modern Experimental Biochemistry*. (2nd ed.).
- Westcott, C.C. pH Measurement, U.K., London: Academic Press.
- Wilson, K. & Walker, J. (2000). *Principles and Techniques of Biochemistry and Molecular Biology*. U.S.A., New York: Cambridge University Press.

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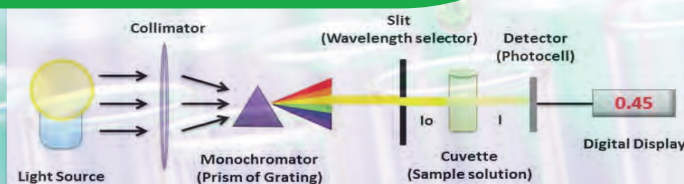
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USE OF SPECTROPHOTOMETER IN QUANTITATIVE ESTIMATION



1. Aim

To demonstrate the functioning of spectrophotometer.

2. Introduction

The quantitative measurement of the absorbance or transmission properties of a material as a function of wavelength is referred to spectrophotometry. This technique involves electromagnetic radiations in the visible, near UV and near infrared regions IR. These radiations are absorbed / transmitted differently by different material. Spectrophotometer is the instrument used to measure the fraction of incident light transmitted through a solution. It is designed to transmit light of narrow wavelength ranges and the intensity of the absorption is measured as a function of frequency and/or concentration of the test material.

The relationship of absorbance and concentration can be explained by Lambert-Beer law, absorbance of a light absorbing material is proportional to its concentration in solution and pathlength of the incident light.

$$A = \epsilon lc$$

Where:

ϵ = the extinction coefficient of the substance, has units of $M^{-1} \text{ cm}^{-1}$ (unique for each substance)

l = the sample path length measured in centimetres (i.e. the width of the cuvette—almost always 1 cm)

c = the molar concentration of the solution (you must express concentration in terms of molarity)

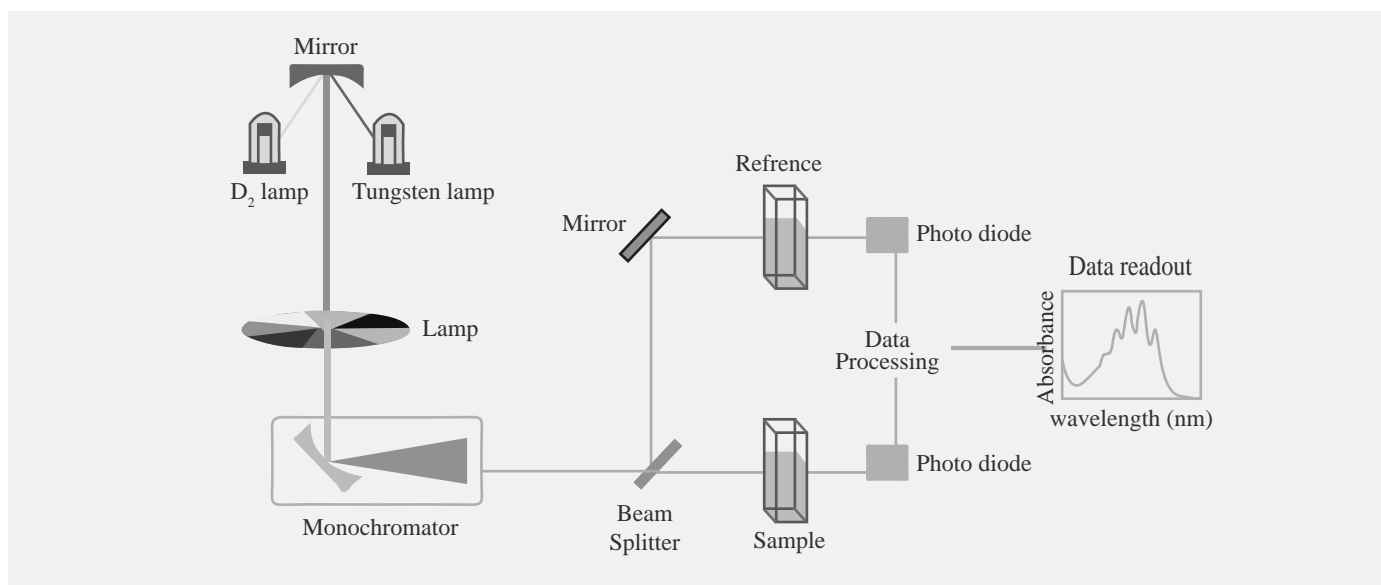


Fig. 1: The components of a UV-Visible spectrophotometer

A colorimeter uses only visible wavelength of light (400 – 700 nm) and the monochromatic light is obtained using a colour filter. A colour filter can only give a band of wavelengths (generally a band width of 10 nm) and therefore, the light is not truly monochromatic. In a UV-visible spectrophotometer, both ultra violet and visible wavelengths are used. There are two light sources, a deuterium (D₂) lamp for UV rays and a tungsten lamp for visible light. Instead of colour filters, a dispersion grating is used to obtain monochromatic light as narrow as 0.1 nm bandwidth. This not only improved the accuracy of measurements but also the sensitivity of the measurement. Unlike the colorimeter, spectrophotometer uses square cuvettes made of quartz which is UV transparent. The incident light is usually split into two beams and both the blank and sample are simultaneously compared in a double beam spectrophotometer. This further improves the accuracy and measurements are faster allowing scanning of the sample for the entire length of the spectrum (generally, 200 – 800 nm).

3. Materials Required

- 3.1. **Chemicals/Reagents:** Bromophenol blue (20 μM), solution of bromophenol blue of unknown concentration, double distilled water (DDW)
- 3.2. **Equipment:** UV-Vis Spectrophotometer, micro pipette, vortex mixer.
- 3.3. **Glassware/Plastic ware:** Cuvettes, test tubes.

4. Procedure

4.1. Preparation of solutions:

- i. Label four test tubes as 1 to 4. Using a micropipette add 2 mL DDW in each tube.
- ii. Add 2 mL of 20 μM bromophenol blue in tube one. Prepare a serial dilution in 1:1 ratio in the tubes 2 to 4, such that tube 2, 3 and 4 has 10 μM , 5 μM and 2.5 μM bromophenol blue concentrations respectively.
- iii. Mix the solutions in tubes using a vortex mixer.

4.2 Estimating λ_{max} of Bromophenol blue:

- i. Switch on the spectrophotometer and set it in scanning mode.
- ii. Select the range from 200 nm to 800 nm.
- iii. Take two paired cuvettes.
- iv. Clean both the cuvettes with DDW and blot it with a tissue paper.
- v. Add 2 mL of DDW in both the cuvettes.
- vi. Place one of the cuvettes in the reference position and the other in the sample position (both positions will now have blank solutions (DDW) only).
- vii. Carry out “Auto Zero” / “Baseline correction” for the spectrophotometer. The spectrophotometer will now adjust DDW for 100% transmittance for all wavelengths from 200 nm to 800 nm.
- viii. Remove the cuvette from the sample position.
- ix. Drain the DDW and replace it with 2 mL of the 20 μM bromophenol blue.
- x. Place the cuvette back in the sample position (Reference position will now have DDW and the sample position will have bromophenol solutions).

- xi. Start the scanning procedure. The spectrophotometer will now scan (record the absorbance from 200 nm till 800 nm) bromophenol blue for the entire range of spectrum specified.
- xii. From the data obtained / the spectrum obtained, note the wavelengths at which bromophenol shows maximum absorbance (λ_{max}).

4.3 Estimating unknown concentration of bromophenol from a solution:

- i. Switch on the spectrophotometer and set the wavelength at 593 nm (wavelength at which bromophenol blue shows maximum absorbance and hence known as absorption maxima or λ_{max}).
- ii. Clean the cuvette with DDW and blot it with a tissue paper. Add 2 mL of DDW in the cuvette and set as “blank”. Place the “blank” cuvette at the reference position.
- iii. Now in the other cuvette add 2ml of bromophenol solution from the test tube 4 (smallest concentration of bromophenol blue).
- iv. Place this cuvette at the sample position and read the absorbance.
- v. Remove the cuvette from the sample position and drain the solution. Invert it on a wad of tissue paper to drain the solution fully.
- vi. Now take 1 mL of solution from tube No.3 and rinse the cuvette. Drain the solution.
- vii. Add fresh 2 mL of solution from tube No. 3 and wipe the sides of the cuvette with tissue paper.
- viii. Place the cuvette back into the sample position and read the absorbance.
- ix. Remove the cuvette and repeat the steps for rest of the test tubes starting from the next smaller concentration. Note down the absorbance for each concentration.
- x. Finally, follow the same steps and read the absorbance for the bromophenol blue sample with unknown concentration.

4.4 Linearity curve and Regression Analysis:

- i. Plot a graph with concentration (μM) on X-axis versus absorbance (optical density) on Y-axis.
- ii. Draw a straight line such that it passes through or nearly touches maximum number of points. (This line may not pass through the origin.)
- iii. Taking two points on the line, calculate the slope of the line.
- iv. Note the Y-intercept of the line.
- v. Obtain the equation of the line as $Y = (\text{Slope}) \cdot X + (\text{Y-Intercept})$
- vi. Substitute the absorbance of unknown solution as Y in the equation and calculate the unknown concentration.
- vii. Compare the calculated concentration with the value obtained by extrapolation on the graph.

Suggested Reading(s)

- i. Wilson, K. & Walker, J. *Principles and Techniques of Biochemistry and Molecular Biology*. U.S.A, New York: Cambridge University Press

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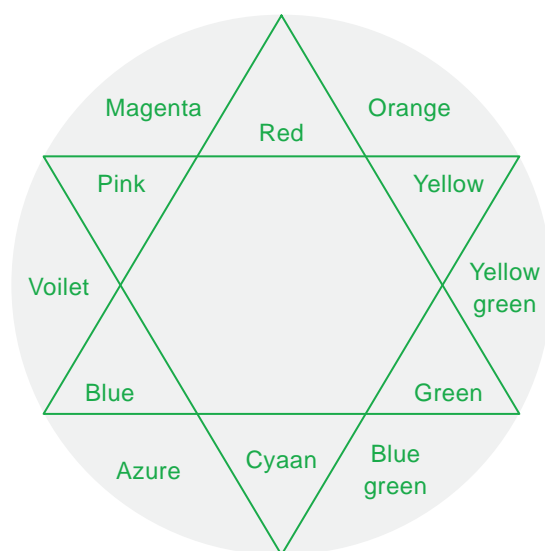
Additional information

Spectrum bands and their wavelength (approx.) used for UV-Vis spectrometry are listed below;

Wavelength in nm	Spectrum band
200 – 280	Ultraviolet C
280 – 315	Ultraviolet B
315 – 400	Ultraviolet A
400 – 435	Violet
435 – 500	Blue
500 – 520	Cyan
520 – 565	Green
565 – 590	Yellow
590 – 625	Orange
625 – 750	Red
750 – 800	Near-Infrared

Spectroscopy is the study of interactions between electromagnetic radiation (energy that is transmitted through space at very high velocity) and matter. When a photon of electromagnetic radiation hits a molecule, energy is transferred to the molecule, shifting it from a ground state to an excited state.

When electromagnetic radiation interacts with molecules, the wavelengths of energy absorbed are a function of the structure of the molecule. Therefore, each compound has a characteristic absorption spectrum, or pattern of absorption. In the visible wavelengths, the absorption spectrum is responsible for the colour of the compound. The wavelengths of light that you see are wavelengths that are not absorbed by interaction with the bonding electrons in the compound.



COLOUR WHEEL
showing
Complementary Colours

Red, green and blue are considered primary colours of light. Mixing these coloured lights give white light (*additive colours*). Cyan, pink and yellow are basic pigment colours and mixing them gives black colour (*subtractive colours*). The mixture of chlorophyll molecules found in leaves, for example, absorbs several wavelengths of visible light, with distinct absorbance peaks in the blue range (400–500 nm) and in the yellow-red range (600–700 nm). The green colour that human eye sees is a combination of visible light that is not absorbed. As various wavelengths of visible light (colours) are absorbed by a compound, only the complementary colours to the colours that are absorbed reach the human eye. We see colour of the compound as a combination of the complementary colours that are not absorbed. Colour is therefore a function of the pattern of wavelengths that are absorbed and wavelengths that are not absorbed. (If no wavelengths of light in the visible region are absorbed, the compound is colourless).

ESTIMATION OF PROTEIN BY LOWRY'S METHOD



1. Aim

To determine the protein content in a given food sample by Lowry's Method.

2. Introduction

Lowry's Method is often cited assay for protein estimated. By this method, the concentration of protein present in the given sample can be determined. Under alkaline conditions copper ions and peptide bonds of given sample reduce the phosphomolybdate in phosphotungstic acid (Folin reagent) to molybdenum blue. At the end, it gives a blue colour complex. The concentrations of protein in the sample are determined by the absorbance of the end product taken at 660 nm against a standard curve of Bovine Serum Albumin (BSA) solutions. The pH range of the sample solutions should be between 9 - 10.5 as this method is very sensitive. The Lowry method is sensitive to low concentrations of protein (0.01 mg of protein/mL).

3. Materials Required

3.1. Chemical/Reagents: BSA stock solution (1 mg/mL), reagent A (mix 50 mL of 2% sodium carbonate (w/v) with 50 mL of 0.1 N NaOH solution), reagent B (mix 10 mL of 1.56% copper sulphate solution with 10 mL of 2.37% sodium potassium tartrate solution), reagent C (mix 2 mL of reagent B with 100 mL of reagent A), Folin-Ciocalteu reagent solution 1 N (mix 2 mL of commercial reagent (2 N) with equal volume of water just before use).

3.2. Equipment: UV- Visible spectrophotometer.

3.3. Glassware/Plastic ware: Cuvettes.

4. Procedure

- i. Dilute the BSA stock solutions to different concentrations by adding distilled water and make volume up to 5 mL.
- ii. Take 0.2 mL of sample protein solution in different test tubes. Add 2 mL of alkaline Reagent C. Mix them properly.
- iii. Now, incubate for 10 minutes in RT (room temperature).
- iv. Add 0.2 mL of Folin-Ciocalteu solution to each of the tubes.
- v. Incubate for 30 minutes at RT.
- vi. Measure the absorbance at RT and plot the standard curve from the absorbance value against protein concentration.
- vii. Calculate the protein content of the given sample from the standard curve.
- viii. A blank is prepared using water as the sample which is set to zero.

5. Observations

BSA (mL)	Water (mL)	Sample concentration (mg/mL)	Sample volume (mL)	Reagent C (mL)	Incubation for 10 min at RT	Folin-Ciocalteu solution (mL)	Incubation for 30 min at RT	O.D. at 660 nm
0	5.00	-	0.2	2		0.2		
0.25	4.75	0.05	0.2	2		0.2		
0.5	4.5	0.1	0.2	2		0.2		
1	4	0.2	0.2	2		0.2		
2	3	0.4	0.2	2		0.2		
3	2	0.6	0.2	2		0.2		
4	1	0.8	0.2	2		0.2		
5	0	1.0	0.2	2		0.2		

6. Result

The amount of protein present in the given sample was found to be _____.

7. Precautions

- i. The Lowry assay is sensitive to pH changes and therefore, the pH of assay solution should be maintained at pH 9-10 .
- ii. A variety of compounds interfere with Lowry's procedure. Substances like ammonium ions, detergents, buffers, drugs and sugars are to be removed before the start of the assay.

Suggested Reading(s)

- i. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J.Biol.Chem* 193: 265-75.
- ii. Hartree, E.E. (1972). Determination of protein: A modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* 48:422.
- iii. Wilson, K. and Walker, J. (2000). *Principles and Techniques of Biochemistry and Molecular Biology.*, U.S.A., New York: Cambridge University Press.

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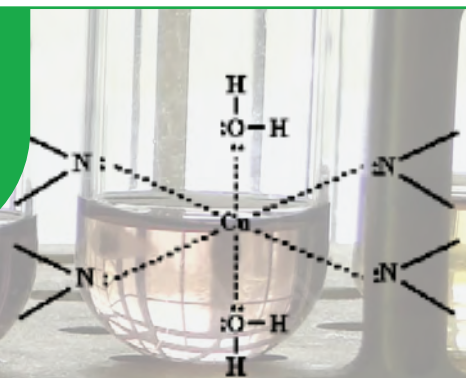
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ESTIMATION OF PROTEIN BY BIURET METHOD



1. Aim

To estimate the amount of protein in the given solution by using Biuret reagent .

2. Introduction

Biuret reagent (Sodium potassium tartarate + Alkaline CuSO_4 solution) forms violet coloured complex with compound containing two adjacent peptide bonds. Intensity of the coloured complex can be measured by using green filter at 540 nm. It is directly proportional to the amount of protein in the unknown sample. The Biuret method is sensitive to low concentration of protein (1-2 mg of protein/mL).

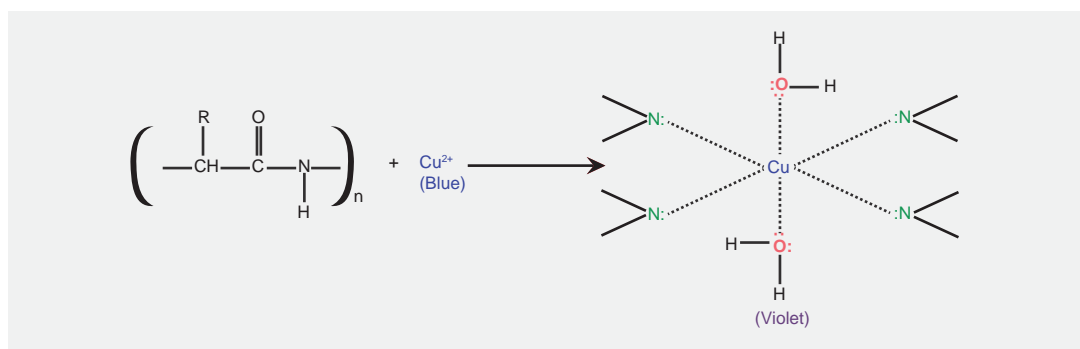


Fig. 1: Formation of biuret complex

3. Materials Required

3.1. Glass wares/Plastic ware: Standard flask (100 mL, 500 mL, 1000 mL), test tubes, beaker, dropper, graduated pipette

3.2 Chemicals/Reagents: 400 mg Bovine serum albumin (BSA), sodium hydroxide (10% w/v), cupric sulphate (0.15% w/v) Sodium potassium tartrate solution (0.6% w/v), 5 g of potassium iodide.

3.3 Equipment: Spectro photometer (Spectronic 20D+).

4. Procedure

- Pipette out 0.5 to 2.5 mL of standard bovine serum albumin in test tubes (label S1 to S5) with concentration ranging from 2 to 10 mg. Make up the solutions to 2.5 mL with distilled water.
- Add 2.5 mL of Biuret reagent to all the test tubes and incubate at room temperature for 10 minutes. Make up the given unknown solution to 100 mL with distilled water. From that 1 and 2 mL of unknown solution is taken in different test tubes and treated similarly.
- As the violet colour develops, measure the intensity at 540 nm with the help of spectrophotometer. A standard graph is drawn by taking optical density along Y-axis and the concentration along X-axis. From the graph, calculate the amount of protein present in the unknown solution.

5. Expected Observations and Result

As the concentration of the protein increases the intensity of the colour produced will increase and the optical density increases linearly.

S. No	Particulars	Blank	Working Standard					Unknown Sample	
			S1	S2	S3	S4	S5	U1	U2
1.	Bovine Serum Albumin (mL)	0	0.5	1.0	1.5	2.0	2.5	-	-
2.	Concentration (μg)	0	2	4	6	8	10	-	-
3.	Volume of Unknown (mL)	-	-	-	-	-	-	U1	U2
4.	Volume of water (mL)	2.5	2.0	1.5	1.0	0.5	0	-	-
5.	Biuret reagent (mL)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Incubated at room temperature for 10 minutes									
6.	Optical density at 540 nm	0							

Table: 1 Optical density of the complex formed

6. Conclusion and Interpretation

O.D. obtained of the unknown sample can be used to determine its concentration from standard curve (Fig. 2)

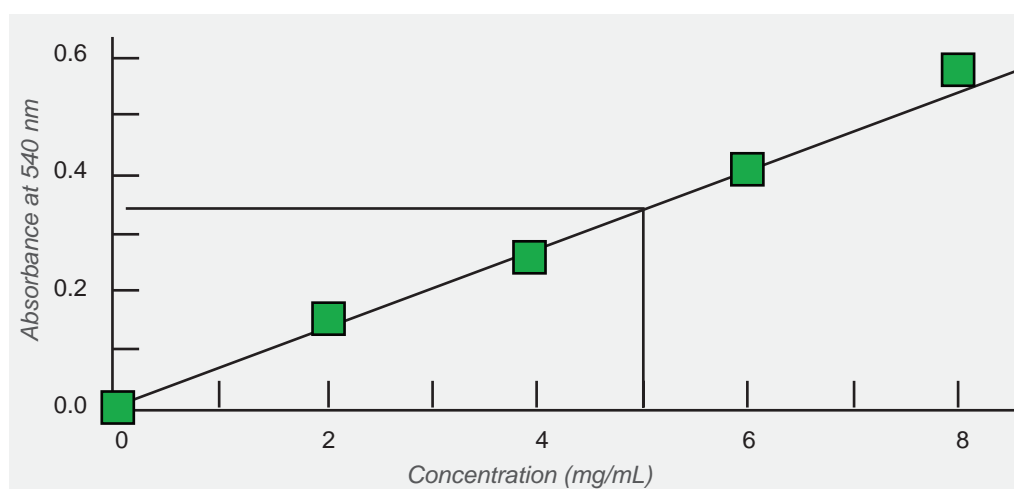


Fig. 2: Model graph for estimation of protein concentration by biuret method

7. Precautions

- i. Prolonged incubation (more than 10 minutes) may lead to structural decomposition of the complex formed.
- ii. Serum albumin being very sensitive, for accuracy and reproducibility of the results all the samples and glassware used should be pure and sterilized.
- iii. Since the sample becomes hygroscopic and hence silinased glassware should be used to prevent the loss of protein as well as its nature.

8. Trouble shooting and bottlenecks expected

- i. Solubility of BSA becomes a trouble shoot during the stock solution preparation.
- ii. BSA solution should be refrigerated properly to avoid protein degradation.

Suggested Reading(s)

- i. David, T. Plummer (1988). *An Introduction to Practical Biochemistry*. Tata McGraw-Hill education.
- ii. www.analiticaweb.com.br/newsletter/16/51859_proteina_biureto.pdf
- iii. www.jbc.org/content/176/3/1421.full.pdf

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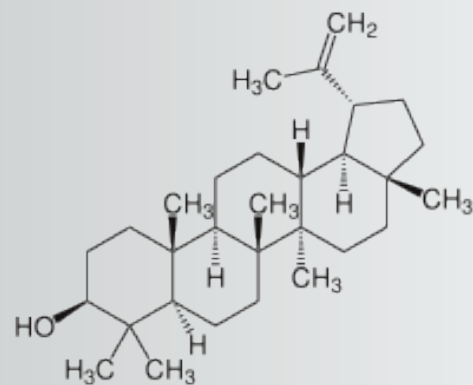
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ESTIMATION OF PHYTOSTEROLS



1. Aim

To estimate the content of phytosterols from the given sample using spectrophotometer.

2. Introduction

Cholesterol is obtained from food and is also synthesized by the body, mainly in hepatic and intestinal cells. It is a metabolic precursor of bile acids, vitamin D and steroid hormones. Its high concentrations are associated with a high risk for vascular accident and apparition of atherosclerosis. In plants, oilseeds are good source of cholesterol.

The determination is based on the method (Allain et al., 1974) of monitoring generated free cholesterol in the reaction catalyzed by cholesterol oxidase. In the commercially available kit, the Enzyme Reagent is a mixture 0.6% m/v $(\text{NH}_4)_2\text{HPO}_4$, 0.334% (m/v) $\text{NH}_4\text{H}_2\text{PO}_4$, 0.017% (m/v) 4-aminoantipyrine, 0.0086% (m/v) sodium cholate, 0.002% (m/v) cholesterol oxidase (Roche, 17.7 U/mg), 0.46·10⁻³% (m/v) horseradish peroxidase (Fluka, 150 U/mg), 0.066% (m/v) DHBSA (Sigma-Aldrich) water solutions and 0.02% (v/v) detergent Triton X-100 (20%). Cholesterol is measured enzymatically in a series of coupled reactions that hydrolyze cholesteryl esters and oxidize the 3-OH group of cholesterol. Cholesterol is oxidized to release proportionate amount of H_2O_2 which is measured quantitatively (colored complex quinoneimine) using spectrophotometer.

Cholesterol esterase



Cholesterol Oxidase



Peroxidase



3. Materials Required

3.1. Biological Materials: Oil (groundnut, flax, soya bean, olive, rice bran etc.).

3.2. Chemicals/Reagents: Cholesterol estimation kit based on cholesteryl esterase oxidase (commercially available), standard cholesterol (from the kit), deionized water, potassium hydroxide (KOH), petroleum ether.

3.3. Equipment: UV-Vis spectrophotometer, autopipettes, weighing balance, incubator.

3.4. Glassware/Plastic ware: Clean and dry test tubes.

3.5. Miscellaneous: Tissue paper.

Procedure

- i. Saponify the samples with a 150 g/L solution of KOH, the sterols extracted with petroleum ether, and aliquots used for direct colorimetric (3) or enzymatic analysis.

Setup clean, dry test tubes labeled as Blank (B), Standard (S) and Test (T) as follows:

Contents	B	S	T
Enzyme Reagent (mL)	1.0	1.0	1.0
Deionized water (mL)	0.01	-	-
Standard (mL)	-	0.01	-
Vegetable oil sample (mg/mL)	-	-	1.0 /10

- ii. Mix the contents in each tube and keep them at 37 °C for 5 min (incubation).
- iii. Adjust the spectrophotometer with blank at 500nm.
- iv. Record the absorbance, optical density (OD) after 5 min incubation.

NOTE: The final colour is stable for at least 1 hour.

5. Observations

Samples	Absorbance at 500 nm
Blank	0.0
Standard	
Test	

5.1. Calculation

$$\text{Cholesterol Conc. in mg\%} = \frac{\text{OD of Test}}{\text{OD of Standard}} \times 200 \text{ (Conc. of Standard)}$$

NOTE: The concentration of the standard cholesterol is 200 mg%

6. Result

The content of cholesterol in the sample is;

S. No.	Sample Identity	Concentration of cholesterol (mg %)

7. Precautions:

- i. Allow the lamps and electronics of the spectrophotometer to warm up (15 min) before use.
- ii. Use the correct wavelength.
- iii. Wipe the outer sides of the cuvette before measuring, using a smooth tissue paper.
- iv. Carry out the procedure in the correct order.
- v. Check the calibration of the spectrophotometer.
- vi. Close the door of the cuvette compartment before recording the OD.
- vii. Do not spill any solution inside the cuvette compartment.
- viii. Adjust the temperature of the incubator accurately.

Suggested Reading(s)

- i. Charles, C. Allain; Lucy, S. Poon; Cicely, S. G.; Chan, W. Richmond; Paul, C. Fu. (1974). Enzymatic determination of total serum cholesterol. *Clinical Chemistry*. 20, 20(4):470-5

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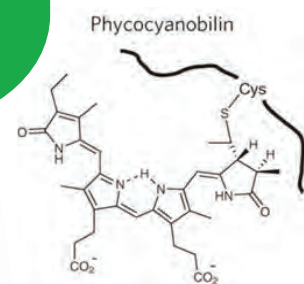
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SPECTROSCOPIC STUDIES ON PLANT PIGMENTS



1. Aim

To study the absorption spectrum of different plant pigments.

2. Introduction

Plants contain many different molecules directly or indirectly involved with photosynthesis, which may also impart color to the plant. Chlorophyll contains a porphyrin ring in its structure with a magnesium ion which accounts for the molecule's light absorbance.

Carotenoids are accessory pigments associated with many colors. Carrots get their color, which is often orange but is not restricted to orange, from carotene. Another type of carotenoid is anthocyanin. These molecules provide much of the red color in autumn foliage. They also contribute to blue/purple color of flowers, fruits and vegetables. The purplish color of a red cabbage is a result of the presence of anthocyanins. Anthocyanins change color with changes in pH, absorb UV light, which is used by plants to attract insects, and as a "sunscreen".

In this experiment, pigments from different plant samples are extracted and their ultraviolet-visible absorbance spectra are recorded with a spectrometer.

3. Materials Required

- 3.1. **Biological Materials:** Three plant materials (*Tradescantia leaves*, Spinach leaves, Marigold petals).
- 3.2. **Chemicals/Reagents:** Absolute ethanol.
- 3.3. **Equipment:** UV-Visible spectrophotometer.
- 3.4. **Glassware/ Plasticware:** Glass funnel, glass test tubes.
- 3.5. **Miscellaneous:** Cuvette, filter papers, test tube stand.

4. Procedure

Reagents/ Media preparations

4.1. Part One – Preparation of ethanol extracts:

- i. Take 5g (approx.) of each plant material (A, B and C) into three separate pestle and mortar.
- ii. Mark the pestle and mortar correspondingly as A, B and C.
- iii. Dry grind the plant material into a paste. Add a small portion (upto 10 mL) from the 30 mL ethanol to assist in grinding.
- iv. Pour the remaining ethanol to the mortar and mix the contents well using the pestle and keep aside for 5 min.

4.2. Part Two – Filtration of extracts:

- i. Arrange three test tubes marked A, B and C in the test tube stand.
- ii. Place the glass funnel with folded filter paper in each test tube.
- iii. Gently pour the coloured supernatant from each mortar into the funnel so that the filtrate will be collected in the corresponding labeled test tube.

4.3. Part Three – Spectrophotometry:

- i. Take the clear filtrate collected in each test tube to the spectrophotometer.
- ii. Set the spectrophotometer for baseline correction using ethanol as blank.
- iii. Pour the filtrate into the cuvette and record the spectrum (wavelength range 200 nm to 800 nm).
- iv. Obtain the print of the spectrum.

4.4. Part Four – Evaluation of Results:

- i. Read the spectrum carefully and understand the X, Y coordinates and the scale.
- ii. Notice that the spectrum is a continuous curve with peaks and valleys.
- iii. The peaks denote higher absorption of corresponding wavelengths while the valleys denote lesser absorption of corresponding wavelengths.
- iv. The highest point in the peak corresponds to the maximum absorption wavelength and is denoted as λ_{\max} . Each compound has its characteristic λ_{\max} . Since the extracts are a mixture of compounds, the spectrum will have more than one peak.
- v. With a pencil, draw a perpendicular line from each peak in the spectrum to the X axis so as to cut the X axis.
- vi. Using a graduated ruler, note the corresponding wave length at each intersection on the X axis (approximate to the nearest ten).

6. Observation

Record the λ_{\max} obtained for each of the spectrum in the following table (enter the wavelengths in the increasing order);

λ_{\max}	Sample A (Wavelength in nm)	Sample B (Wavelength in nm)	Sample C (Wavelength in nm)
1			
2			
3			
4			
5			
6			

7. Results

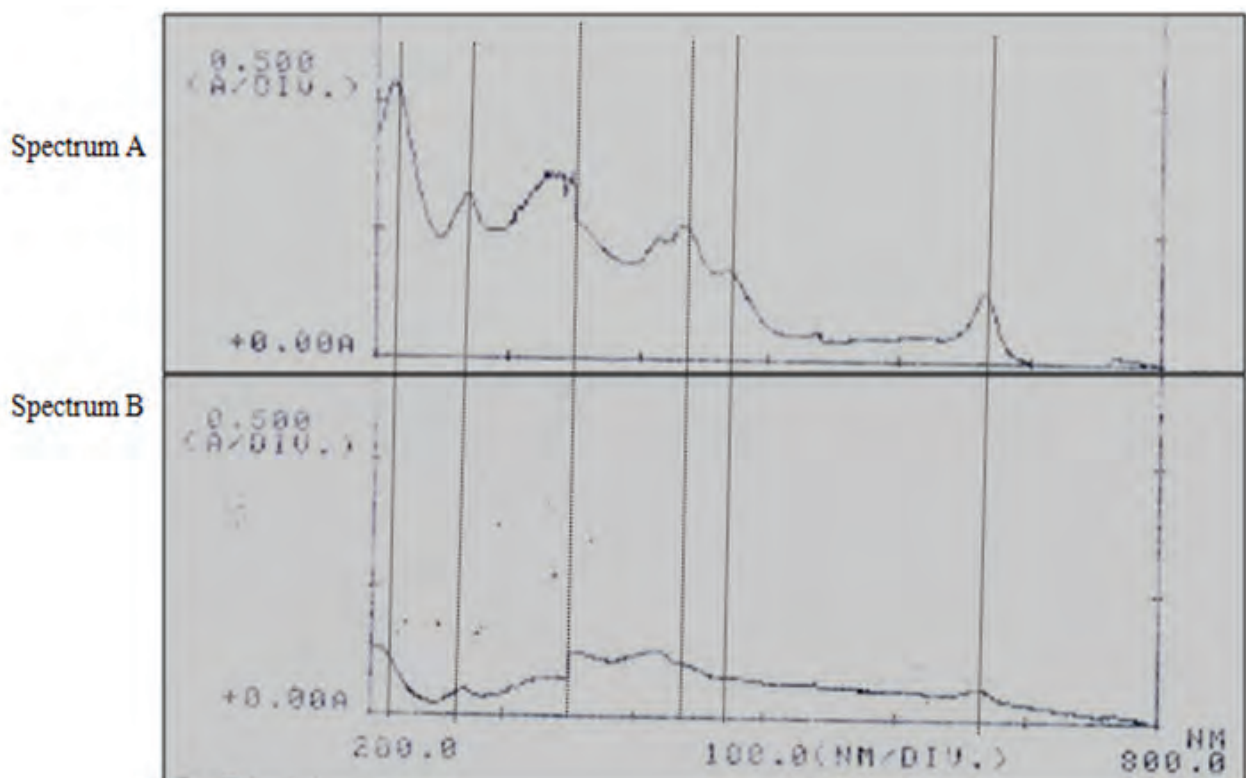
- i. The sample that has the maximum UV absorption is _____.
- ii. The sample that has the maximum absorption in red band is _____.
- iii. The sample that probably has the maximum chlorophyll content is _____.
- iv. The sample that has maximum UV protecting pigments is _____.
- v. The probable natural colour of the plant material could be;
 1. Sample A _____.
 2. Sample B _____.
 3. Sample C _____.

8. Precautions

- i. Grind the plant material well to obtain good extraction of pigments.
- ii. Keep all test tubes with extracts covered to avoid loss of solvents by evaporation.
- iii. Allow the lamps and electronics of the spectrophotometer to warm up (15 min.) before use.
- iv. Use the correct range of wavelength.
- v. Wipe the outer sides of the cuvette before measuring using a smooth tissue paper.
- vi. Check the calibration of the spectrophotometer.
- vii. Close the door of the cuvette compartment before recording the OD.
- viii. Do not spill any solution inside the cuvette compartment

9. Extension Activities

Given below are two spectrums of pigment extracts from the same leaf. Study them carefully and answer the questions;



Spectrum A is the spectrum of ethanol extract from leaves while spectrum B is the spectrum after the ethanol extract is treated with HCl. Select the most appropriate answer(s) for the following;

- A. Significant variations in the spectrum observed after HCl treatment is / are;
- Loss of absorption in the UV range
 - Loss of absorption in the yellow-red range
 - Loss of absorption in the blue range
 - All the above
- B. Variations in the spectrum after HCl treatment indicate
- Loss of nutrients from leaves
 - Loss of UV protection for the leaves
 - Higher transpiration rate
 - Loss of photosynthetic ability of leaves
 - Loss of natural colour of the leaves

Suggested Reading(s)

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- Biehler, E., Mayer, F., Hoffmann, L., Krause, E., & Bohn, T. (2010). Comparison of 3 spectrophotometric methods of carotenoid determination in fruits and vegetables. *J. Food Sci.* 75(1):C55-61.

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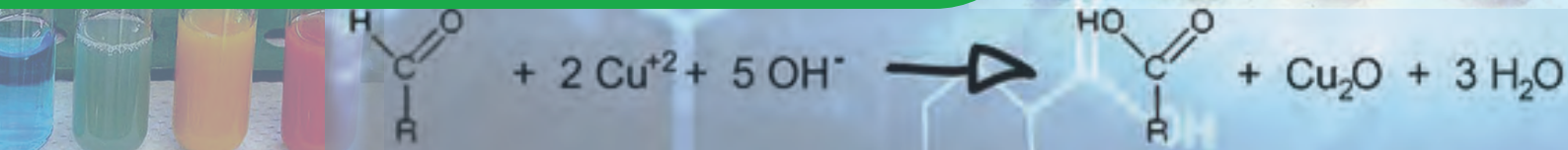
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Backdrop title picture: <http://en.es-static.us>
Title picture shows the structure of phycocyanobilin.
<https://images.nature.com>

QUALITATIVE ANALYSIS OF SUGARS



1. Aim

To analyze the given unknown carbohydrate compound on the basis of performing varied specific tests.

2. Introduction

2.1. Carbohydrates:

Carbohydrates are polyhydroxy aldehydes and ketones or substances that hydrolyze to yield polyhydroxy aldehydes and ketones. Aldehydes ($-\text{CHO}$) and ketones ($=\text{CO}$) constitute the major groups in carbohydrates.

Carbohydrates are mainly divided into monosaccharides, disaccharides and polysaccharides. The commonly occurring monosaccharides include glucose, fructose, galactose, ribose, etc. The two monosaccharides combine together to form disaccharides which include sucrose, lactose and maltose. Starch and cellulose fall into the category of polysaccharides, which consist of many monosaccharide residues.

2.1.1. Molisch's Test:

This is a common test for all carbohydrates larger than tetroses. The test is on the basis that pentoses and hexoses are dehydrated by conc. Sulphuric acid to form furfural or hydroxyl methyl furfural, respectively. These products condense with α -naphthol to form purple condensation product.

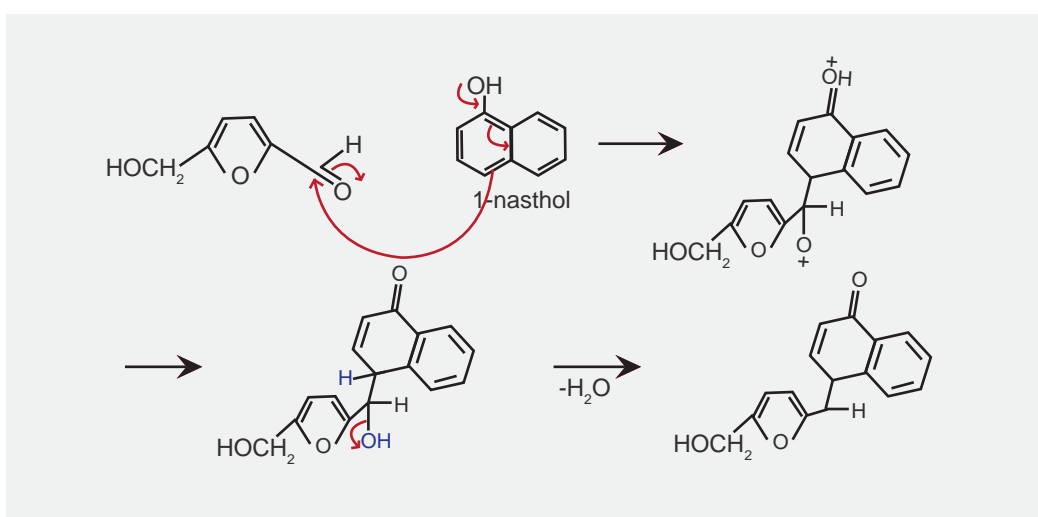


Fig. 1: Mechanism of Molisch's Test

2.1.2. Fehling's Test:

This forms the reduction test of carbohydrates. Fehling's solution contains blue alkaline cupric hydroxide solution, heated with reducing sugars gets reduced to yellow or red cuprous oxide and is precipitated. Hence, formation of the yellow or brownish-red colored precipitate helps in the detection of reducing sugars in the test solution.

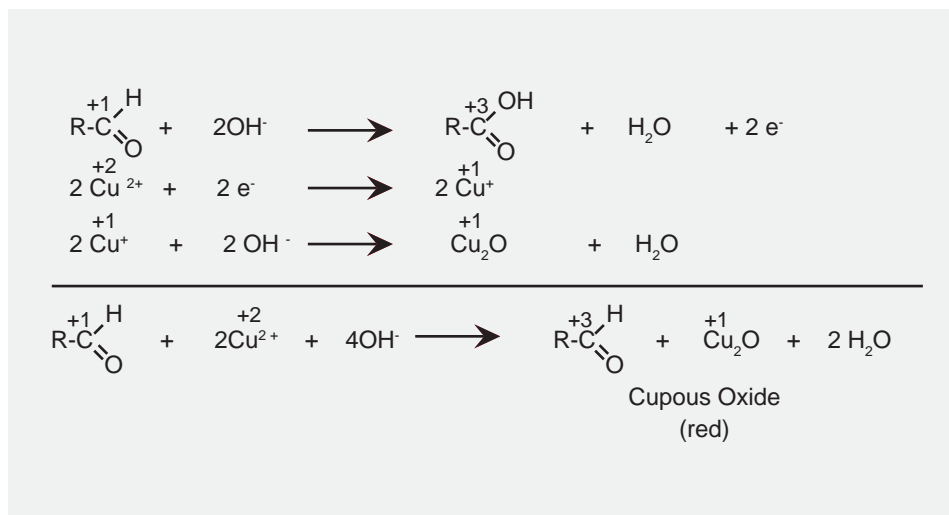


Fig. 2: Mechanism of Fehling's Test

2.1.3. Benedict's Test:

Free aldehyde or keto group in the reducing sugars reduce cupric hydroxide in alkaline medium to red colored cuprous oxide. Depending on the concentration of sugars, yellow to green color is developed. All monosaccharides are reducing sugars as they all have a free reactive carbonyl group. Some disaccharides, like maltose, have exposed carbonyl groups and are also reducing sugars, but less reactive than monosaccharides.

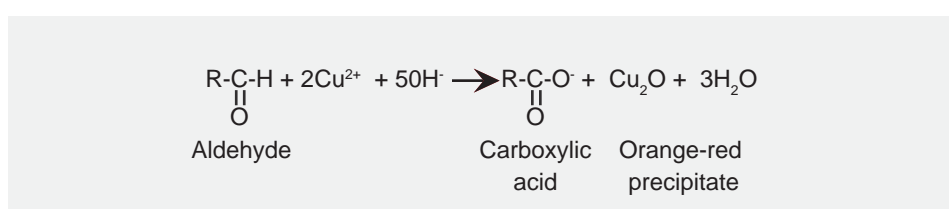


Fig. 3: Benedict's Reaction

2.1.4. Barfoed's Test:

Barfoed's test is used to detect the presence of monosaccharide (reducing) sugars in solution. Barfoed's reagent, a mixture of ethanoic (acetic) acid and copper (II) acetate, is combined with the test solution and boiled. A red copper (II) oxide precipitate is formed will indicate the presence of reducing sugar. The reaction will be negative in the presence of disaccharide sugars because they are weaker reducing agents. This test is specific for monosaccharides. Due to the weakly acidic nature of Bardfoed's reagent, it is reduced only by monosaccharides.

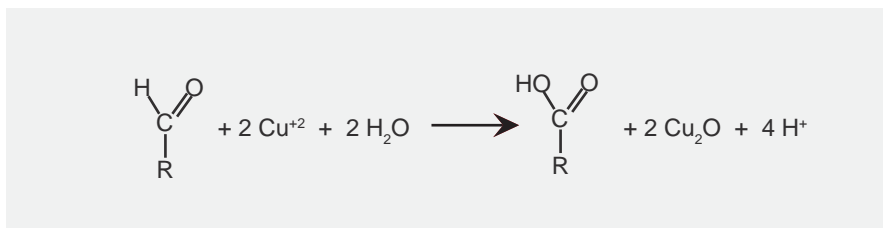


Fig. 4: Barfoed's Test

2.1.5. Seliwanoff's Test:

It is a color reaction specific for ketoses. When conc. HCl is added, ketoses undergo dehydration to yield furfural derivatives more rapidly than aldoses. These derivatives form complexes with resorcinol to yield deep red color. The test reagent causes the dehydration of ketohexoses to form 5-hydroxymethyl furfural. 5-hydroxy methyl furfural reacts with resorcinol present in the test reagent to produce a red product within two minutes (reaction not shown). Aldohexoses reacts so more slowly to form the same product.

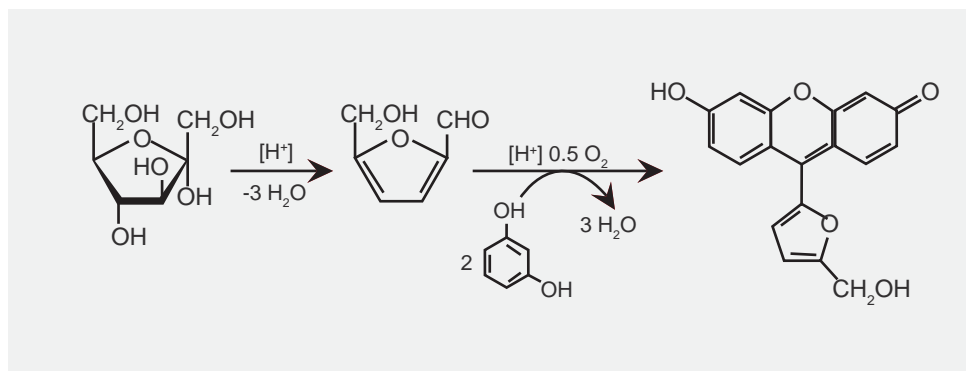


Fig. 5: Seliwanoff's Test

2.1.6. Bial's Test:

Bial's test is used to distinguish between pentoses and hexoses. They react with Bial's reagent and are converted to furfural. Orcinol and furfural condense in the presence of ferric ion to form a colored product. Appearance of green colour or precipitate indicates the presence of pentoses and formation of muddy brown precipitate shows the presence of hexoses.

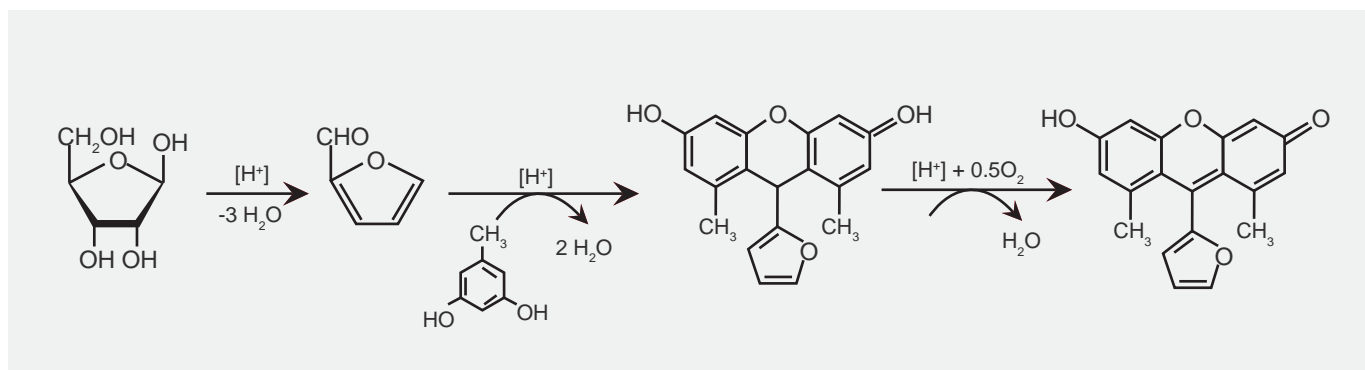
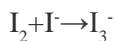


Fig. 6: Bial's Test

2.1.7. Iodine Test:

This test is used for the detection of starch in the solution. The blue-black colour is due to the formation of starch-iodine complex. Starch contains polymer of α -amylose and amylopectin which forms a complex with iodine to give the blue black colour.



Iodine slides into starch coil to give blue black colour

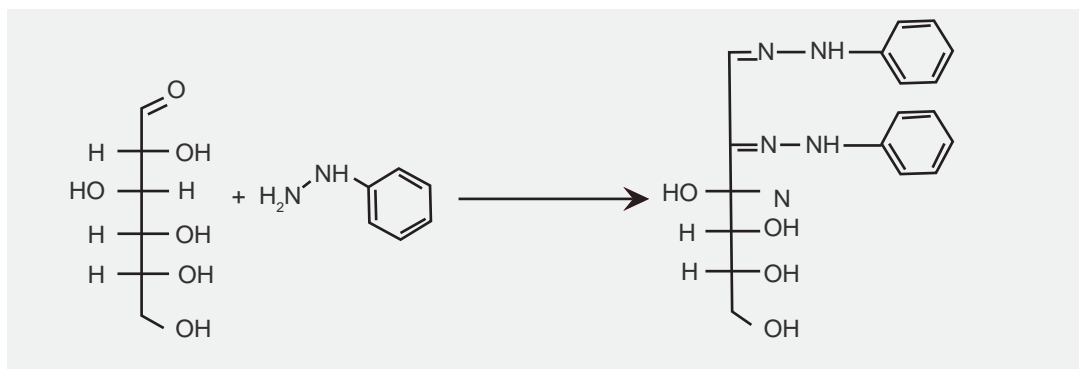


Fig. 7: Iodine Test

3. Materials Required

3.1. Chemicals/Reagents: Molisch's reagent, iodine solution, Fehling's reagent A, Fehling's reagent B, Benedict's qualitative reagent, Barfoed's reagent, Seliwanoff's reagent, Bial's reagent, phenylhydrazine hydrochloride, sodium acetate, glacial acetic acid, conc. H_2SO_4 , glucose, fructose, sucrose, starch.

3.2. Glassware/Plastic ware: Test tubes, test tube holder, water bath, nickel spatula, semi micro dropper, microscopic slides, watch glass.

3.3. Equipment: Weswox microscope with halogen lamp.

4. Procedure

Qualitative Analysis of Sugar

S. No	Experiment	Observation	Inference
1.	Solubility Test: A pinch of sugar is dissolved in 2 mL of distilled water.	a. Soluble in cold water b. Insoluble in cold water	Presence of Monosaccharides Presence of Polysaccharides
2.	Molisch's Test: Add 2 drops of Moloch's reagent to 2 mL of test solution. Pour 1 mL of conc. H_2SO_4 above the sides of the test tube.	Violet colour ring is formed at the junction of the two liquids	Presence of carbohydrates.

3.	Iodine Test: Add 1 to 2 drops of Iodine solution to 2-3 mL of sugar solution.	a. Blue colour is obtained b. Red colour is obtained c. Brown colour is obtained d. No characteristic change	a. Presence of Starch b. Presence of Dextrin c. Presence of Glycogen d. Absence of polysaccharides
4.	Fehling's Test: Add a few drops of sugar solution at a time to 5 mL of Fehling's reagent and heat it in boiling water bath.	Brownish red colour is obtained.	Presence of reducing sugar.
5.	Benedict's Test: 1 mL of Benedict solution and 1 mL of test solution is heated in a boiling water bath.	Brick red colour is obtained	Presence of reducing sugar.
6.	Barfoed's Test: To 1 mL of sugar solution add 2 mL of Barfoed's reagent and heat in a water bath.	Reddish tinch is formed at the bottom of the tube within 5 min of heating	Presence of Monosaccharides
7.	Bial's Test: To 1 mL of sugar solution add 2 mL of Bial's reagent and heat it in boiling water bath.	Green colour is obtained	Presence of Pentose sugar
8.	Seliwanoff's Test: To 1 mL of sugar solution add 2 mL of Seliwanoff's reagent and heat in boiling water bath. Prolonged heating is avoided.	Cherry red colour is obtained	Presence of Keto sugar
9.	Osazone Test: Add 1 mL of glacial acetic acid to 5 mL of sugar solution. Then add a pinch of phenyl hydrazine hydrochloride and double the amount of sodium acetate. Mix well and keep it in a boiling water bath until the solid dissolves. Filter in another test tube and keep the filtrate in a boiling water bath. Note the time taken for the formation of crystals and observe the crystals under a microscope.	a. Needle shaped crystals are formed after 10 min b. Needle shaped crystals are formed within 5 to 10 minutes of heating c. Broad glass piece shaped crystals are formed within 30 min d. Sunflower (star) shaped crystals are formed within 30 min e. Hedge-hog (Puff) shaped crystals are formed after 45 minutes f. White coloured irregular shaped crystals are formed after 45 minutes	a. Presence of Glucose is confirmed. b. Presence of Fructose is confirmed. c. Presence of Galactose is confirmed. d. Presence of Maltose is confirmed. e. Presence of Lactose is confirmed. f. Presence of Mannose is confirmed.

5. Expected Observations and Results

S.No	Test	Observation	Result
1.	Molisch Test	Purple ring at the junction of two liquids	All carbohydrates
2.	Benedict's Test	Brick red precipitate	Reducing carbohydrate
3.	Barfoed's Test	Scanty red precipitate at the bottom of test tube	Reducing mono saccharide
4.	Seliwanoff's Test	Cherry red color is not observed	Ketohexose containing disaccharide
5.	Osazone Test	a. Needle shaped crystals, b. Rhombic plate shaped crystals c. Sunflower shaped crystals d. Powderpuff/hedgehog crystals.	a. Confirmatory test for glucose, fructose and mannose. b. Confirmatory test for Galactose. c. Confirmatory test for Maltose. d. Confirmatory test for Lactose.

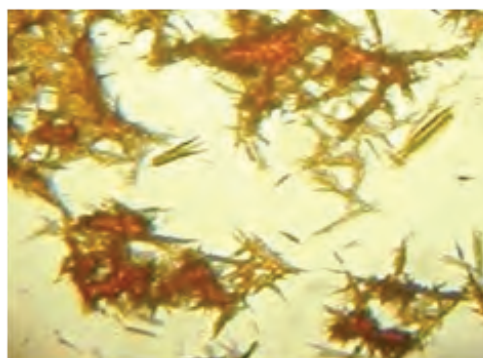
6. Result and Interpretation

The given unknown sugar is found to be _____.

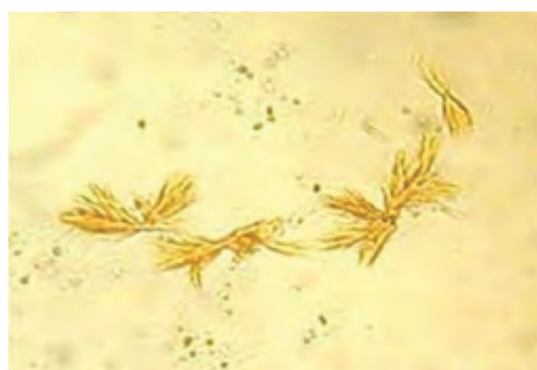
In this exercise, students explore a diversity of carbohydrate chemistry. The tests are helpful in classifying as the reducing/non-reducing sugars viz., Benedict's (reducing sugars), Barfoed's (monosaccharides), Seliwanoff's (ketoses) etc.



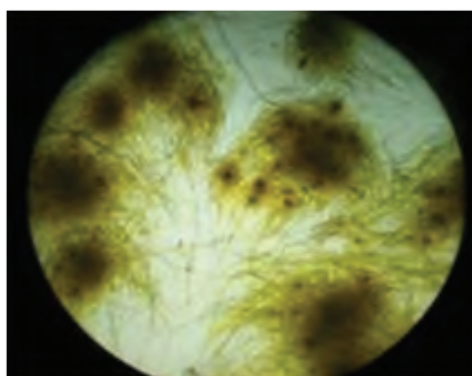
Needle shaped Crystal



Rhombic plate Crystal



Sun flower shaped Crystal



Powderpuff / hedgehog Crystal

Fig. 8: The shape of different crystals pertaining to various carbohydrates

7. Precautions

- i. Always wear lab coat and gloves when you are in the lab. Confirm the availability of all the reagents required for the experiment before performing the tests. If not available, prepare the reagents using appropriate chemicals needed for reagent preparation.
- ii. Care should be taken while handling caustic acids like conc. sulphuric acid [H₂SO₄]. The acid should be opened and used in FUMEHOOD only. Accidental spill will cause severe burns and itching. If so, wash the spilled area with cold water and inform the lab assistant immediately.
- iii. Make sure that the water level in the water bath is sufficient [nearly half the volume], then switch on the water bath and adjust to the required temperature. Always use test tube holder to hold the test tubes.
- iv. Care should be taken to add the reagents and the test solutions in required proportions to obtain the exact result within the time period. The droppers used should not be mixed between the reagents, always use separate droppers for each reagent.
- v. The color formed will depend upon the quality of the reagents. If commercially available reagents are used, ensure that it is not kept open for a long time.
- vi. Clean the test tubes and glassware with soap and distilled water. Recap the reagent bottles once the experiment is completed.

8. Trouble shooting and bottlenecks expected

- i. Problem - False Positive or False Negative results.
- ii. Possible Cause-Procedure was not carried out properly.
- iii. Solution –The entire procedure should be followed carefully.
- iv. Molisch's test-Addition of conc. H₂SO₄ at an upright position of the test tube leads to non appearance of violet coloured ring.

Suggested Reading(s)

- i. Rao, B. S., & Anshan, V. D. (2006). *Experimental biochemistry: a student companion*.
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- iii. <http://himedialabs.com/TD/HTBC002.pdf>
- iv. <http://www.harpercollege.edu/tm-ps/chm/100/dgodambe/thedisk/carbo/molisch/molisch.htm>
- v. <https://biochemistryisagoodthing.wordpress.com/2013/02/17/lab-review-1/>
- vi. <https://www.healthaliciousness.com/articles/foods-highest-in-carbohydrates.php>

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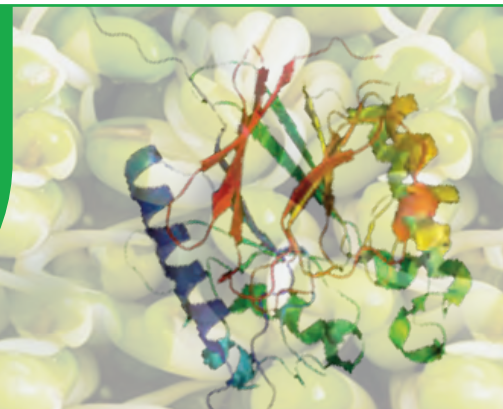
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PURIFICATION OF ACID PHOSPHATASE FROM SPROUTED MOONG



- A. Preparation of crude extract from sprouted moong beans and assay of enzyme activity.
- B. Partial purification of acid phosphatase using ammonium sulphate fractionation.
- C. Purification of acid phosphatase by ion exchange chromatography.

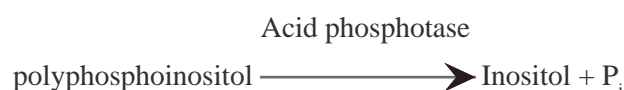
A. Preparation of crude extract from moong beans and assay of enzyme activity.

A.1. Aim

To prepare crude extract from sprouted moong beans and assay of enzyme activity.

A.2. Introduction

Acid phosphatase is a phosphohydrolase. It is active at acidic pH. The enzyme commission number for acid phosphatase is 3.1.3.1. The molecular mass of wheat germ acid phosphatase is 58 KDa. It is a fairly nonspecific enzyme. The substrate for acid phosphatase in many bean sprouts is polyphosphoinositol which is present in the seed coat. Acid phosphatases (APases) catalyze the hydrolysis of inorganic phosphate (P_i) from a broad range of P_i -monoesters with an acidic pH optimum. The liberated P_i is reassimilated into cellular metabolism via mitochondrial or chloroplastic ATP synthases of respiration or photosynthesis, respectively. Eukaryotic APases exist as a wide variety of tissue- and/or cellular compartment-specific isozymes that display marked differences in their physical and kinetic properties. Increases in intracellular (vacuolar) and secreted APase activities are useful biochemical markers of plant nutritional P_i deficiency. The protocols for protein extraction, APase activity determination and measurement of soluble protein concentration from plant tissues or cell suspension cultures are presented.



For enzyme assay, the substrate used is para nitro phenol phosphate



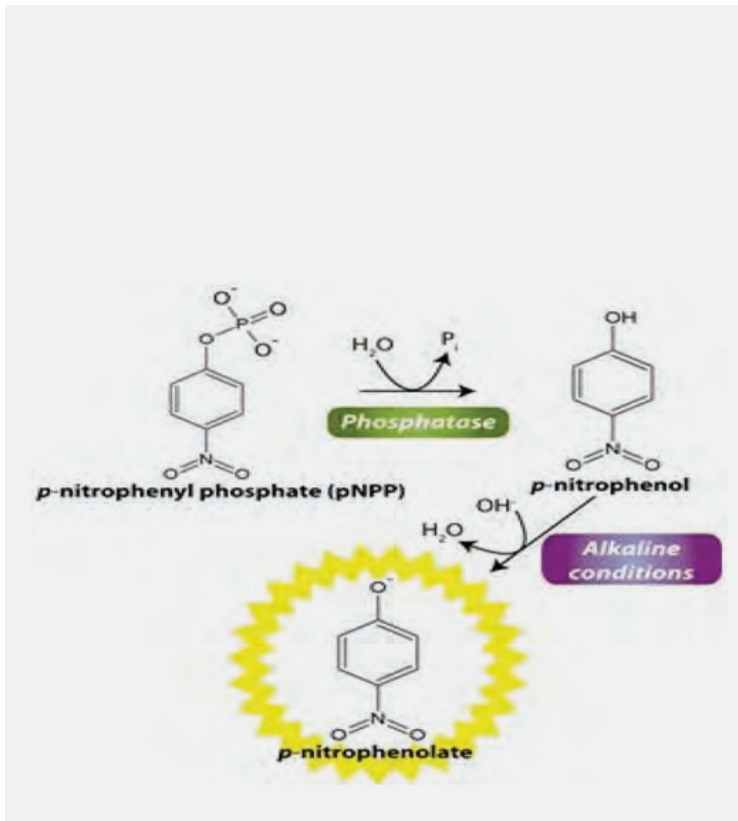


Fig. 1: Diagram showing steps taking place during reaction source (<https://www.gbiosciences.com/Bioassays/Phosphatase-Assay>)

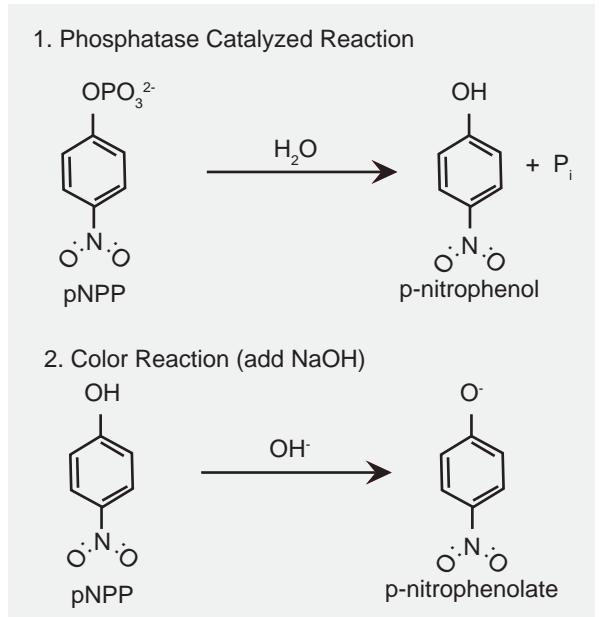


Fig. 2: (1) APase activity is often assayed spectrophotometrically at 405-410 nm by determining the amount of pNP produced following the hydrolysis of P_i from pNPP. (2) Addition of KOH/NaOH after a specified assay time (e.g., 10 min) serves to stop the APase reaction while simultaneously converting the product p-nitrophenol into the yellow colored p-nitrophenolate ($\lambda_{\text{max}} = 405-41$ nm)

(Source: <http://www.bio-protocol.org/e889>)

2.1 Enzyme Assays:

A useful enzyme assay must meet four criteria:

- i. Absolute specificity
- ii. High sensitivity
- iii. High precision
- iv. Convenience

Enzyme assays are of two types:

- i. Continuous assay
- ii. Discontinuous assay

2.2 Continuous Assays:

Continuous assays are most convenient, with one assay giving the rate of reaction with no further work necessary. Here, the reaction is not terminated, however the absorbance is measured at regular intervals. The cuvette with enzyme and substrate is placed in the spectrophotometer and the absorbance is measured at λ_{max} .

2.3 Discontinuous Assays:

Here, the reaction is terminated at one step by adding inhibitor after incubation and then the absorbance is measured. The concentration of product formed can be calculated using the standard curve.

2.4 Protein Purification:

The deep study of many biological systems has involved purification of one or more of the system components. The five basic steps of purification are as follow:

- i. Development of suitable procedures.
- ii. Selection of the best source from which the molecule may be purified.
- iii. Solubilization of the desired molecule.
- iv. Stabilization of the molecule repeatedly at each stage of its purification.
- v. Development of a series of isolation and concentration procedure.

2.5 Method of Solubilization:

Solubilization is required for any protein to be purified, because all the isolation procedures commonly used operate only in aqueous solution. A wide range of solubilization methods exist, and the one chosen must take into account the characteristics of the cells to be broken and the nature of the proteins to be subsequently isolated. A second consideration that strongly influences the choice of solubilization procedures is the sample size. The methods used to break a few milligrams of cultured animal cells are likely to be of little use in processing several pounds of yeast or beef liver.

2.8 Ultrasonic waves:

Sonic oscillators provide a very efficient means of breaking cells or organelles. They are effective against bacteria and yeast at sufficiently long periods of application. The times may be shortened, however by including glass beads in the cell suspension. Sonicators are usually composed of 2 main units: an electric generator which produces an ultrasonic signal of high intensity and a transducer which transmits these waves into the solution in contact with it. It is the shock and vibration set up by these sound waves that brings about tissue destruction or moves the five glass beads. The major drawback is the large amount of heat generated at the transducer.

2.9 Presser:

There are 3 major types of presses that may be used to break open microorganisms or cells. They are the Hughes, French and Eton presses. All 3 of these instruments function by placing a cell suspension of small volume (5-50 mL) under 4000-8000 lb pressure and forcing it through a small opening. Breakage is brought about by shearing of the cells as they pass through the small onfice. This method is both gentle and thorough, but its application is restricted by its limited sample size range.

A.3. Materials Required

3.1 Biological Material: Moong beans for crude extract.

3.2 Chemicals/Reagents: Acetate buffer 1 M (stock solution) and 0.1 M (working) pH 5.5, 0.5 M KOH, PNPP (p-nitrophenylphosphate).

3.3 Equipment: Centrifuge, UV- Visible spectrophotometer, pipettes.

3.4 Glassware/Plastic ware: Cuvette, test tubes, beaker, conical flask.

3.5 Miscellaneous: Tissue paper, mortar and pestle.

A.4. Procedure

4.1 Enzyme extract preparation:

- Prepare the 0.1 M acetate buffer (pH 5.5) from stock of 1 M acetate buffer.
- Prepare the enzyme extract in 0.1 M acetate buffer (pH 5.5) by grinding the moong beans sprouts using a mortar and pestle.
- Homogenize the moong beans in acetate buffer and centrifuge it at 5000 rpm for 10-15 min.
- Separate the pellet and the supernatant. The supernatant is collected as crude extract, as supernatant contains enzyme.

4.2 acid phosphatase assay:

- Add 2.7 mL of Acetate buffer 0.1 M concentration in test tubes along with 0.1 mL of enzyme and add 0.2 mL of substrate.
- Add 2 mL 0.5 M KOH in the control test tube prior to incubation to cease enzyme activity.
- Incubate for 10 minutes 37 °C.
- Add 2 mL of 0.5 M KOH solution after incubation to each test tube labeled Test.
- Use 1:10 and 1:100 dilutions of the enzyme. Make 1: 100 dilution of enzyme in both acetate buffer and water. Observe variation enzyme activity.
- Measure the absorbance and calculate the enzyme activity.

A.5. Observation

Observation table:

Blank solution = 3 mL buffer + 2 mL KOH

S.No	Sample	Acetate Buffer (mL)	Substrate (PNPP) (mL)	Enzyme (mL)		0.5 M KOH	Absorbance for PNP (at 405 nm)	Absorbance T-C
1	Blank	2.7	0.2	0.1	Incubate at 37° C for 10 min	2 mL		
2	Neat Test Control							
3	Dilution in buffer 1:100 (T) 1:100 (C)							
4	Dilution in buffer 1:10 (T) 1:10 (C)							

* T= Test, C= Control

A.6. Calculation

$$\text{Enzyme Activity} = \frac{\text{Optical Density (T - C)} \times \text{Dilution factor} \times \text{Assay volume} \times 10^3}{\text{Molar extinction coefficient} \times \text{Time} \times \text{Vol. of enzyme}}$$

Activity is measured in $\mu\text{M}/\text{min} = 1 \text{ IU}$ (international Unit)

1 IU is the amount of enzyme required to convert 1 μM concentration of substrate into the product per minute under optimum conditions (of pressure, pH and temperature).

$$\text{SI unit} = \text{Katal} = \frac{\text{(moles of substrate)}}{\text{second}}$$

Where 1 Katal = 6×10^7 I.U.

A.7. Result

- The enzyme activity of crude sample of mung beans was found to be _____ $\mu\text{moles ml}^{-1} \text{ min}^{-1}$.
- Enzyme activity for 1: 100 dilution in acetate buffer was found to be _____ $\mu\text{moles ml}^{-1} \text{ min}^{-1}$
- Enzyme activity for 1: 10 dilution in acetate buffer was found to be _____ $\mu\text{moles ml}^{-1} \text{ min}^{-1}$

A.8. Precautions

- Clean the cuvette thoroughly after every reading.
- Pipetting should be done carefully.
- Set the appropriate filter of the Spectrophotometer.
- Calibration must be done using the blank solution.

B. Partial purification of acid phosphatase using ammonium sulphate fractionation.

B.1. Aim

To partially purify acid phosphatase from crude extract using ammonium sulphate fractionation and dialysis of fractionated samples.

B.2. Introduction

2.1. Salt Precipitation:

The solubility of the protein varies with the ionic strength of the solution. Ionic strength is related to the concentration of salt in the solution.

On addition of salt two phenomena can take place:-

- Salting in
- Salting out

2.2. Salting In:

At very low concentration of salt, the salt ions interact with the protein molecules. This way, they shield the counter ions on protein molecules and prevent aggregation of the protein, thus increasing the solubility of the protein in the solvent. This process is known as salting in.

2.3. Salting Out:

In the interaction between the polar amino acids and the solvent can be prevented, thus destabilizing the solution (solvation shell), then the protein molecules will aggregate and this way, the protein can be precipitated out. Salt like ammonium sulphate will quench the solvent molecules and thus, there will not be enough solvent for protein molecules to dissolve.

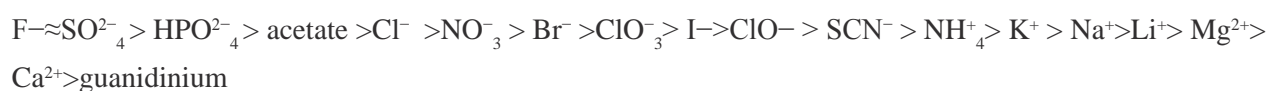
Hydrophobic amino acids that are present in the core are exposed on the surface and hydrophobic interactions between the protein molecules cause aggregation which leads to precipitation of the protein. This process is referred to as salting out.

2.4. Desalting:

Once the protein has precipitated out, the salt molecules need to be removed before proceeding to the next step of purification. Removal of salt molecules refers to desalting. If a solution of proteins is separated from bathing solution by a semipermeable membrane, small molecules and ions can pass through the semipermeable membrane to equilibrate between protein solution and bathing solution, called dialysis bath or dialysate. This method is useful and therefore helpful in desalting. For desalting, the solution should be kept overnight and the buffer should be changed every 4-5 hours.

2.5. Hofmeister Series:

The Hofmeister series or lyotropic series is a classification of ions in order of their ability to salt out or salt in proteins. The effects of these charges were first worked out by Franz Hofmeister, who studied the effects of cations and anions on the solubility of proteins. Anions appear to have a larger effect than cations, and are usually ordered as follows:

**2.6. Nomogram:**

It is a table that gives an idea of the amount of salt that has to be added in per liter of solution to obtain desired concentration for fractionation of proteins. It is an optimized table. Most proteins get precipitated in 30%-40% range of $(\text{NH}_4)_2\text{SO}_4$. (See Annexure Table 1: for nomogram)

2.7. Determination of Enzyme Activity:

Ammonium sulphate fractionation can be used for purification of enzymes. The proteins which are unwanted other than acid phosphatase will be removed by precipitating with different concentrations of ammonium sulphate. After centrifugation the pellets which are obtained are named as P_1 and P_2 .

Before fractionation enzyme activity = E

Ideally, assuming no loss of enzyme activity during fractionation,

$$E = P_1 + P_2 + \text{supernatant}$$

However, this never happens as some amount of enzyme is always lost during purification process.

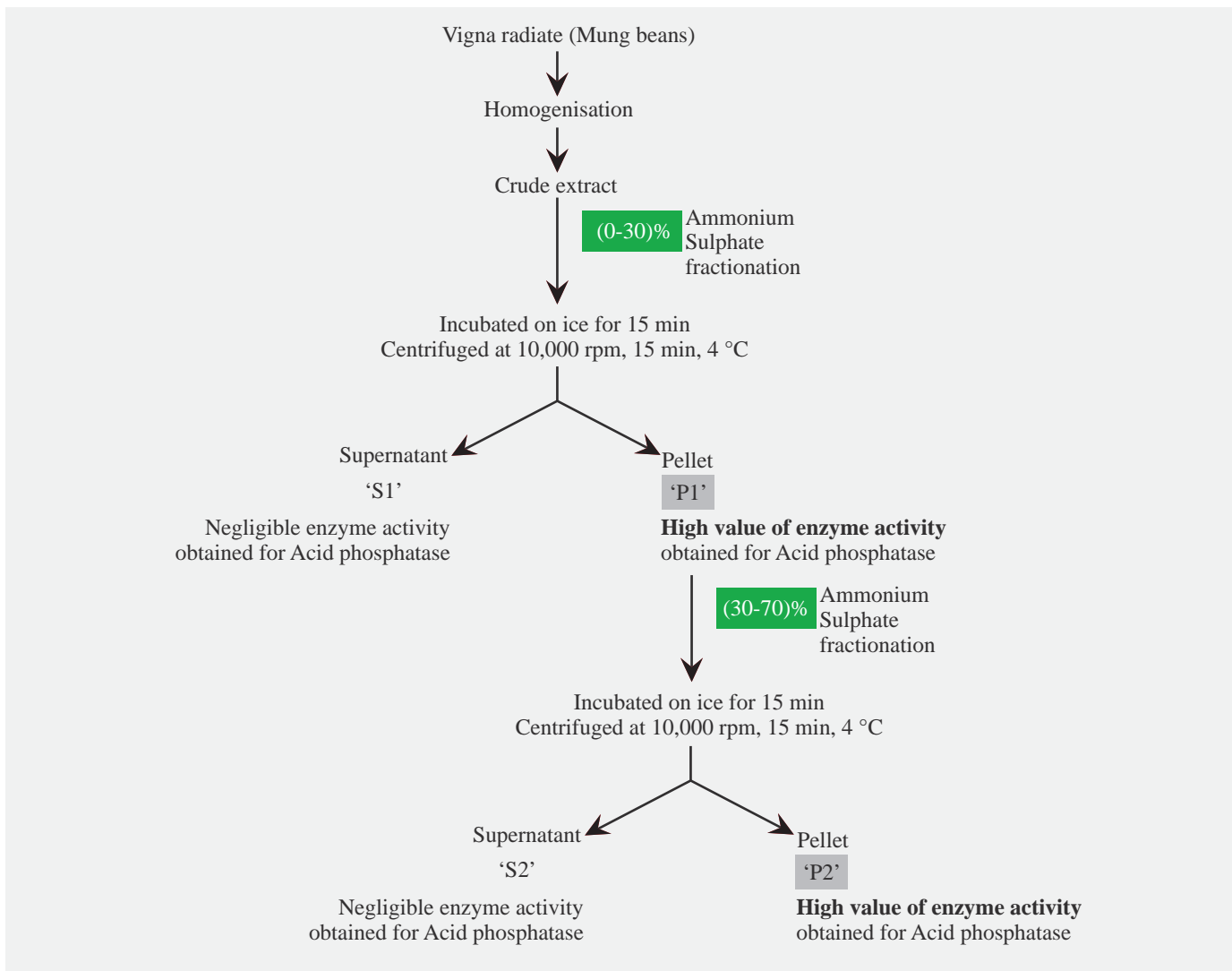


Fig. 3: Flow chart for salting out

2.8. Specific Activity:

It is the activity of enzyme per milligram protein in the sample.

$$\text{Specific activity} = \frac{\text{Total activity } \mu\text{mol min}^{-1}\text{mg}^{-1}}{\text{mg protein in the sample}}$$

- In every step of purification, contaminating proteins are eliminated, so the amount of protein decreases.
- Therefore, specific activity would increase at each step as some proteins are being removed.
- Increasing specific activity

2.9. Protein Estimation (Lowry's Method):

Protein is estimated from samples by Lowry's method.

2.10. Dialysis:

Dialysis is a technique to remove the salt ions present in protein sample which are employed during purification process. It is the random, thermal movement of molecules in solution that leads to the net movement of molecules from an area of higher concentration to a lower concentration until equilibrium is reached.

In dialysis, a sample and buffer solution (called dialysate), separated by a semi permeable membrane that causes differential diffusion patterns, thereby permitting the separation of molecules in both the sample and dialysate. Due to the pore size, large molecules in the sample cannot pass through the membrane, thereby restricting their diffusion from the sample chamber. To enhance the separation of the proteins in the bag (tube) from other impurities such as salt we can also take advantage of the equilibrium constant. In an equilibrium environment, the salt will flow through the membrane, until the concentration outside the dialysis tube is equal to the concentration inside. At this point there is no net flow of the salt through the membrane because equilibrium reached. But if we add in a new solution buffer, then the remaining amount of salt will flow out of the dialysis tube until the concentration of salt in the new buffer equals the concentration in the tube. If we keep replacing the buffer, their will enhance the purification of the proteins inside.

B.3. Materials Required

3.1. Biological Material: Moong beans extract, samples for dialysis.

3.2. Chemicals/Reagents: Ammonium sulphate, acetate buffer 0.1 M, 1 M (for dialysis).

3.3. Equipment: Centrifuge, UV- Visible spectrophotometer, pipettes, magnetic stirrer.

3.4. Glassware/Plastic ware: Cuvette, test tubes, beaker, conical flask, glass rod, dialysis tube.

3.5. Miscellaneous: Tissue paper, water, scissors.

B.4. Procedure

4.1. Ammonium sulphate fractionation:

- i. Prepare the enzyme extract as given in the step “Enzyme extract preparation”.
- ii. Place the crude sample on ice and add $(\text{NH}_4)_2\text{SO}_4$ gradually for 0-30% precipitation.
 - a. For fraction with 0-30% salt saturation 176 g of $(\text{NH}_4)_2\text{SO}_4$ in 1 L solution (from nomogram), hence
 $1 \text{ mL} = 0.176 \text{ g of } (\text{NH}_4)_2\text{SO}_4$
 For desired vol. (mL) of crude sample = $0.176 \times \text{vol.} = \underline{\hspace{2cm}} \text{ g.}$
 - b. For fraction with 30-70% salt saturation 273 g of $(\text{NH}_4)_2\text{SO}_4$ in 1 L solution (from nomogram), hence
 $1 \text{ mL} = 0.273 \text{ g of } (\text{NH}_4)_2\text{SO}_4$
 For desired vol. (mL) of S1 (Supernatant) = $0.273 \times \text{vol.} = \underline{\hspace{2cm}} \text{ g.}$

NOTE: See Annexure Table 1: Nomogram, at the end of the protocol.

- iii. Add respective amount of $(\text{NH}_4)_2\text{SO}_4$ to crude extract of acid phosphatase. Stir properly. Incubate on ice for 15 min.
- iv. Centrifuge the solution at 10,000 rpm at 4 °C. Separate the supernatant (S_1). Collect the pellet (P_1) as well in acetate buffer.
- v. Add the required amount of salt for 30%-70% concentration to the supernatant (S_1). After adding the salt, centrifuge the solution. Collect S_2 and P_1 separately.
- vi. Once all the fractions are obtained, calculate the enzyme activity for each fraction.
- vii. Carry out the protein estimation using Lowry’s method and determine the amount of protein in each fraction
- viii. Determine the specific activity and percentage yield and recovery of enzyme and protein.

4.2. For Dialysis:

- i. Wash the dialysis tube thoroughly with distilled water and boil in H₂O for 30 mins.
- ii. After boiling, let the tube to cool down at normal temperature and wash with acetate buffer thoroughly to pretreat the tubes.
- iii. After washing the tube with acetate buffer for 3-4 times tie the tube from one end tightly using thread.
- iv. Check the knot for leakage using blotting paper.
- v. Then transfer the sample in the tube using autopipettes.
- vi. After transferring the samples from previous experiment in separate tubes, tie the other end also tightly
- vii. Cross check the tube to prevent any leakage of the samples.
- viii. Label the tubes as P₁, P₂, S₁, and S₂.
- ix. Tie the dialysis tubes with glass rod to suspend the tubes in 0.1 M buffer on the magnetic stirrer for dialysis over night.
- x. Next morning, remove the samples (tubes) from the 0.1 M acetate buffer and place them in 1 M acetate buffer, leave for dialysis for another 5 hrs on magnetic stirrer.
- xi. Take the samples out from dialysis tubes and transfer them in the eppendorfs, label them.

B.5. Observations

5.1. Ammonium Sulphate Fractionation:

- i. Volume of crude = -----mL
For 0-30% fractionation, (NH₄)₂SO₄ required = 0.176g/mL of extract
For -----mL, (NH₄)₂SO₄ required = -----g
- ii. Volume of S₁ (supernatant) = -----mL
For 30%-70% fractionation, (NH₄)₂SO₄ required = 0.273g/mL of extract
For -----mL, (NH₄)₂SO₄ required =-----g

5.2. Lowry's method for Protein Estimation:

BSA stock: - 100µg/mL

S.No	BSA conc. (µg/mL)	Vol. (mL)	Vol. of H ₂ O (mL)	Analytical Reagent (mL)		Folin's Reagent (mL)		Abs. (660 nm)	Ab. (660 nm) Sample
1	Blank	0		2.5	Incubate at room temperature for 10 min	0.25	Incubate at room temperature for 30 min		Crude__
2	10							Crude__	
3	20							P1_____	
4	40							P2_____	
5	60							S1_____	
6	80							S2_____	
7	100								

5.3 Enzyme Activity of Acid Phosphatase

S.No	Sample	Acetate Buffer (mL)	Substrate PNPP (mL)	Enzyme (mL)		0.5 M KOH (mL)	Absorbance (405 nm)	Enzyme Activity ($\mu\text{moles ml}^{-1} \text{min}^{-1}$)
1	Blank	3			Incubate At 37o C For 10 minutes	2.0		
2	Crude(T)	2.7	0.2	0.1				
3	Crude(C)							
4	P1 (T)							
5	P1 (C)							
6	S1 (T)							
7	S1 (C)							
8	S2 (T)							
9	S2 (C)							
10	P2 (T)							
11	P2 (C)							

*1:10 Dilution of P₂ may be used and other samples may be used in neat form according to the need.

*In control tubes, add KOH before incubation

$$\text{Enzyme Activity} = \frac{\text{O.D.} \times \text{Dilution factor} \times \text{Assay volume} \times 10^3}{\text{Molar extinction coefficient} \times \text{Time} \times \text{Vol. of enzyme}}$$

$$\text{Percentage yield} = \frac{\text{Total Activity} \times 100}{\text{Total activity of crude}}$$

$$\text{Percentage recovery} = \frac{\text{Total Protein} \times 100}{\text{Crude Protein}}$$

$$\text{Fold Purification} = \frac{\text{Specific Activity}}{\text{Specific activity of crude}}$$

5.4. Calculation Table:

Sample	Total Volume	Activity	Total Activity	Protein mgmL ⁻¹	Total Protein	Specific Activity	% yield	% Recovery	Fold purification
Crude									
(0-30%) P1 S1									
(30-70%) P2 S2									

5.5. Table for dialyzed samples:

Sample	Total Volume	Activity	Total Activity	Protein mgmL ⁻¹	Total Protein	Specific Activity	% yield	% Recovery	Fold purification
Undialyzed									
Dialyzed									

B.6. Result.

6.1. Ammonium sulphate fractionation is carried out to purify acid phosphatase from moong beans extract.

Following observations can be made over the course of the experiment:-

- In the crude, total protein is _____ mg and total activity is _____ $\mu\text{moles mL}^{-1}\text{min}^{-1}$.
- In the 1st round of Ammonium Sulphate Fractionation (0-30%), however the total protein decreases to _____ mg and the total activity decreases to _____ $\mu\text{moles mL}^{-1}\text{min}^{-1}$. This indicates that acid phosphatase is not present in the fraction.
- In the 2nd round of Ammonium Sulphate Fractionation, the total activity was found to be _____ $\mu\text{moles mL}^{-1}\text{min}^{-1}$. The high activity P₂ indicates that Acid phosphatase has precipitated in this fraction. The fold purification is _____ for this round of precipitation.
- Also, before dialysis, the total protein in P₂ was estimated to be _____ mg whereas after dialysis, it decreases to _____ mg. This implies that a major loss of protein takes place during dialysis.

6.2. The samples were successfully dialyzed to remove salt ions present in them, and comparative analysis was made between dialyzed and undialyzed samples.

- Undialyzed sample:
Yield = _____.
Recovery = _____.
Fold purification = _____.

- ii. Dilyzed sample:
Yield = _____.
Recovery = _____.
Fold purification = _____.

B.7. Precautions:

- i. Addition of Ammonium Sulphate must be slow and gradual.
ii. All dilutions must be made accurately.

C. Purification of acid phosphatase by ion exchnage chromatography

C.1. Aim:

To purify acid phosphatase by ion exchange chromatography from fractionated samples

C.2. Introduction:

Ion exchange may be defined as the reversible exchange of ions solution with ions electrochemically bound to some sort of insoluble support medium. The ion exchanger is the inert support medium to which a covalently bound positive (in the case of anion exchanger) or negative (in the case of cation exchanger) functional groups are bound. Any ion electrostatically bound to the exchanger is referred to as a counter ion. The value of this technique in the isolation and separation of charged compound is that conditions can be found under which some compounds are electrochemically bound to the ion exchanger whereas other are not.

At a specific pH, the isoelectric point, the molecule contains an equal number of positive and negative charges. This amphoteric nature of proteins may be manipulated to great advantage when this method is used for purification purpose. For example, the pH of a protein mixture may be lowered to the point where the desired protein behaves as a cation. If the preparation is chromatographed on a cation exchange column, under these conditions of pH, many of the anionic protein species are lost. If this is followed by an increase in pH to convert the desired protein to its anionic form, the preparation may be chromatographed on an anionic exchanger column with the concomitant loss of many of the cationic species. It should be pointed out that anion and cation exchange chromatography used sequentially often afford a large degree of purification even if the pH of preparation cannot be varied.

The selection of an exchanger that is best suited to a particular application is largely an empirical process. The nature of the supporting matrix usually determines its flow properties, ion accessibility, chemical and mechanical stability. Charged groups that are covalently bonded to the matrix determine the type and strength of binding. The size of the resin/ beads determines the flow rate, equilibration time and capacity of the exchanger. The larger the mesh size (decrease in the bead size), the larger the capacity and counterion equilibration time but lower the flow rate.

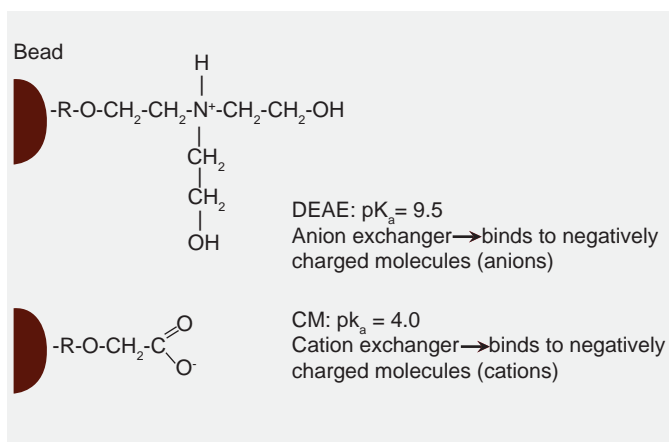


Fig. 4: Showing the charges on DEAE cellulose and CM beads

(Source: Taken from Pranav Kumar (2014). *Fundamentals and techniques of biophysics & molecular biology*. New Delhi: Pathfinder publication)

Diethylaminoethyl (DEAE) has positive charge and carboxymethyl (CM) has negative charge on them. Positive charge of DEAE attracts negatively charged molecules. CM is suitable for binding with positively charged molecules (Fig. 4).

Column materials used for ion exchange chromatography contain charged groups covalently linked to the surface of an insoluble matrix. The charged groups of the matrix can be positively or negatively charged. Anion exchangers have positively charged groups that will attract negatively charged anions. When a mixture of solutes is loaded into the anion exchanger, negatively charged solutes bind to the exchanger. Beads have positive charged functional groups hence after loading the mixture of the sample (solute) into the column separation of the solutes take place according to the charges on them as shown in (Fig. 5).

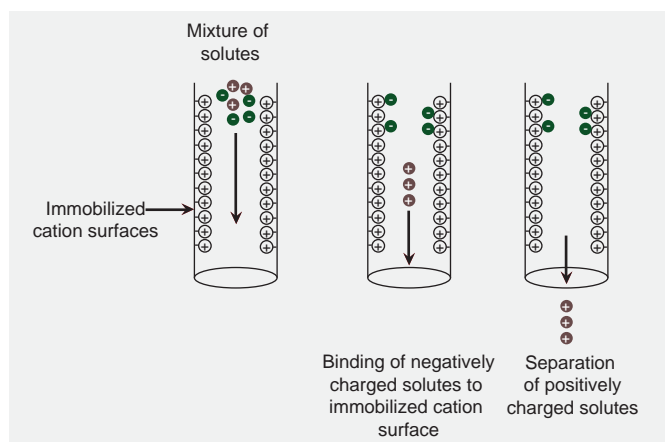


Fig. 5: Separation of charges in the column

When the mixture of solutes is loaded into the column containing the beads which have covalently attached charged groups with them, separation of the solutes takes place according to the charge present on the solutes. If the bead is DEAE which is an anion exchanger that binds to negatively charged molecules separation of anions takes place depending upon the buffers of different concentration that is used for the elution of solutes (Fig. 6).

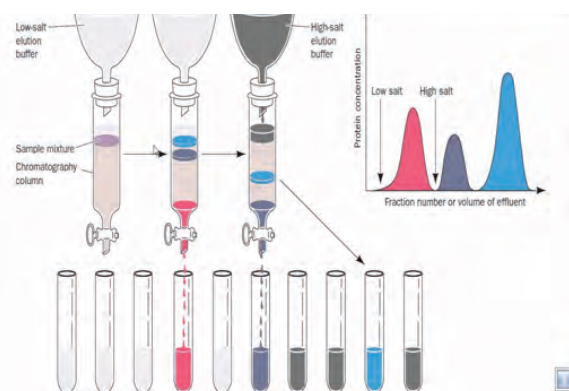


Fig. 6: Showing the elution of different solutes by applying buffers of different salt concentration for the successive elution of solutes.

(Source: <https://www3.nd.edu/~aseriann/CHAP6B.html/sld005.htm>)

2.1 Preparation of the Exchange medium:

There are four basic procedure involved in preparing an exchanger:-

- Removal of impurities that result from incomplete purification of the exchanger by the manufacture.
- Swelling the medium (termed precycling) so that a greater percentage of the exchangers charged groups are exposed to the suspending solution.
- Removal of the fines (very small particles of exchanger).
- Conversion of the counter ion of the exchanger to that desired for a given application.

The process of precycling an exchanger is to expose the charged groups that are bound to the matrix. At the molecular level, cellulose exchangers are carbohydrate polymers that have substituted hydroxyl groups. During the drying step of the manufacturing process, water is removed from the exchanger and extensive intermolecular hydrogen bonding of the hydroxyl groups occurs. Such bonding results in very dense packing of the carbohydrate polymers, which in turn results in burial of many of the charged functional groups. Suspending on exchanger in water breaks a small volume of these hydrogen bonds but much stronger treatment is required to fully swell the matrix and hence break the remaining hydrogen bonds. In the case of DEAE, an anion exchanger, treatment with HCL results in the conversion of all the diethylamino ethyl groups to their charged species ($C_2H_4N+H(C_2H_5)_2$). Placing like positive charges on the functional groups results in mutual repulsion and hence maximum amount of swelling.

Failure to precycle an exchanger results in drastically reduced capacity and resolution. The loss in resolution becomes more acute as the size and charge densities of molecules being separated increases. There are a large number of buffers that may be selected to maintain the pH of a chromatographic medium. Four general considerations pertinent to this condition are given below.

- i. Cation buffers should be used with anion exchangers and vice versa.
- ii. The pKa of the chosen buffer should lie within ± 0.7 units of the pH at which the system will be buffered.
- iii. The pH of the buffer system should be chosen so that the ions to be separated possess the same charge as the counter ions of the exchanger.
- iv. The buffer system selected must be such that it does not interfere with analysis of the fractions yield.

Four things must be considered in the chromatography of a group of ions, have given below:

- i. The volume and shape of the column.
- ii. The shape of the gradient to be employed.
- iii. The rate of elution.
- iv. The size of the fractions to be collected.

Water Regain:

It is the volume upto which 1g of resin will swell when kept in water overnight.

Water regain for DEAE = 10 -15ml/g

Binding Capacity:

It is the mass of solute to which the resin can bind.

Binding capacity of DEAE = 0.6 mg/g

Bed Volume:

It is the volume occupied by the gel in the column.

C.3. Materials Required

3.1. Biological Material: Sample of acid phosphatase.

3.2. Chemicals/Reagents: Equilibration buffer, 0.1 M acetate buffer (pH 5.5), 0.5 N HCL, 0.5 N NaOH, DEAE cellulose, 50 mM NaCl, 100 mM NaCl, 200 mM NaCl.

3.3. Glassware/Plastic ware: Beaker, eppendorfs.

3.5. Miscellaneous: Cuvette, wash bottle, pipettes, stand, dropper.

C.4. Procedure

4.1 Column Preparation:

- i. Swelling in water: - weigh the required amount of DEAE and keep in water overnight for swelling to take place.
- ii. Precycling: - This is done to expose the charged groups. This is done by washing first with 0.5 N HCL and then with 0.5 N NaOH.
- iii. Packing into column: - Place the glass wool first at the bottom/mouth of the column so that gel beads do not come out. Then equilibrate with a suitable equilibration buffer.

4.2 Equilibration and Elution:

- i. Equilibrate the column by pouring 0.1 M acetate buffer (pH 5.5) through the column. Run the equilibration buffer till the buffer eluting out of the column has the same pH i.e. 5.5.
- ii. Set the flow rate for elution.
- iii. Load 750 μ L of the sample.
- iv. Keep adding the equilibration buffer for the elution of first 10 fractions.
- v. Obtain the next 10 fractions with buffer of 50 mM NaCl.
- vi. Obtain the subsequent fractions with solutions of ionic strength of 100 mM and 200 mM NaCl respectively.

4.3 Detection

- i. Measure the absorbance of all fraction at $\lambda = 280$ nm. Plot the graph of fraction number vs. absorbance.
- ii. To detect the presence of acid phosphatase, perform the enzyme assay of alternate fraction.
- iii. For the fractions that need to be pooled, carry out Lowry's estimation method to determine the amount of protein.
- iv. Calculate the percentage recovery and fold purification for the fractions.

C.5. Observations

5.1 Observation Table:

Fraction No.	Absorbance at (280 nm)	Fraction No.	Absorbance at (280 nm)
Unbound (1-10) fractions		100 mM (1-10) fractions	
50mM(1- 10)			

5.2 Enzyme Assay of Fractions:

Sample No	Absorbance	Optical Density
i. Unbound		
ii. 50 mM NaCl		
iii. 100 mM NaCl		
iv. 200 mM NaCl		

5.3 Estimation of Activity, Specific Activity, percentage recovery

Sample	Total Volume	Activity	Total Activity	Protein mgmL ⁻¹	Total Protein	Specific Activity	% yield	% Recovery	Fold purification
Crude									
50 mM pool									
Unbound pool									
Unbound fraction									

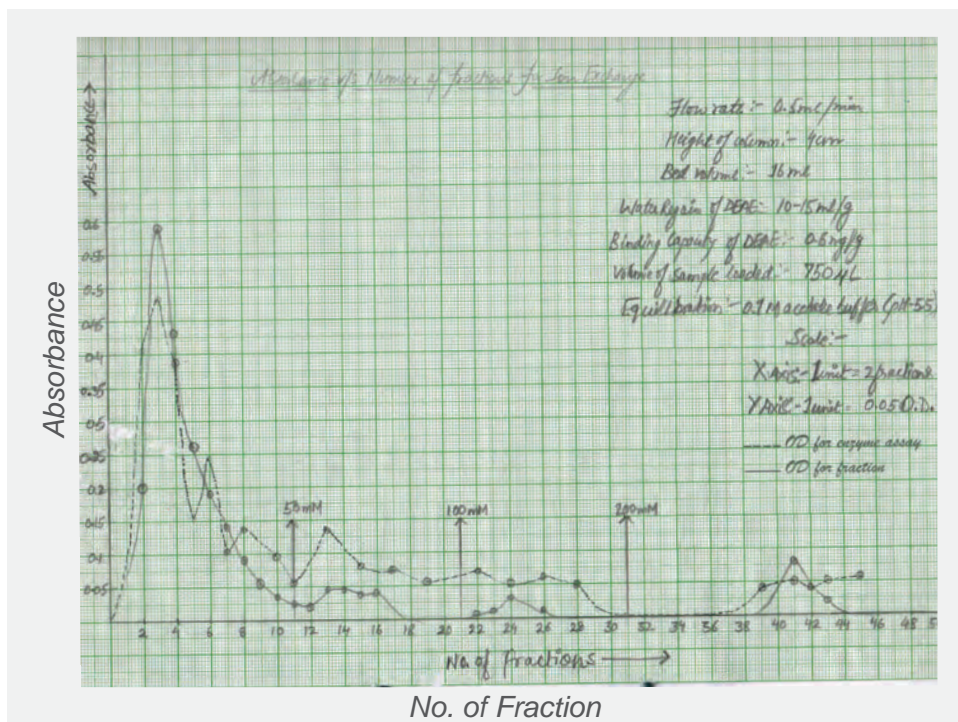


Fig. 7: Graph for purification of acid phosphatase by ion exchange chromatography

C.6. Result

- i. The fold purification of P₂ after Ammonium Sulphate Fractionation was found to be _____.
- ii. The fold purification of the three samples is as follow.
50 mM pool = _____.
Unbound pool = _____.
Unbound fraction = _____.

C.7 Precautions

- i. Packing of the column should be done with accuracy and precision. No air bubble should be present in the column.
- ii. Selling and precycling should be done properly.
- iii. Pipetting for enzyme assay and protein estimation should be done accurately.
- iv. The filter of the spectrophotometer should be set in accordance with the required wavelength and calibrate the spectrophotometer with the suitable buffer solution (Blank).
- v. Keep adding the required buffer until all the fraction are collected otherwise column will get dried and gel bed may be broken.

Suggested Reading(s)

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- ii. Richard, J. Simpson. *Purifying Proteins for Proteomics*. CSHL Press.
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- iv. Wilson, K., & Walker, J. (2008). *Biochemistry and Molecular Biology*. (6th ed.). Cambridge University Press.
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- **Dr. P. Hemalatha Reddy**, Principal, Sri Venkateswara College. University of Delhi, New Delhi.

Source

Backdrop title picture: <http://boroughmarket.org.uk>
Title picture: <https://en.wikipedia.org>

ANNEXURE:

Table 1: Nomogram

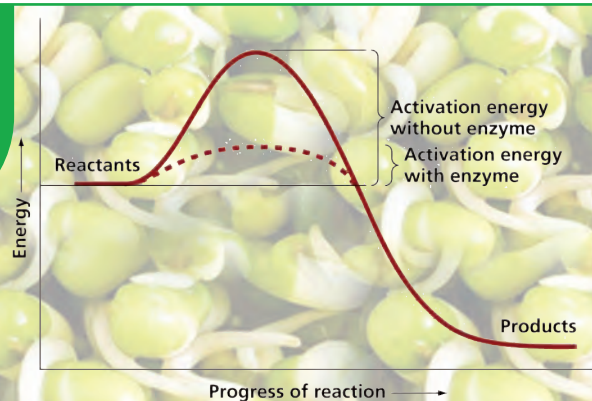
Ammonium sulfate precipitation of proteins

Proteins are precipitated from aqueous solutions by dissolving ammonium sulfate into the protein solution. The following table shows the weight (g) of ammonium sulfate to be added to one litre of solution to produce a desired change in the concentration (% saturation) of ammonium sulfate. Precipitation of proteins is conventionally carried out at 0°C to avoid possible denaturation of proteins, although the % saturation values shown in the table are based on the values at 25°C described by Green and Hughes in *Methods Enzymol.* 1, 67-90 (1955).

%	10	15	20	25	30	33	35	40	45	50	55	60	65	70	75	80	85	90	95	100		
0	56	84	114	144	176	196	209	243	277	313	351	390	430	472	516	561	610	662	713	767		
10		28	57	86	118	137	150	183	216	251	288	326	365	406	449	494	540	592	640	694		
15			28	57	88	107	120	153	185	220	256	294	333	373	415	459	506	556	605	657		
20				29	59	78	91	123	155	189	225	262	300	340	382	424	471	520	569	619		
25					30	49	61	93	125	158	193	230	267	307	348	390	436	485	533	583		
30						19	30	62	94	127	162	198	235	273	314	356	401	449	496	546		
33							12	43	74	107	142	177	214	252	292	333	378	426	472	522		
35								31	63	94	129	164	200	238	278	319	364	411	457	506		
40									31	63	97	132	168	205	245	285	328	375	420	469		
45										32	65	99	134	171	210	250	293	339	383	431		
50											33	66	101	137	176	214	256	302	345	392		
55												33	67	103	141	179	220	264	307	353		
60													34	69	105	143	183	227	269	314		
65														34	70	107	147	190	232	275		
70															35	72	110	153	194	237		
75																36	74	115	155	198		
80																	38	77	117	157		
85																		39	77	118		
90																				38	77	
95																						39

Source: siraisi@kuchem.kyoto-u.ac.jp
 (http://kuchem.kyoto-u.ac.jp/sieka/shiraishi/protocols/as_precipitation.html)

ENZYME KINETICS OF ACID PHOSPHATASE



- A. To plot the progress curve for enzyme acid phosphatase.
- B. To determine the effect of pH on enzyme activity for acid phosphatase.
- C. To determine the effect of temperature on enzyme activity of acid phosphatase.
- D. To determine the K_m and V_{max} of acid phosphatase using Michaelis-Menten curve.

A. To plot the progress curve for enzyme acid phosphatase

A.1. Aim

To plot the progress curve for enzyme acid phosphatase.

A.2. Introduction

In an enzyme catalyzed reaction, the formation of product increases with increase in time initially but does not remain a simple linear function of the incubation time for extended periods. This is because as the reaction proceeds, more and more substrate molecules are used up and converted to product.

Also most proteins suffer denaturation and thus loss of their catalytic activity with time. This may also contribute to decrease in enzyme activity with time. Therefore, selecting an optimum incubation time requires compromise between various factors. The incubation time should be long enough to permit required amount of product formation but should not allow levelling of time curve for enzyme assay. The rate of reaction is determined only when the enzyme is active at a constant rate throughout the reaction.

A.3. Materials Required

3.1. Biological Material: Moong bean sprouts extract (source of acid phosphatase).

3.2. Chemicals/Reagents: 1 M Acetate buffer (stock) and 0.1 M (working) pH 5.5, p-nitrophenylphosphate (PNPP), 0.5 M KOH solution .

3.3. Equipment: UV-Vis spectrophotometer, centrifuge, micropipette.

3.4. Glassware/Plastic ware: Cuvettes, test tubes, test tube stand.

3.5. Miscellaneous: Mortar and pestle.

A.4. Procedure

- i. Please refer previous protocol for enzyme extract preparation (step “Enzyme extract preparation”).
- ii. Prepare 0.1 M acetate buffer pH 5.5 (Please refer previous protocol for preparation of assay mixture).
- iii. Label test tubes as T, C for 1:5 dilution and S, D for 1:10 dilution. Add enzyme extract, substrate and acetate buffer of required volume in two sets of 1:5 and 1:10 enzyme dilutions respectively.

NOTE: Add 2 mL of 0.5 M KOH in control tubes prior to addition of substrate to inhibit enzyme activity.

- iv. Incubate at 37 °C in water bath for respective time duration at an interval of 10 min and note the time.
- v. After completion of time duration, post incubation add 2 mL of 0.5 M KOH to all reaction tubes to terminate enzyme reaction.
- vi. Determine the absorbance of all solutions using a spectrophotometer, record the absorbance. Calculate the enzyme activity for respective time courses and plot the graph:-
 - a) Absorbance vs time (please refer sample graph given in discussion).
 - b) Enzyme activity vs time, for both sets of enzyme dilutions (please refer sample graph given in discussion).

NOTE: Calculate the enzyme activity.

A.5. Observations

Blank solution = 2 mL KOH + 3 mL acetate buffer

λ_{max} for PNP = 405 nm

Observation Table:

Sample	Time course (min) for incubation	Acetate buffer (mL)	Substrate (mL)	Enzyme (mL)		Volume 0.5 M KOH (mL)	Abs. at 405 nm (OD)	Abs. due to product (OD) (T-C)/(S-D)*	Enzyme activity ($\mu\text{moles mL}^{-1} \text{min}^{-1}$)
SET 1: with 1:5 enzyme dilution									
	0	2.7	0.2	0.1	Incubation at 37 °C for respective time	2			
	10								
	20								
	30								
	40								
	50								
	60								

SET 2: with 1:10 enzyme dilution									
	0				Incubation at 37 °C for respective time				
	10								
	20								
	30	2.7	0.2	0.1					
	40								
	50								
	60								

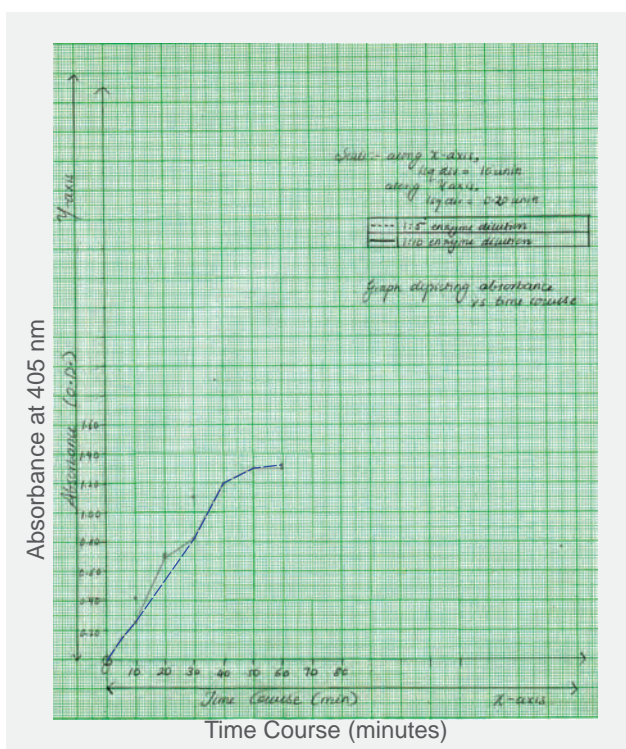
* T = Test, C = control for set 1; S = Test, D = Control for set 2

A.6. Result

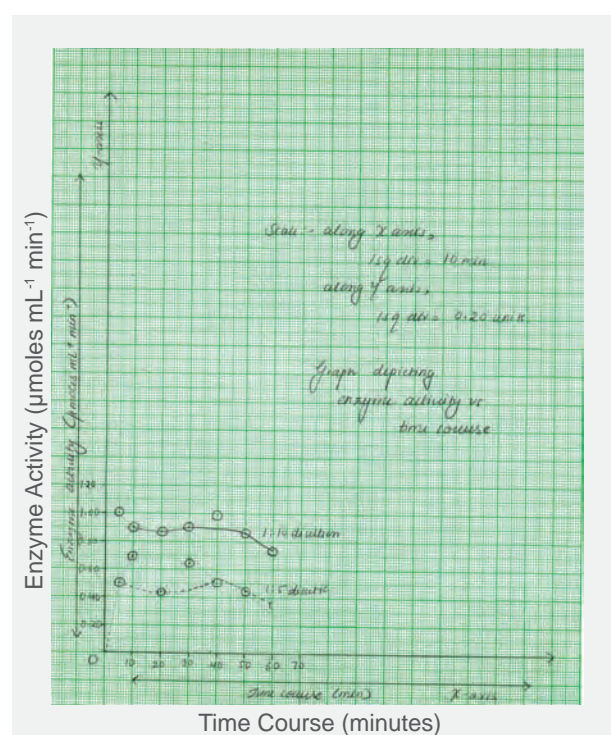
Thus, the effect of time course on enzyme activity was successfully studied and progress curve of acid phosphatase was plotted.

A.7. Discussion

From the graph (Fig. 1) absorbance v/s time course, it was found that slope of the graph increases linearly, indicating that amount of product formed increases with time duration. However, after a particular time, the slope of the graph begins to plateau. This indicates the point at which product formation is not linear and enzyme activity becomes constant. A dip in the slope in the graph of enzyme activity v/s time course shows regulatory inhibition due to product formation, leading to a decline in apparent enzyme activity with further time progression.



Graph of absorbance v/s time course



Graph of enzyme activity v/s time course

Fig. 1: Effect of time on enzyme activity

B. To determine the effect of pH on enzyme activity for acid phosphatase.

B. 1. Aim

To determine the effect of pH on activity of acid phosphatase.

B.2. Introduction

The pH of a solution can have several effects on the structure and activity of enzymes. For example, pH can have an effect on the state of ionization of acidic or basic amino acids. Acidic amino acids have carboxyl functional groups in their side chains. Some basic amino acids have amine functional groups in their side chains. If the state of ionization of amino acids in a protein is altered then the ionic bonds that help to determine the 3-D shape of the protein can be altered. This can lead to altered protein recognition or an enzyme might become inactive at extreme of pH.

Changes in pH may not only affect the shape of an enzyme but it may also change the shape or charge properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis. In general enzymes have an optimum pH for their folding in such a manner that they are active in that optimum pH. However the optimum pH is not the same for all enzymes.

For example Fig. 2 below represents a situation in which two different enzymes might have very different pH optima. The one depicted by the green curve represents the pH optimum for the enzyme pepsin which degraded proteins (protease) in the acidic lumen of the wall the stomach. The second curve (in red) represents the pH optimum of the enzyme carbonic anhydrase that works in the neutral pH of cytosol.

Optimum pH level:

Changes in pH have influence on enzyme activity. The pH at which enzyme shows maximum activity is called as optimum pH. This is the point that the enzyme is most active. Extremely high or low pH values generally result in complete loss of activity for most enzymes.

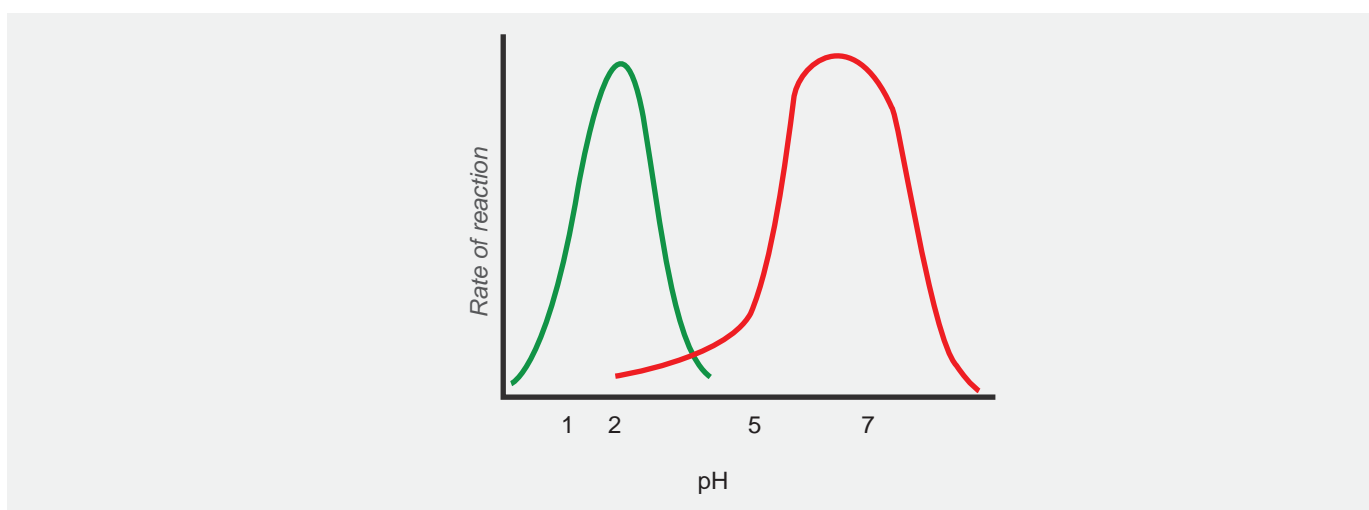


Fig. 1: Optima of pH for pepsin (green) and carbonic anhydrase (red)

Enzymes typically are most active in a pH range of 5.0 - 9.0. This is due to the fact that proteins function in an environment that reflects this pH. There are a variety of reasons as to why proteins have a narrow pH range. A variety of amino acid residues as well as the carboxyl and amide termini of proteins have a pK_a range in the range of intracellular pH. As a result, a change in pH can protonate or deprotonate a side group, thereby changing its chemical features. For example, carboxyl termini, under deprotonated, could potentially lose an interaction with an adjacent subunit, changing the enzyme conformation. In conclusion, this conformation could cause a decrease in substrate affinity. A more drastic pH change can change the protein folding, thereby completely deactivating the enzyme or cause irreversible denaturation. Thus, pH change can potentially effect the enzyme substrate complex formation in the following ways:

- i. The binding of the substrate to enzyme.
- ii. The ionization states that the amino acid residues of the catalytic site of the enzyme have.
- iii. The ionization state of the substrate.
- iv. The variation in protein structure (more significant at extreme pH values).

B.3. Materials Required

3.1. Biological Material: Mung beans sprouts extract (contains acid phosphatase).

3.2. Chemicals/Reagents: 1 M Acetate buffer (stock) and 0.1 M (working) pH 5.5, p-nitrophenylphosphate (PNPP), 0.5 M KOH solution, citrate buffer (pH 2, 2.5, 3, 3.5, and 4), acetate buffer (pH 4.5, 5, 5.5, and 6) and Tris buffer (pH 6.5, 7.8).

3.3. Equipment: UV-Vis spectrophotometer, centrifuge, micropipette.

3.4. Glassware/Plastic ware: Cuvettes, test tubes, test tube stand.

3.5. Miscellaneous: Mortar and pestle.

B.4. Procedure:

- i. Prepare the enzyme extract in acetate buffer.
- ii. Prepare the citrate buffer pH (2, 2.5, 3, 3.5, and 4), acetate buffer pH (4.5, 5, 5.5, and 6) and Tris buffer pH (6.5, 7.8).
- iii. Add the required solutions in required volumes of enzyme extract, substrate PNPP, acetate buffer, citrate buffer and tris of respective pH in labelled test tubes and also make control tubes as given in the observation table.
- iv. Add 2 mL of 0.5 M KOH to the control tubes prior to addition of substrate.
- v. Post incubation; add 2 mL of 0.5 M KOH to the reaction tubes.
- vi. Note the absorbance for each reaction, plot the graph and calculate the enzyme activity.

NOTE: Calculate the enzyme activity as per the formula given in earlier protocol.

5. Observation Table.

Blank solution = 2 mL KOH + 3 mL acetate buffer

λ_{max} for PNP = 405 nm

Dilution of enzyme = 1:10

Molar extinction coefficient (ϵ) of PNP = 1.47×10^4

Sample	pH	Buffer (2.7 mL)	PNPP (mL)	Enzyme (mL)		0.5 M KOH 2 mL	Abs at 405 nm (OD)	Abs. due to product (OD) (T-C)	Enzyme activity ($\mu\text{moles mL}^{-1} \text{min}^{-1}$)
	2	citrate			Incubation for 10 min	Add KOH in all control tubes before incubation, add KOH in all T test tubes post incubation			
	2.5	citrate							
	3	citrate							
	3.5	citrate	0.2	0.1					
	4	citrate							
	4.5	acetate							
	5	acetate							
	5.5	acetate							
	6	acetate							
	6.5	Tris							
	7.8	Tris							

* T = Test, C = control

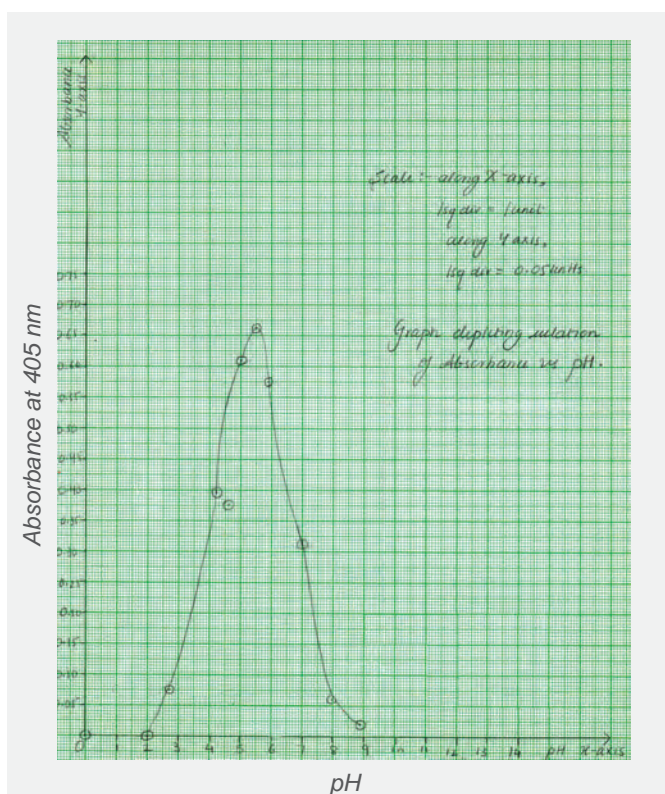
B. 6. Result

Thus, the effect of pH on enzyme activity was successfully studied for acid phosphatase enzyme and optimum pH of acid phosphatase was found to be pH _____.

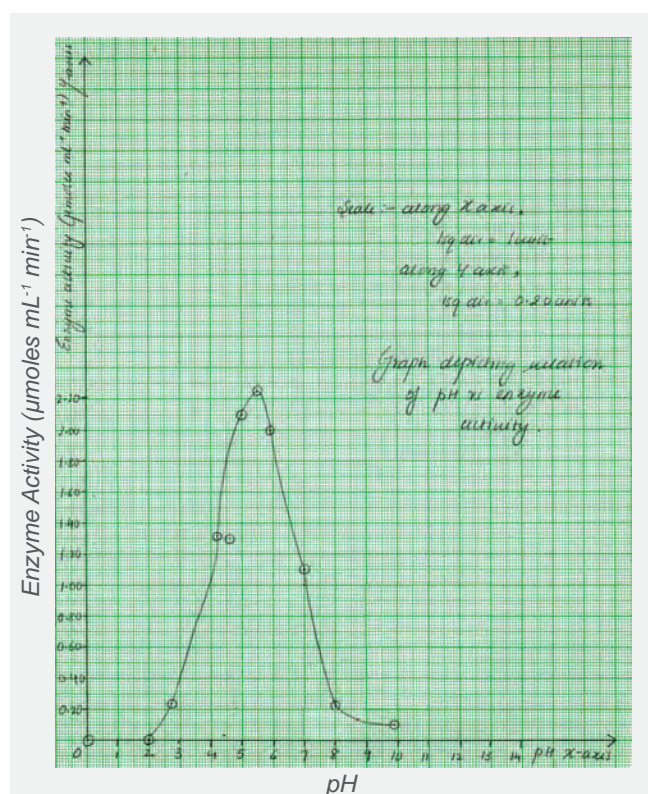
B. 7. Discussion

From the graph, it can be clearly seen that enzyme activity rises with increase in pH, reaches an optimal value of pH and decreases henceforth with further increase in pH. The enzyme activity of acid phosphatase is maximum at a pH of 5.5. This illustrates the fact that the enzyme is active under acidic pH.

Moreover, decline in enzyme activity due to increase in pH beyond optima may be explained by virtue of the fact that changing $[\text{H}^+]$, affects the charge stabilization due to electrostatic interactions between charged amino acid residues. This affects the 3-dimensional conformation of the enzyme, which may even be lost under extreme pH changes.



Graph of absorbance v/s pH



Graph of enzyme activity v/s pH

Fig. 3: Effect of pH on enzyme activity

C. To determine the effect of temperature on enzyme activity of acid phosphatase.

C.1. Aim:

To determine the effect of temperature on the activity of acid phosphatase.

C.2. Introduction

Temperature Effects:

Every enzyme has an optimum temperature at which it shows maximum activity. If the temperature is too low, there can be no noticeable reaction rate since the enzyme is operating at a temperature too below its optimum. If the temperature at which the enzyme is operating at is well above 100 °C, then thermal deactivation can occur. This occurs because as the high temperature produce enough thermal energy to break some of the intramolecular interactions between polar groups (Hydrogen bonding, dipole-dipole attractions, ionic interactions) as well as the hydrophobic forces between the non-polar groups within the enzyme structure. When these interactions are disturbed, the secondary and tertiary levels of the enzyme structure change to a random coiled form that alter the active site's confirmation beyond its ability to bind the substrate molecule as it was -proposed to catalyze. The overall phenomenon is called "Thermal deactivation or Denaturation".

Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases as the temperature is raised. A 10 °C rise in temperature will increase the activity of most enzymes by 50 to 100%. Variations in reaction temperature as small as 1 or 2 degrees may introduce changes of 10 to 20% in the results. In the case of enzymatic reactions, this is complicated by the fact that many enzymes are adversely affected by high temperatures.

As shown in Fig. 4, the reaction rate increases with temperature to a maximum level, then abruptly declines with further increase of temperature. Because most enzymes rapidly become denatured at temperatures above 40 °C, most enzyme determinations are carried out somewhat below that temperature. Over a period of time, enzymes will be deactivated at even moderate temperatures.

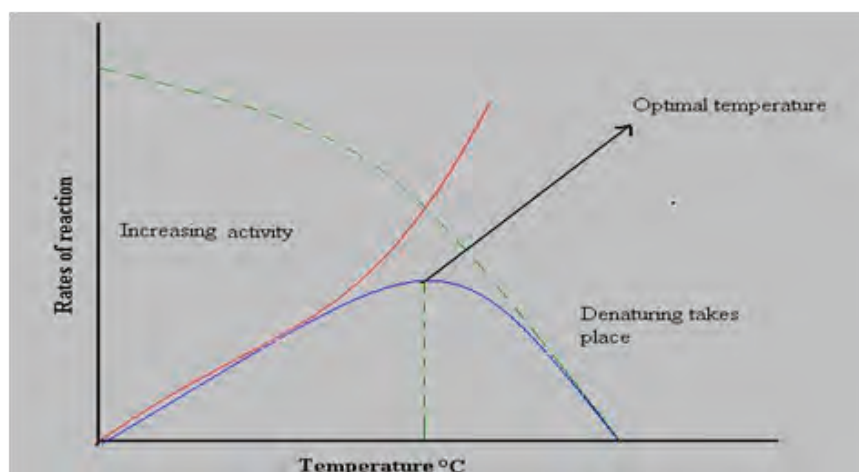


Fig. 4: effect of temperature on reaction rate

(Source: <http://vlab.amrita.edu/?sub=3&brch=64&sim=1342&cnt=1>)

Storage of enzymes at 5 °C or below is generally the most suitable. Some enzymes lose their activity when frozen. Rate of reactions including those catalyzed by enzymes rises with increase in temperature based on the Arrhenius equation $k = Ae^{-E_a/RT}$ where k is the kinetic rate constant for the reaction, A is the Arrhenius constant also known as the frequency factor.

ΔG is the standard free energy of activation which depends on entropic and enthalpic factors, R is the universal gas constant and T is the absolute temperature. Kinetic energy (K.E.) of the system increases with increase in temperature of a system results from the increase in the kinetic energy of the system. When a molecule collides, the K.E. of the molecules is converted into chemical potential energy of the molecules. Thus greater K.E. of the molecules in a system automatically increases the resulting chemical potential energy. As the temperature increases it is possible that more molecules per unit time will attain the activation energy. Thus the rate of the reaction may increase. In order to convert substrate into product, enzymes must collide with and bind to the substrate at its active site. Increase in temperature of a system will increase the number of collisions of the enzyme and substrate per unit time. Thus within the limits, the rate of the reaction will increase.

Optimum Temperature:

Each enzyme has a temperature that it works optimally in, which in humans is around 98.6 °F / 37 °C i.e. the normal body temperature for humans. However, some enzymes work really well at lower temperatures like 39 °F / 4 °C, and some work really well at higher temperatures. For instance, animals from the Arctic have enzymes adapted to have lower optimum temperatures while animals in desert climates have enzymes adapted to higher temperatures. While higher temperatures do increase the activity of enzymes and the rate of reactions, enzymes are still proteins, and as with all proteins, temperatures above 104 °F / 40 °C, will start to break them down. So, the two ends of the activity range for an enzyme are determined by what temperature starts the activity and what temperature starts to break down the protein.

Temperature vs. Enzyme Activity:

Collisions between all molecules increase as temperature increases. This is due to the increase in velocity and kinetic energy that follows temperature increases. With faster velocities, there will be less time between collisions. This results in more molecules attaining the activation energy, which increases the rate of the reactions. Since the molecules are also moving faster, collisions between enzymes and substrates also increase.

C.3. Materials Required

3.1. Biological Material: Mung bean sprouts extract (source of acid phosphatase).

3.2. Chemicals/Reagents: 1 M Acetate buffer (stock) and 0.1 M (working) pH 5.5, p-nitrophenylphosphate (PNPP), 0.5 M KOH solution.

3.3. Equipment: UV-Vis spectrophotometer, centrifuge, micropipette.

3.4. Glassware/Plastic ware: Cuvettes, test tubes, test tube stand.

3.5. Miscellaneous: Mortar and pestle.

C.4. Procedure

- i. Prepare the enzyme extract.
- ii. Prepare the 0.1 M acetate buffer pH 5.5 as described earlier.
- iii. Add acetate buffer, substrate PNPP, enzyme extract in labelled test tubes and also make control tubes.
- iv. Add 2 mL of 0.5 M KOH to the control tubes prior to addition of substrate for each reaction mixture.
- v. Set the test tubes at various temperatures (4, 10, 25, 37, 50, 60 and 70) °C and incubate for equal time duration of 10 min for each test tube.
- vi. Post incubation; add 2 mL of KOH to the reaction tubes.
- vii. Note the absorbance for each reaction; calculate the enzyme activity and plot the graph between temperature and enzyme activity.

NOTE: Calculate the enzyme activity as per the given formula.

C.5. Observation

Observation Table

Blank solution = 2 mL KOH + 3 mL acetate buffer

λ_{\max} for PNP = 405 nm

Dilution of enzyme = 1:10

Sample	Acetate buffer (mL)	PNPP (mL)	Enzyme (mL)	Temp. for incubation (°C)		0.5 M KOH 2 mL	Abs. at 405 nm (OD)	Abs. due to product (OD) (T-C)	Enzyme activity ($\mu\text{moles mL}^{-1} \text{min}^{-1}$)
				4	Incubation for 10 min	Add KOH in all control tubes before incubation, add KOH in all 'T' test tubes post incubation			
				10					
				25					
	2.7	0.2	0.1	37					
				50					
				60					
				70					

* T = Test, C = control

C.6. Result

The effect of temperature on enzyme activity was successfully studied for acid phosphatase and maximum enzyme activity was observed at _____ °C.

C.7. Discussion

From the graph it can clearly be determined that enzymatic activity increases with increase in temperature. This can be mainly because, on increasing temperature thermal energy increases and the kinetic energy which imparts greater tendency among molecules to undergo effective collision. Thus, increasing kinetic energy imparts chemical potential to overcome activation energy barrier, enabling facilitated product formation, and hence marked by increased enzyme activity. At low temperature kinetic energy is reduced which reduces collisions, thus reducing enzyme activity. Under physiological conditions, a temperature of 37 °C is optimum and high temperature in a range of 70 °C rarely exist. Further increase in temperature would result in loss of enzyme activity. This is because at extremely high temperature, enzymes (being protein in nature) get denatured and lose their conformation in an irreversible manner.

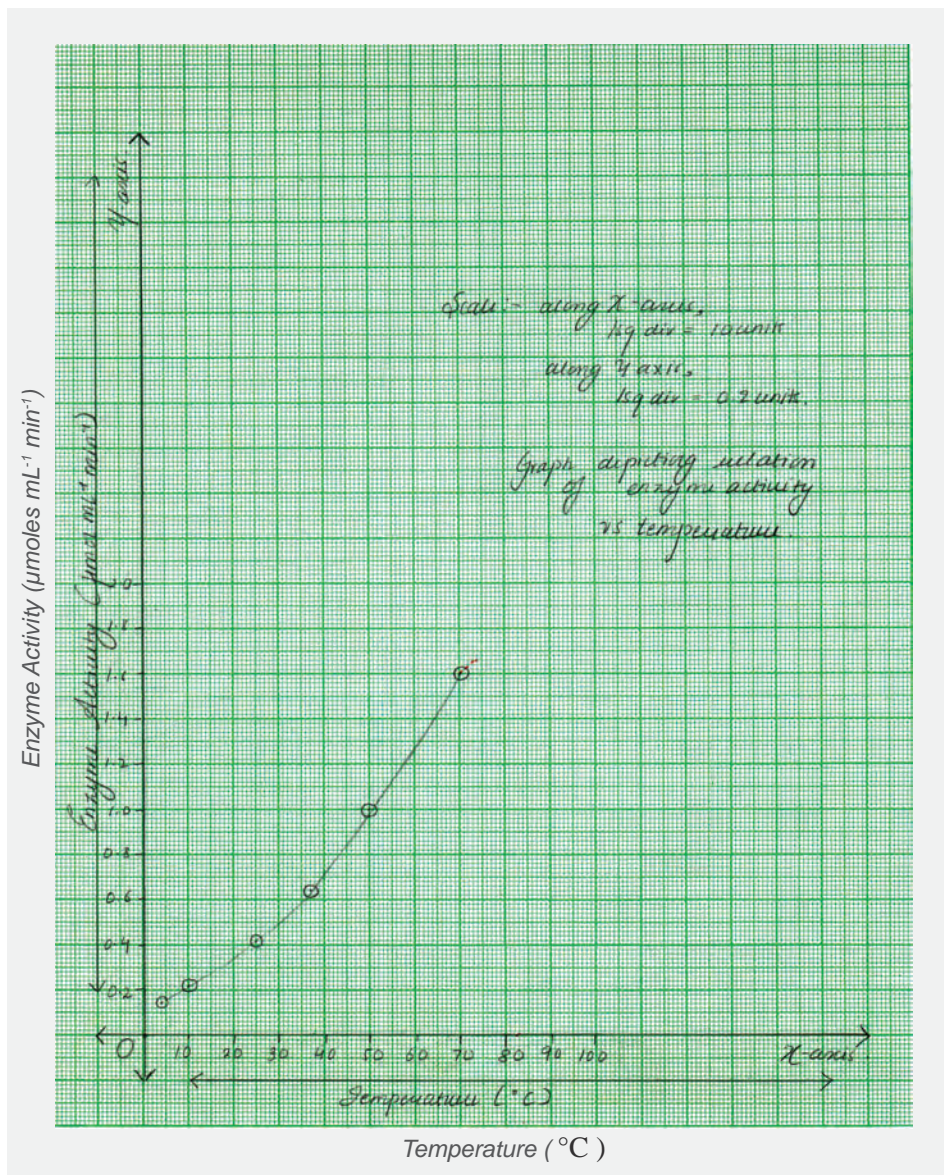


Fig. 5: Effect of temperature on enzyme activity

D. To determine the K_m and V_{max} of acid phosphatase using Michaelis-Menten curve.

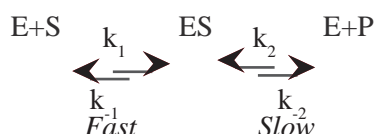
D.1. Aim

To determine the K_m and V_{max} of acid phosphatase using Michaelis-Menten curve.

D.2. Introduction

i. The Michaelis-Menten equation:

Consider an enzyme catalysed reaction:-



V_o is determined by the breakdown of ES to form product which is determined by [ES]

$$\frac{d[P]}{dt} = V_o = k_2[ES] \text{----- (i)}$$

$$\text{Rate of formation ES complex} = k_1[E][S] \text{----- (ii)}$$

$$= k_1[[E_t] - [ES]][S] \text{----- (iii)}$$

$$\text{Rate of breakdown of ES complex} = k_2[ES] + k_{-1}[ES] \text{----- (iv)}$$

Assuming a steady state,

Rate of formation of ES = rate of breakdown of ES,

$$\text{Therefore, } k_1([E_t] - [ES])[S] = k_2[ES] + k_{-1}[ES] \text{----- (v)}$$

$$k_1[E_t][S] - k_1[ES][S] = k_2[ES] + k_{-1}[ES]$$

$$k_1[E_t][S] = k_1[ES][S] + k_2[ES] + k_{-1}[ES]$$

$$k_1[E_t][S] = [ES] [k_2 + k_{-1} + k_1[S]]$$

Dividing both sides by k_1 ,

$$[E_t][S] = [ES] \left[\frac{k_2}{k_1} + \frac{k_{-1}}{k_1} + [S] \right]$$

$$[E_t][S] = \left[\left(\frac{k_2 + k_{-1}}{k_1} \right) + [S] \right] [ES] \text{----- (vi)}$$

The term $\frac{k_2 + k_{-1}}{k_1}$ is defined as Michaelis Constant K_m

Substituting this into equation (vi) and solving for [ES] simplifies the equation to

$$[ES] = \frac{[E_t][S]}{[K_m] + [S]} \text{----- (vii)}$$

Substituting in (i), we have

$$V_o = \text{initial velocity} = \frac{d[P]}{dt} = k_2[ES]$$

$$V_o = \frac{k_2[E_t][S]}{K_m + [S]} \text{-----(viii)}$$

This is the Michaelis-Menten equation, the rate equation for one-substrate enzyme - catalyzed reaction.

When enzyme is saturated with substrate,

$$V_o = V_{\max} = k_2[E_t]$$

Also, during substrate saturation, all enzyme exists as ES complex and $[E] = 0$,

Therefore $[E_t] = [ES]$

$$V_{\max} = k_2[E_t]$$

Assuming that $V_o = \frac{V_{\max}}{2}$

$$V_o = \frac{V_{\max} [S]}{K_m + [S]}$$

$$\frac{V_{\max}}{2} = \frac{V_{\max} [S]}{K_m + [S]}$$

$$K_m + [S] = 2[S]$$

$$K_m = [S]$$

ii. K_m Michaelis Constant

K_m is the concentration of the substrate at which velocity of an enzyme- catalyzed reaction is half of the maximum velocity obtained.

Assumptions made while deriving Michaelis–Menten equation:

- Assumption of steady state: - Concentration of all reaction intermediates is a constant.
- Rate kinetics depend upon the slowest step.
- Rate of backward reaction can be ignored since initially rate of forward reaction predominates due to small $[ES]$ concentration.

iii. Lineweaver-Burk equation

Since Michaelis–Menten equation fails to determine the exact value of V_{\max} and K_m , reciprocal of Michaelis-Menten equation gives the Lineweaver- Burk equation

$$\frac{1}{V_o} = \frac{K_m}{V_{\max} [S]} + \frac{1}{V_{\max}}$$

iv. Eadie-Hofstee equation

Lineweaver-Burk plot yields inaccurate values of V_{\max} and K_m due to involvement of large errors. Thus, multiplying it by V_o , V_{\max} yields the Eadie-Hofstee equation.

$$V_o = K_m \frac{V_o}{[S]} + V_{\max}$$

v. Hanes equation

Multiplying both sides of Lineweaver-Burk equation by $[S]$

$$\frac{[S]}{V_o} = \frac{K_m}{V_{\max}} + \frac{[S]}{V_{\max}}$$

D.3. Materials Required

3.1. Biological Material: Mung bean sprouts extract (source of acid phosphatase).

3.2. Chemicals/Reagents: 1 M Acetate buffer (stock) and 0.1 M (working) pH 5.5, p-nitrophenylphosphate (PNPP), 0.5 M KOH solution.

NOTE: Make different concentrations of p-nitrophenolphosphate (PNPP) solution (50 mM, 10 mM, 1 mM).

3.3. Equipment: UV-Vis spectrophotometer, centrifuge, micropipette.

3.4. Glassware/Plastic ware: Cuvettes, test tubes, test tube stand.

3.5. Miscellaneous: Mortar and pestle.

D.4. Procedure

- i. Make different concentrations of PNPP using stock solution of PNPP (1 mM, 10 mM, 50 mM) as given below by diluting against required volume of acetate buffer.
(0.025, 0.05, 0.1, 0.2, 0.3, 0.5, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 8, 10, 20, 30, 40) mM
- ii. Add 0.1 mL of enzyme to all test tubes.
- iii. Incubate the test tubes at 37 °C for 10 min.
- v. Add 2 mL of KOH to control tube prior to addition of the enzyme.
- vi. Add 2 mL of 0.5 KOH to the test tubes to stop the reaction (post incubation).
- vii. Note the absorbance for each reaction, calculate V_o and plot the Michaelis-Menten equation
- viii. Calculate V_o values and make the plots of Michaelis-Menten (please refer sample graph of Michaelis-Menten curve given in the discussion), Lineweaver-Burk and Eadie-Hofstee appropriately to calculate the V_{\max} and K_m values.

D.5. Observations

Observation Table:

Enzyme dilution: 1:10

Sample	Concentration [S] PNPP (mM)	Concentration of stock PNPP	Stock PNPP (mL)	Acetate Buffer (mL)	Enzyme (mL)		0.5 M KOH (mL)	Abs. At 405 Nm (OD)	Vo ($\mu\text{moles mL}^{-1} \text{min}^{-1}$)	$\frac{1}{V_o}$	$\frac{1}{[S]}$	$\frac{V_o}{[S]}$			
Blank				3											
1	0.025	1 mM			0.1	Incubate at 37 °C for 10 min	2								
2	0.05														
3	0.1														
4	0.2														
5	0.3														
6	0.5														
7	0.6	10 mM													
8	0.8														
9	1														
10	2														
11	3														
12	4														
13	5	50 mM													
14	6														
15	8														
16	10														
17	20														
18	30														
19	40														

D.6. Result

The values of K_m and V_{max} of acid phosphatase were determined as follow:-

a. $K_m =$ _____, b. $V_{max} =$ _____.

D.7. Discussion

The knowledge of binding affinity helps to design various drugs with the similar affinity with the respective binding sites. a shortcoming of Michaelis–Menten curve is that V_{max} is attained only when $[S]$ is equivalent to infinity. Thus an accurate value of V_{max} can not be determined.

Thus, a small error in $1/V_0$ and $1/[S]$ values yields a large error in the values of V_{\max} and K_m .

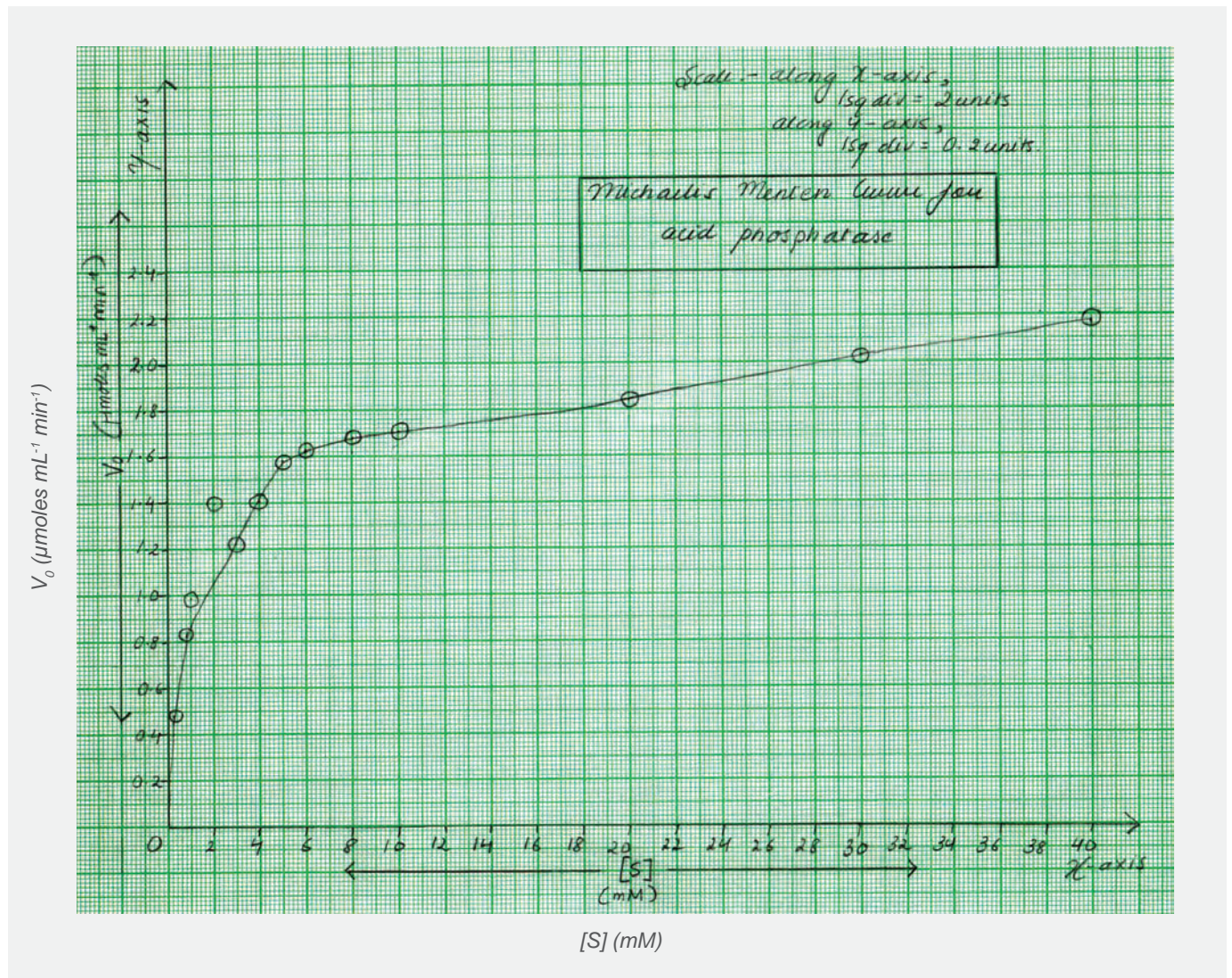


Fig. 6: Michaelis-Menten curve for acid phosphatase

7. Precautions

- i. Pipetting to be done carefully and accurately.
- ii. Calibrate the spectrophotometer before taking readings.
- iii. Incubation time should not be exceeded.

Suggested Reading(s)

- i. T. Palmer & B. L. Bonner (2007). *ENZYMES: Biochemistry, Biotechnology, Clinical Chemistry*. (2nd ed). Woodhead Publishing. eBook ISBN: 9780857099921, Paperback ISBN: 9781904275275

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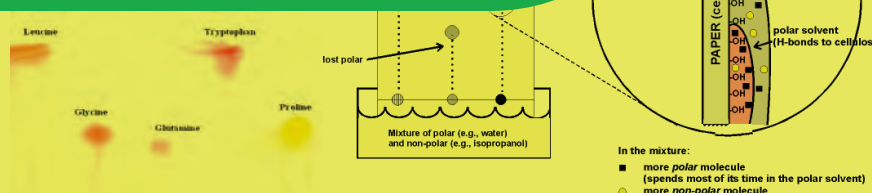
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PAPER CHROMATOGRAPHY BY ASCENDING METHOD



1. Aim

To separate amino acids by paper chromatography by ascending method.

2. Introduction

Chromatography is a separation technique that relies on the differential solubility of solutes in solvents. The separation of components from a mixture can therefore be achieved if the solutes are partitioned between two or more phases. Paper chromatography is thus a type of partition chromatography in which the differences in the partition coefficients of the substances to be separated are used for separating them. When solvent system containing both hydrophilic and hydrophobic components (mobile phase) migrates on a paper, the hydrophilic solvent is absorbed on the cellulose, which acts as the stationary phase whereas the hydrophobic one does not. Thus, a phase separation takes place at the micro level. When the solvent front reaches the spot where the compounds have been spotted, the compounds get partitioned as the solvent front migrates further by capillary action and separation is achieved. The separated amino acids are visualized by spraying a solution of ninhydrin (trihydrindene hydrate) and heating the paper. The amino acid spots appear purple.

The two amino acids proline and hydroxyl proline appear yellow. Ninhydrin deaminates and decarboxylates the amino acids and at the same time gets reduced to hydrindantin. This hydrindantin then combines with another molecule of ninhydrin and one molecule of ammonia to form a purple coloured complex (Ruhemann's purple reaction) (Fig.1).

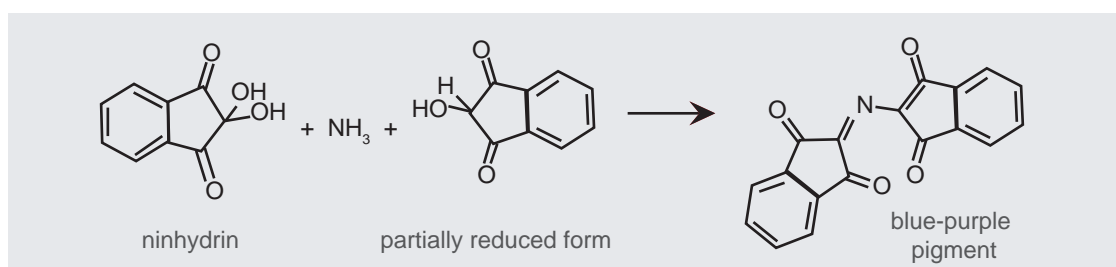


Fig.1: Reaction of amino acid with ninhydrin

(<https://biochembro.wordpress.com/2013/02/18/yellow-result-from-ninhydrin-test/>)

Ratio of distance traveled by the solute to the distance traveled by the solvent from the origin of the spot is termed as R_f value (resolution front). Different substances have different characteristic R_f values.

3. Materials Required

- 3.1. Chemicals/Reagents:** Reference amino acids (leucine, lysine and proline), mixture of unknown amino acids, solvent system, butanol: acetic acid: distilled water in a ratio of 4:1:5, 0.1 % ninhydrin in acetone.
- 3.2. Equipment:** Chromatographic chamber.
- 3.3. Glassware/Plastic ware:** Capillary tubes, hot plate.
- 3.4. Miscellaneous:** Pencil, scale, strip of whatman no.1 chromatography paper (about 15 mm x 200 mm).

4. Procedure

- i. Fill the chromatographic chamber with solvent system to height of about 1 cm.
- ii. Close the lid and allow equilibrating for at least 30 minutes.
- iii. On the end of the chromatographic paper, draw a light horizontal line with pencil about 1.5 cm from the bottom. (This is the base line)
- iv. On this line mark 4 equidistant spots. Label these spots as Le (for leucine), Ly (for lysine), P (for proline), and M (for mixture).
- v. With the help of capillary tubes, transfer a very small drop of appropriate solution to the spots. Allow to dry.
- vi. Insert the chromatographic paper into the chamber in such a way that the end near the spot is immersed in the solvent system and the spots are about 5-6 mm above the solvent level. Close the chamber and allow the solvent to run. Take care not to allow the paper to touch either the bottom or the sides of the chamber.
- vii. When the solvent has migrated to about 10-15 cm, remove it from the chamber and mark the solvent.
- viii. Dry the paper.
- ix. Spray ninhydrin and heat on a hot plate or can be placed in a heated oven.
- x. When the spots develop, mark their outlines tightly with pencil.
- xi. Measure the distance from the origin (base line) to the solvent front (distance traveled by solvent).
- xii. Mark centre of spots, measure the distance traveled by solute and tabulate them.

5. Observations

It is found that:-

- i. Leucine with the highest position coefficient migrates the longest distance.
- ii. Lysine with the lowest partition coefficient migrates the minimum distance.
- iii. Proline occupies a position in between the two on spraying with ninhydrin.
- iv. Leucine and lysine develop into dark purple spots with strong heat.
- v. Proline which is an amino acid develops yellow colour.
- vi. Measure the distance travelled by the solvent system and each amino acid and tabulate.

Name of the aminoacid	Leucine	Lysine	Proline	Mixture
Distance travelled by the solvent				
Distance travelled by the aminoacid				

6. Calculations

Calculate the R_f values of each amino acids

$$R_f = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

7. Results

- i. R_f value of Leucine =
- ii. R_f value of Lysine =
- iii. R_f value of Proline =

8. Conclusion

- i. R_f value of given mixture matches with R_f value of reference amino acids such as leucine, lysine and proline.
- ii. Leucine is a neutral, hydrophobic amino acid and migrates maximum distance because it has maximum solubility in butanol and a high partition coefficient.
- iii. Proline migrates a relatively closer distance because it has partial positive charge.
- iv. Lysine is a hydrophilic amino acid and migrate minimum distance as it is highly soluble in aqueous medium and least soluble in organic solvent.

8. Precautions

- i. Use clean glassware for setting up the experiment.
- ii. Do not touch the chromatography paper with hands.
- iii. Be very careful while spraying Ninhydrin on the chromatogram (avoid skin contact).

Suggested Reading(s)

- i. Plummer, Mu., Plummer, D. T. (1988). *An introduction to practical biochemistry*. Tata McGraW-Hill Education.
- ii. Sherma, J., Zweig, G. (1971). *Paper chromatography*. (1sted.). Academic Press.

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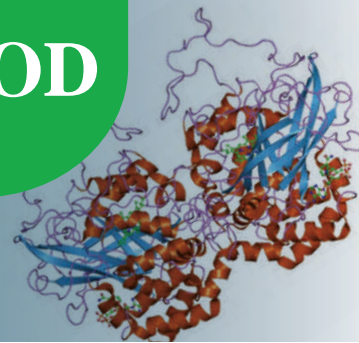
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EVALUATING CATALASE ACTIVITY OF DIFFERENT FOOD SOURCES USING PAPER DISC

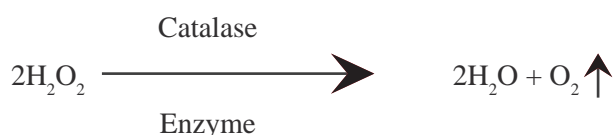


1. Aim

To study activity of catalase in different food sources like potato, yeast and wheat sprouts.

2. Introduction

Hydrogen peroxide is a normal by-product of several metabolic processes (fatty acid oxidation, cholesterol synthesis, and bile acid production) and since it is a very strong oxidizing agent, cannot be allowed to build up in the cell. Higher concentrations of hydrogen peroxide can kill cells instantly. Therefore, hydrogen peroxide needs to be broken down as soon as it is produced and catalyse that. Catalase is an enzyme that speeds up the breakdown of hydrogen peroxide. The turnover rate for catalase is one of the highest recorded: one molecule of catalase can convert one hundred thousand hydrogen peroxide molecules into water and oxygen in one second. Catalase is found in abundance in the liver (main detoxification organ) and in blood. In plants H_2O_2 is mainly produced in peroxisomes, chloroplasts and mitochondria. Plants have several enzymes to metabolize peroxides but the major ones are catalase and peroxidases. Catalases regulate reactive oxygen species (ROS) which are produced more under conditions of stress.



In this experiment, different food sources are compared for catalase activity. Each food which is the enzyme source will be taken on a paper disc separately and dropped into a beaker containing hydrogen peroxide solution. The time taken for the paper disc to float back to surface is used as the measure of enzyme activity.

3. Materials Required

3.1. Biological Materials: Fresh potato, yeast and sprouted wheat

3.2. Chemicals/Reagents: 40 mL 1% hydrogen peroxide solution, distilled water.

3.4. Glassware/ Plastic wares: Three glass beakers (50 mL capacity), eppendorf tubes labelled as A, B and C.

3.5. Miscellaneous: Punched discs of whatman filter paper placed in a petridish, normal filter paper, strips, forcep, stop watch, pestle and mortar, ice-cold distilled water.

4. Reagents/Media Preparations

1% hydrogen peroxide solution – add 20 mL stock H_2O_2 solution in 80 mL D/W (use hydrogen peroxide available at a chemist's shop which is 5% w/v, as stock).

4. Procedure

- i. Dice the plant material (potato and sprouted wheat) into small pieces and take 10 g of the diced plant material in a clean mortar.
- ii. Take 100 mL of ice-cold distilled water. Pour small portions of ice-cold distilled water and crush the plant material into a smooth pulp. Add the remaining ice-cold water and mix well.
- iii. Filter the paste through a whatman filter paper and collect the filtrate into an eppendorf tube. Label it appropriately and keep aside.
- iv. Take 10 g of baker's yeast and suspend in 100 mL of warm water (60 °C) to form slurry.
- v. Filter the slurry through a whatman filter paper and collect the filtrate into an eppendorf tube. Label it appropriately and keep aside.
- vi. Keep the stop watch ready for use by adjusting it to zero.
- vii. Pour 40 mL of hydrogen peroxide solution into glass beaker marked "A".
- viii. Gently pick up a single paper disc with the help of forceps.
- ix. Dip the paper disc inside the eppendorf tube marked "A".
- x. Drain off excess fluid from filter paper by touching the edge on a normal filter paper strip.
- xi. Drop the disc into the hydrogen peroxide solution in glass beaker marked "A".
- xii. Allow the paper disc to reach the bottom. As soon as it touches the bottom, start the stop watch.
- xiii. Stop the watch as soon as the paper disc reaches the surface.
- xiv. Note the time taken (in seconds) for the paper disc, to reach the top of the solution, after it touched the bottom. (NOTE: If the paper disc rises too fast, within 5 seconds, then dilute the enzyme source and try again. All enzyme sources must be diluted to the same concentration.)
- xv. Record your observation in the table given below, against sample A.
- xvi. Repeat the steps no. viii – xiv, two times with fresh paper discs to obtain three readings for the plant material.
- xvii. Repeat the above steps (no. vii – xv) with samples B and C separately with fresh paper discs.
- xviii. Calculate the mean time for each plant sample.

6. Observations

Time recorded (in seconds)	Sample A	Sample B	Sample C
Trial 1			
Trial 2			
Trial 3			
Mean Time			

7. Results

- i. The best source of catalase activity is found in _____.
- ii. The food source showed decreasing catalase activity in the following order:
_____.
- iii. The food source with better antioxidant activity is _____.
- iv. The paper discs rose to the surface due to _____.
- v. The gas seen as bubbles attached to the paper disc is _____.

8. Precautions

- i. Hydrogen peroxide can damage clothes. Rinse any spills with water immediately.
- ii. Store stock hydrogen peroxide in the refrigerator between 5 °C and 10 °C.
- iii. Use freshly prepared dilutions of hydrogen peroxide for each food source.
- iv. Use ice-cold distilled water for preparing the plant pulp (enzyme source).
- v. Store the plant pulp in refrigerator but carry out assay within two hours of preparation of enzyme source.
- vi. Use the same stop watch for all replicates.
- vii. The paper discs should be made of same size and from the same quality of filter paper.
- viii. Be precise in starting and stopping the start watch to record the time accurately.

9. Extension Activity

- i. Use the method to compare catalase activities in various plant sources including fresh, processed and cooked.
- ii. The method can be extended to study various parameters that affect the enzymatic reaction of catalase. Use any one enzyme source and apply the same technique to evaluate the effect of pH, concentration of hydrogen peroxide, presence of inhibitor like hydroxylamine on catalase activity.

Suggested Reading(s)

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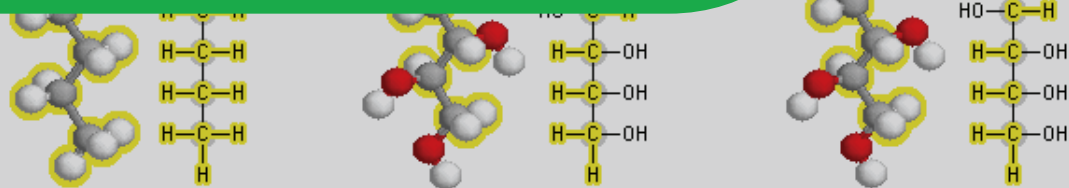
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ESTIMATION OF SUGAR BY NELSON-SOMOGY'S METHOD



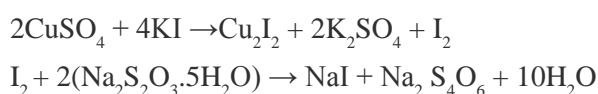
1. Aim

To estimate concentration of sugar by Nelson-Somogy's method.

2. Introduction

In general, all the monosaccharide's and oligosaccharides are collectively known as sugars, some sugars are reducing while others are non-reducing sugars which have the ability to reduce Fehling's or Benedict's solutions are referred to as reducing sugars and other are non-reducing. The reducing sugar converts cupric hydroxide [$\text{Cu}(\text{OH})_2$] present in the solution to cuprous oxide [Cu_2O]. The reducing property of sugar is due to the presence of aldehyde ($-\text{CHO}$) and Keto ($=\text{CO}$) group in the molecule.

Somogy's method is the most accurate and sensitive method for the determination of reducing sugar (glucose) quantitatively by Idiometric technique up to the concentration from 0.01 to 3.0g. The Idiometric technique is based on the following reaction:



The above reaction showed that when KI is added to cupric salt, Cu_2I_2 is quantitatively precipitated and simultaneously corresponding to each atom of Cu present, one atom of I_2 is liberated. Then, iodine liberated is titrated against a standard thiosulphate solution in presence of starch indicator.

If some amount of reducing sugar is added to the Cu_4SO_4 solution before addition of KI, the liberation of I_2 will be less due to reduction of CuSO_4 to Cu_2O by the sugar. As a result, the amount of thiosulphate required for the titration will be less. [The difference of this two readings will give the amount of Cu reduce by sugar present in the unknown solution from which the percentage of reducing sugar can be calculated.]

3. Materials Required

3.1. Biological Material: Ripe banana.

3.2. Chemicals/Reagents: Somogy's reagent, 5% $\text{Ba}(\text{OH})_2$, 5% ZnSO_4 , 2.3% KI, 2 N H_2SO_4 , 0.005 N sodium thiosulphate, 10% NaOH, 1 N KIO_3 , starch indicator, distilled water.

3.3. Glassware/Plastic ware: Volumetric flask (250 mL, 100 mL, 500 mL), conical flask, beakers, 6 test tubes with stopper, measuring cylinder, burette and pipette, mortar and pestle,

3.4. Miscellaneous: Cotton, water bath, test tube holder, chemical balance and weight box, rods, filter paper.

4. Preparation of reagents

4.1. Preparation of Somogy's reagents:

(a) SOLUTION A

- i. Sodium Potassium Tartarate: 12 g
- ii. Sodium Carbonate (Na_2CO_3): 24 g
- iii. Sodium Bicarbonate (NaHCO_3): 16 g
- iv. Sodium Sulphate (Na_2SO_4): 144 g
- v. Distilled Water: 800 mL

(b) SOLUTION B

- i. Copper Sulphate ($\text{Cu}_4\text{SO}_4 \cdot 5\text{H}_2\text{O}$): 4 g
- ii. Sodium Sulphate (Na_2SO_4): 36 g
- iii. Distilled Water: 200 mL

Before using, Solution A and Solution B are mixed at a ratio of 4:1 which is required Somogy's reagent for the experiment. 20 mL of KIO_3 of 1 M solution is added per liter of Somogy's reagent.

5. Procedure

5.1. Extraction and purification:

The outer coating of banana is removed. Then, 10 g is weighed and grind in a mortar. The grinded mass is mixed with distilled water and filtered through a piece of cotton or with cloth. After cooling the volume of the filtrate is made up to 250 mL in a volumetric flask with distilled water. This is the stock solution. From this stock solution, 2.5 mL of solution is taken and to this 10 mL of 5% ZnSO_4 and 10 mL of 5% $\text{Ba}(\text{OH})_2$ is added to purify the taken stock solution of ripe banana. It is filtered and the volume is made up to 100 mL with distilled water. Then, 5 mL of the purified sugar solution and 5 mL of somogy's reagent is taken in a test tube. Three such test tubes with the mixture are taken (marked as simple). Another such set of 3 test tube are taken and prepared by taking 5 mL of Somogy's reagent and 3 mL distilled water (marked as blank). All the six test tube are covered with stopper and immersed in boiling water for 20 minutes. After required time, the mixture of the sample turns brown and those of the test tube are coded and titrated with 0.005 N solution of sodium thiosulphate.

5.2. Titration:

The standard thiosulphate solution is taken in a burette. 1 mL or 2 mL of 10% NaOH is added to the burette having thiosulphate solution to prevent atmospheric oxidation. Now, the solution of the test tube is taken in a conical flask (100 mL) and added 2 mL of KI solution (already prepared). After a few minutes, 1.5 mL of 2 N H_2SO_4 is added to the solution which is followed by immediate addition of a few mL of thiosulphate from the burette to prevent liberation of I_2 from KI solution. The color of the mixture turns blue. Thus, the solution is titrated against the thiosulphate until the blue color just disappeared, which is the end point. The amount of thiosulphate required for neutralization is noted, the same is repeated for the remaining tubes.

6. Observations

6.1. Estimation of Reducing Sugar:

SAMPLE (sugar + Somogy's reagent)

No. of Observations	Initial Reading (mL)	Final Reading (mL)	Difference	Mean

BLANK (distilled water + somogy's reagent)

No. of Observations	Initial Reading (mL)	Final Reading (mL)	Difference	Mean

6.2. Estimation of total sugar:

For estimation of total sugar, the purified sugar solution is to be hydrolyzed. 10 mL of the purified sugar solution is hydrolyzed by adding 2 mL of 2 N HCl and boiled the solution. Then the solution was neutralized by adding Na_2CO_3 crystals and then it was slightly acidified by adding 2-3 drops of 0.5 N acetic acid. The volume of the solution was made upto 100 mL. This hydrolyzed extract is now ready for estimation as was done for reducing sugar.

Samples:

No. of Observations	Initial Reading (mL)	Final Reading (mL)	Difference	Mean

6.3. Estimation of reducing sugar:

Plant Extract taken= 5 mL.

Blank reading= 'a' mL

Sample reading= 'b' mL

Therefore, Difference in reading= (a-b) mL

= 'A' mL

1 mL of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ neutralizes 0.135 mg of reducing sugars.

Therefore, 'A' mL of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ neutralizes = $0.135 \times 'A'$ mg
= 'B' mg of reducing sugars.

i.e., 5 mL of purified sugar solution contains = 'B' mg of reducing sugars.

Therefore, 100 mL of purified sugar solution contains = mg of reducing sugars.
= 'C' mg of Reducing Sugar

100 mL of purified sugar solution was made from 10 mL of stock solution.

Therefore, 10 mL of purified sugar solution contains = 'C' mg of Reducing sugar.

100 mL of stock solution contains = mg of Reducing sugars.

= 'D' mg of reducing sugars

100 mL of stock solution was prepared from = 5 gm. of the plant sample.

5 gm of plant sample contains = 'D' mg of Reducing sugars

Therefore, 100gms of plant sample contains = $(D \div 5) \times 100$ mg of Reducing sugars.

= 'E' mg of reducing sugar.

= $(E \div 1000)$ gm. of reducing sugar.

= F

Therefore, The % of Reducing Sugar in the plant sample = 'F' %

6.4. Estimation of total sugar:

Blank reading = 'a' mL.

Sample reading = 'b' mL.

Difference in reading = (a-b) mL

= 'A' mL.

i.e., 'A' mL of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ was utilized in neutralization of 5 mL hydrolysed plant extract.

1 mL of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ neutralizes 0.135 mg of sugar.

Therefore, 'A' mL of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ neutralizes = $0.135 \times 'A'$ mg of sugar.

= 'B' mg of sugar.

5 mL of the hydrolysed plant extract contains = 'B' mg of total sugar.

Therefore, 100 mL of the hydrolysed plant extract contains = $(B \div 5) \times 100$ mg total sugar.

= 'C' mg total sugar.

100 mL of hydrolysed solution was prepared from 10 mL of purified sugar solution.

Therefore, 10 mL of the purified sugar solution contains 'C' mg total sugar.

100 mL of the purified sugar solution contains = $(C \div 10) \times 100$ mg total sugar.

= 'D' mg total sugar

100 mL of purified sugar solution was prepared from 10 mL of stock solution.

Therefore, 10 mL stock solution contains = 'D' mg total sugar

100 mL stock solution contains = $(D \div 10) \times 100$ mg total sugar.

= 'E' mg total sugar.

100 mL stock solution was prepared from 5 gm. of plant sample.

5 gms of plant sample contains = 'E' mg total sugar.

Therefore, 100 gms of plant sample contains= $(E \div 5) \times 100$ mg of total sugar.

= 'F' mg of total sugar.

= $(F \div 1000)$ gm of total sugar.

= 'G' gm of total sugar.

So, the % of total sugar in the given plant sample is 'G' %.

7. Precautions

- i. All solutions are needed to be prepared fresh.
- ii. Distilled water is to be used for preparation of all solutions.
- iii. End point for titration is to be calculated by taking mean value of at least three readings.

Suggested Reading(s)

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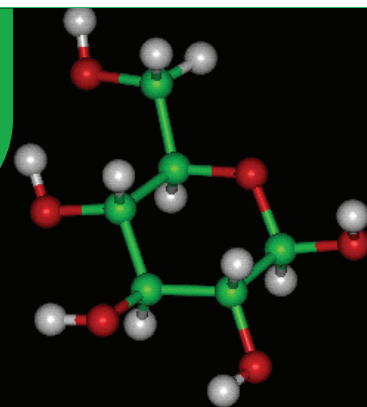
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ESTIMATION OF GLUCOSE BY GOD-POD METHOD



1. Aim

To estimate the amount of glucose from samples by GOD - POD method.

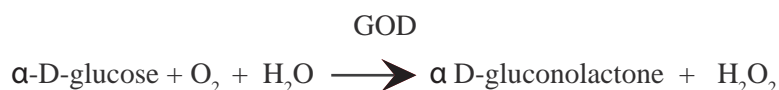
2. Introduction

Enzymatic methods of estimation of glucose are preferred over chemical methods because of their reliability and safety. In this experiment, the glucose estimation is based on Trinder's method (Lott and Turner, 1975; Trinder, 1969) in which Glucose Oxidase (GOD) and Peroxidase (POD) enzymes are used along with the chromogen 4-Aminoantipyrine and phenol. The method is simple and rapid. It does not have any interference due to reducing substances and other matrix components.

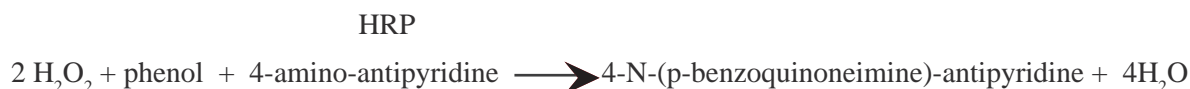
Glucose is oxidized by the enzyme GOD to give D-gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of the enzyme POD oxidizes phenol which combines with 4-Aminoantipyrine to produce a red colored quinoneimine dye. The intensity of the color developed is proportional to glucose concentration in the sample.

The commercially available kit has Glucose Reagent which is a mixture of freeze-dried powder of GOD; >12,000 U, POD; > 650 U and 4-aminoantipyrine (80 mg) reconstituted in buffer of pH 7.4 (0.42 M Potassium phosphate, 0.35 M p-hydroxybenzoic acid and sodium azide (0.64% w/v) as a preservative.

Glucose is oxidized to α -D-gluconolactone in the presence of an enzyme called glucose oxidase (GOD):



To measure the reaction rate, the production of H_2O_2 is coupled to another enzyme-catalyzed reaction. In this case the enzyme is horseradish peroxidase (HRP):



3. Materials Required

3.1. Biological Materials: Fresh fruit juices, packaged fruit juice, honey, beverages etc.

3.2. Chemicals/Reagents: Glucose reagent, distilled water, standards.

3.3. Equipment: UV-Vis Spectrophotometer, autopipettes, weighing balance, incubator.

3.4. Glassware/Plastic Ware: Clean and dry test tubes.

3.5. Miscellaneous: Whatman filter paper no.1, tissue paper.

4. Procedure

4.1. Special instructions:

- i. Use clear, colourless and neutral liquid samples.
- ii. Use directly or diluted solutions of high sugar samples.
- iii. Filter turbid solutions through Whatman filter paper no. 1.
- iv. gas samples containing carbon dioxide by keeping aside for 30 minutes with stirring.
- v. For coloured samples that are used undiluted add 0.2 g of PVPP (Polyvinylpolypyrrolidone) /10 mL sample, shake vigorously for 5 min and filter through Whatman no.1 filter paper to remove colour interference.
- vi. Homogenize solid or semi-solid samples and extract with water or dissolve in water.
- vii. For samples containing fat, extract with hot water.
- viii. Interferences could occur in beverages containing ascorbic acid. Hydrogen peroxide produced through reaction oxidizes ascorbic acid (vitamin C) present in beverages, producing dehydroascorbic acid:



Because this reaction is faster than the HRP-catalyzed reaction, ascorbic acid interferes in end-point assays, resulting in negative errors. Adding ascorbic acid oxidase in the enzyme working solution can eliminate this interference of ascorbic acid.

4.2. Set up clean, dry test tubes labeled Blank (B), Standard (S) and Test (T) as follows:

Contents	B	S	T
Glucose Reagent (mL)	1.0	1.0	1.0
Distilled Water (mL)	0.01	-	-
Standard (mL)	-	0.01	-
Sample (mL)	-	-	0.01

- i. Mix well and incubate for 10 min at 37 °C or incubate the tubes for 20 min at room temperature.
- ii. Adjust the spectrophotometer at 505 nm using the blank.
- iii. Read the absorbance (OD) of standard (S) and Test (T).

4.3. Calculations:

$$\text{Glucose (conc. in mg \%)} = \frac{\text{O.D of (T)} \times \text{Conc. of Standard.}}{\text{O.D of (S)}}$$

5 Observations

Samples	Absorbance at 505 nm

6. Result:

The amount of glucose from plasma sample(s) by GOD-POD method is _____.

7. Precautions:

- Allow the lamps and electronics of the spectrophotometer to warm up (15 min) before use.
- Use the correct wavelength.
- Wipe the outer sides of the cuvette before measuring, using a smooth tissue paper.
- Carry out the procedure in the correct order.
- Check the calibration of the spectrophotometer.
- Close the door of the cuvette compartment before recording the OD.
- Do not spill any solution inside the cuvette compartment.
- Adjust the temperature of the incubator accurately.

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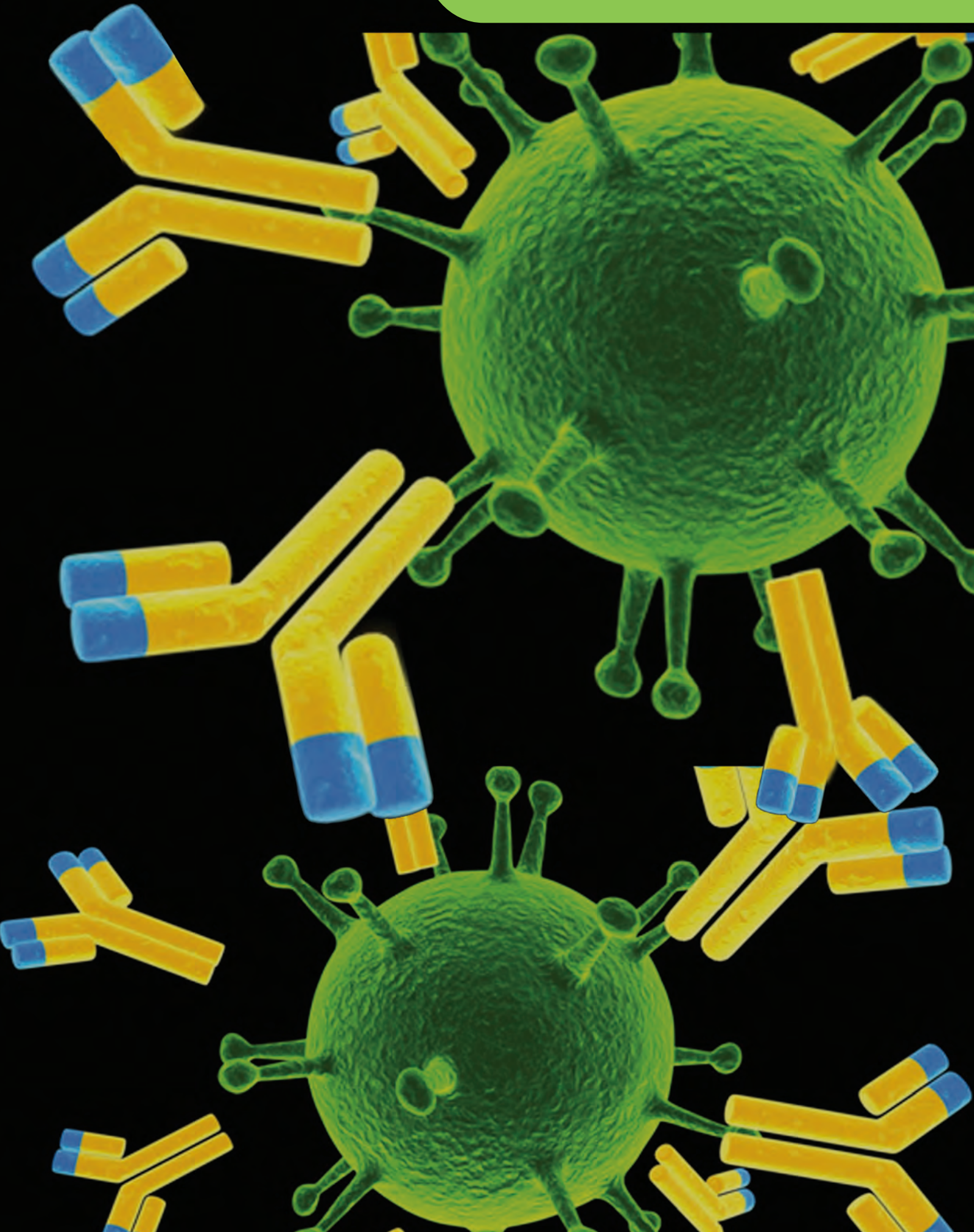
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3D Model of antibody and virus
<http://www.interactive-biology.com>

DOUBLE IMMUNODIFFUSION TECHNIQUE



1. Aim

To demonstrate antigen-antibody interaction by double immunodiffusion technique.

2. Introduction

There are wide varieties of techniques involving antigen-antibody immunodiffusion in gels. The key reaction of these techniques is the binding of antibodies to antigens and forming large macromolecular complexes. The principle behind these techniques is to react an increasing amount of antigen with a fixed amount of antibody. This results in precipitation due to formation of cross links as antibody binds with more than one antigen. The bonds formed between antigen and antibody, include hydrogen bonds, ionic bonds, hydrophobic interactions, and Vander Waals interactions. An immunodiffusion bioassay where both antigen as well as antibody diffuses into the gel is called 'double-diffusion'. Thus, Ouchterlony's double diffusion technique is a free diffusion method and differs in speed and sensitivity: some are strictly qualitative, others are quantitative. It provides a very useful tool for illustrating and clarifying the principles of antibody heterogeneity and specificity and is widely used in diagnosing diseases, monitoring the level of the humoral immune response, and identifying molecules of biological or medical interest.

3. Materials Required

3.1. Biological Materials: Test antigens (Ag1 and Ag2), antiserum

3.2. Chemicals/Reagents: Agarose, 1X assay buffer, alcohol

3.3. Equipment: Micropipette, pipette tips, petri plate, incubator

3.4. Glassware/Plastic ware: Glass slide, conical flask, measuring cylinder

3.5. Miscellaneous: Distilled water, cotton

4. Procedure

4.1. Preparation of agarose plate

- i. Take 5 mL normal saline and add 50 mg of agarose to prepare 1% agarose solution. Heat the solution in boiling water bath or in oven till agarose dissolves completely and no particles of agarose remain in suspension. After complete dissolution, cool the hot agarose solution to 40-50 °C.
- ii. Keep a clean glass microscopic slide on a horizontally level surface.
- iii. Gently pour 4.5 mL agarose solution on the slide using a glass pipette.
- iv. Pour the agarose solution such that it does not flow out of the edges of the slide but remains over the slide surface to form a 3-4 mm thick Layer.

- v. Allow the agarose to solidify at room temperature and keep it aside covered in a Petri dish.
- vi. Store the agarose slide at 4 °C, if not to be used immediately.

4.2. Cutting wells in the agarose

- i. Prepare a paper template for wells using a square white paper or using a graph paper, cut the edges equal to the width of the slide.
- ii. Mark positions of the central well in the centre of the paper and six outer wells at 60° angle and 5 mm away from the edges of the central well.
- iii. Place the slide over the template such that the marking on the paper visible through the agarose layer.
- iv. Before cutting the wells, keep the slide at 2-4 °C for a short period so that edges of the wells do not break when the agarose plugs are removed.
- v. Use a 3 mm cork borer (Fig. 1) to cut wells after the agarose gel is hardened sufficiently,
- vi. Cut 1 well in the center and 6 outer wells on the Gel, as marked on the template.
- vii. Prepare one more set of gel (Set 2) with six wells as explained above.
- viii. Use a marker and label the wells on the lower side of the slide.
- ix. In Set 1 gel, label the central well as Ag (for undiluted antigen), and the six outer wells serially as Ab (for undiluted antibody), and diluted antibody as 1:2, 1:4, 1:8, 1:16, and NS (normal saline) for the sixth well.
- x. In Set 2 gel, label the central well as Ab (undiluted antibody) and the six outer wells serially as Ser (for undiluted serum), and diluted serum as 1:2, 1:4, 1:8, 1:16, and NS (normal saline) for the sixth well.
- xi. Remove the agarose plugs from the wells.

4.3. Filling wells and incubation of agarose slides

- i. Dilute the antibody two-fold (1:2), four-fold (1:4), eight-fold (1:8) and sixteen-fold (1:16) with normal saline.
- ii. In the Set 1 Gel, add 10 µL undiluted antigen in the central well (marked 'Ag') by using a micropipette.
- iii. Add 10 µL of undiluted antibody in the first well (marked 'Ab') and add 10 µL of diluted antibody 1:2, 1:4, 1:8, 1:16 sequentially, in each outer well of agarose slide.
- iv. Add 10 µL of normal saline to the sixth well.
- v. Dilute the serum two-fold (1:2), four-fold (1:4), eight-fold (1:8) and sixteen-fold (1:16) with normal saline.
- vi. In the Set 2 Gel, add 10 µL undiluted antibody in the central well (marked 'Ab') by using a micropipette.
- vii. Add 10 µL of undiluted serum in the first well (marked 'Ser') and add 10 µL of diluted serum 1:2, 1:4, 1:8, 1:16 sequentially, in each outer well of agarose slide.



Fig.1:Cork borer Set
(Source: The Science Company, Lakewood, Colorado)

- viii. Keep the slide in a petri dish lined with filter paper moistened with distilled water.
- ix. Cover the petri dish with its lid and keep at room temperature overnight, undisturbed (lower temperature may slower the formation of precipitation line and prolong the test).
- x. Observe the slide after overnight incubation by keeping it on a black paper or black tile and note the white precipitin line, along the radius, between the edges of central and outer wells.

5. Observations

The precipitation line is seen as white arc between the edges of central well and edges of some of the outer wells. The maximum intensity seen is scored as +++++ (5 pluses) while the minimum intensity is scored as + (1 plus). Score 'Zero' is for no precipitin line.

The presence / absence and the intensity of precipitation line is recorded for each well as follows;

SET: 1	Central Well	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6
Concentration	Undiluted Antigen	Undiluted antibody	1:2 antibody	1:4 antibody	1:8 antibody	1:16 antibody	Normal Saline
Precipitin Intensity							
Titre of antibody is							

SET: 2	Central Well	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6
Concentration	Undiluted Antibody	Undiluted serum	1:2 serum	1:4 serum	1:8 serum	1:16 serum	Normal Saline
Precipitin Intensity							
Titre of antigen in Serum is							

6. Result

The presence of precipitation line (Fig. 2) indicates that antibody added is specific for the antigen and its dilutions. The dilution of the antibody where the precipitin line is no longer observed is the titre value of the antibody. It is the reciprocal of highest dilution of antibody that forms the precipitation line with the antigen

The presence of precipitation line at various dilution of antibody indicates that sufficient antibody is present and the dilution which can form a precipitation line with antigen. The position of the precipitin line depends on the relative concentration of antigen and antibody. The position of the precipitin line would get closer to antigen well as the antibody dilution increases. There should not be any precipitation line between central well and the peripheral well loaded with normal saline.

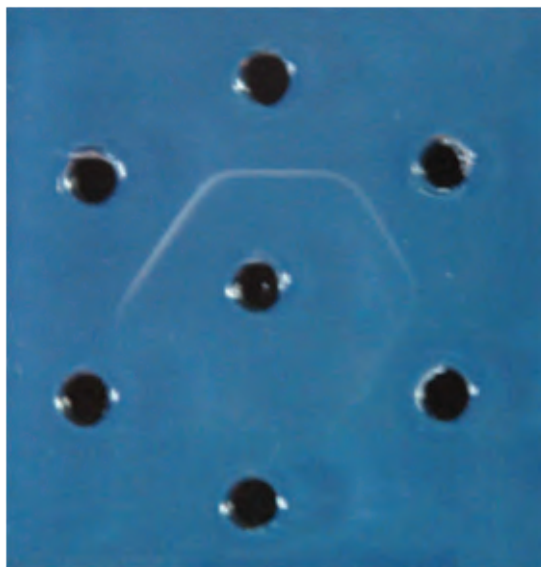


Fig. 2: Representative image of results obtained on antibody dilutions (titre is 1:8)

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TOTAL WHITE BLOOD CELL COUNT



1. Aim

To count total white blood cells (WBC) using Hemocytometer with improved Neubauer Counting Chamber.

2. Introduction

A normal human body has about 5 liters of blood, which is a fluid connective tissue containing large number of different cells (formed elements). Out of all cell types, the most varied type of cells is White Blood Cells (WBCs). These differ in their appearance, nucleus morphology and nature of cytoplasm. The number of leucocytes per liter of blood is known as the leukocyte number or Total WBC count. White blood cell count is made to investigate many diseases like leukemia, leucopenia, unexplained fever, infections and to monitor disease prognosis. Often total leukocyte counts (TLC) are carried out along with differential WBC counts (DLC). The types of WBCs or leucocytes are as follows;

2.1. Granulocytes: These cells contain single nucleus with lobed structures and small granules in cytoplasm. Based on the lobed structure of nucleus and shape of granules they can be classified as-

- Neutrophils:** They have polymorphic nucleus containing 3-5 lobes, cytoplasmic granules which stain pink with neutral dye. They are 10-12 μm in diameter and motile.
- Eosinophils:** They have bilobed nucleus, granules large and stain red with eosin. They are poorly motile and provide primary allergic response against infection by microorganisms.
- Basophils:** These are smaller than neutrophils having a diameter of 8-10 μm . They also have granules, S-shaped nucleus and are stained purple with basic dye.

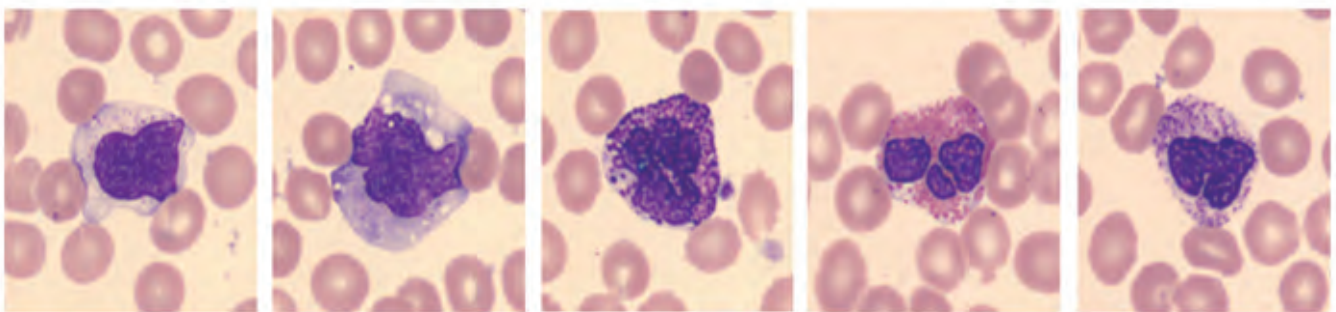


Fig. 1: White blood cells from left to right, lymphocyte, monocyte, eosinophil, basophil, and neutrophil.

(Source: Adapted from: Su et al, 2014)

2.2 Agranulocytes: These cells have homogenous cytoplasm without any granules and large nucleus.

These are (a) Monocytes and (b) Lymphocytes

- a) **Monocytes:** They are large sized cells having a diameter of 16-22 μm containing a large oval or kidney shaped nucleus and cytoplasm that stains greyish blue.
- b) **Lymphocytes:** They are smaller than monocytes about 10–20 μm , spherical with large nucleus and the cytoplasm stains pale blue. They can be differentiated into **T-cells** and **B-cells** which play a key role in providing immunity and protect from microbial and viral infections.

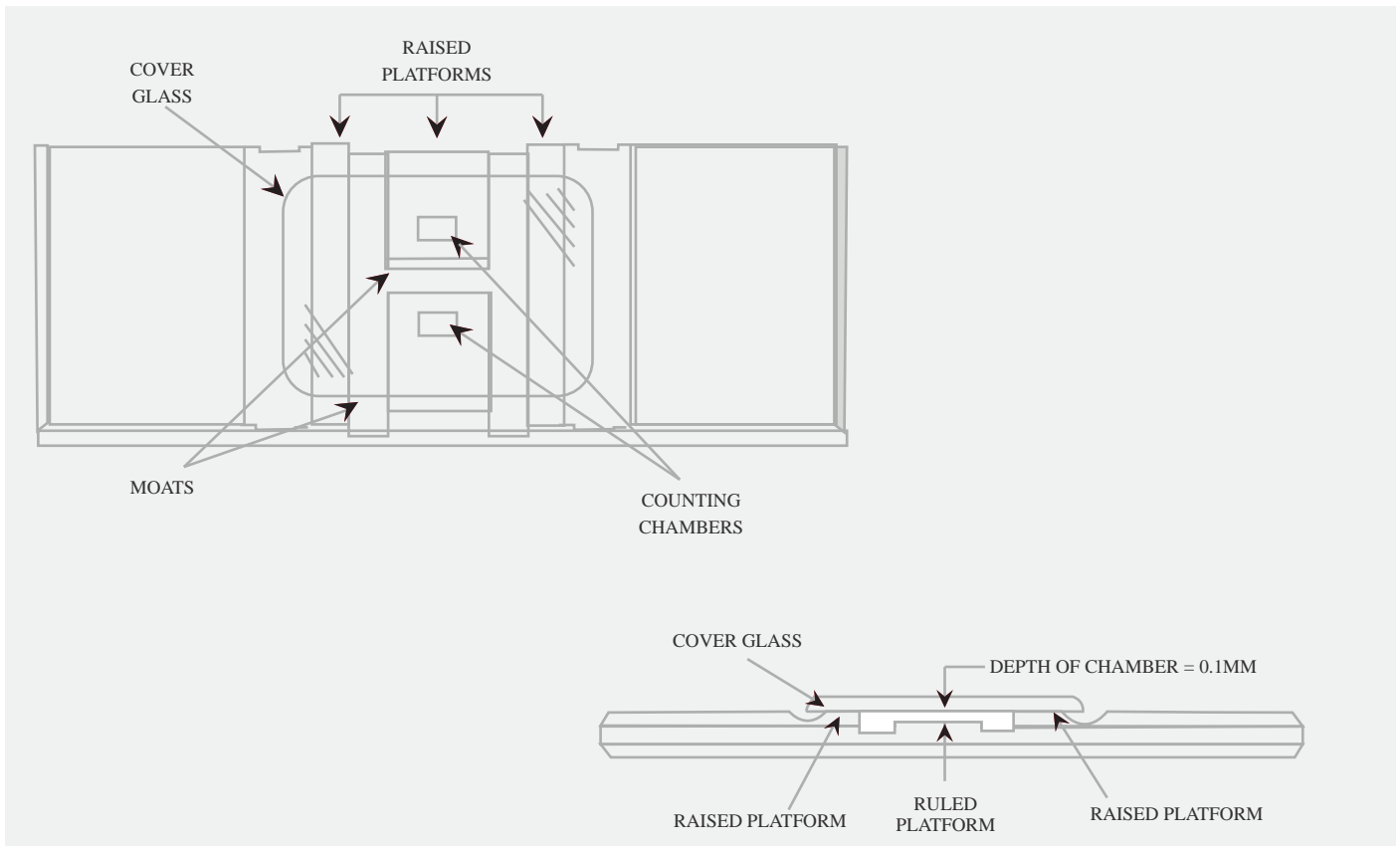


Fig. 2: Top view and Side view of Neubauer Hemocytometer slide

(Source: Integrated Publishing, Inc.)

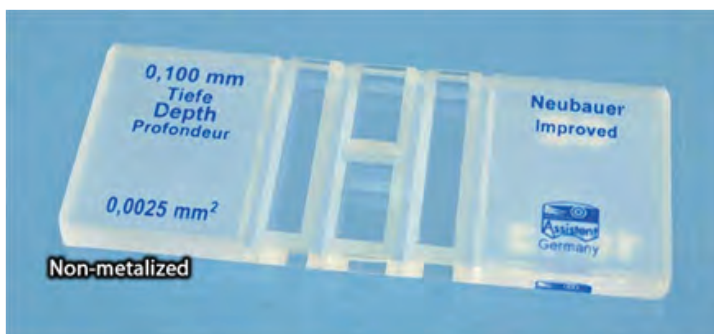


Fig. 3: Improved Neubauer Hemocytometer slide

(Source: Medicine, Science & More)



Fig. 4: White blood cell pipette

(Source: Eisco Labs)

3. Materials Required

3.1. Biological Material: Blood sample.

3.2. Chemicals/Reagents: WBC diluting fluid (Turk's fluid) (contains 8 mL glacial acetic acid, 1 mL of 1% gentian violet and made up to 100 mL with distilled water. Glacial acetic acid lyses RBCs and acts as fixative while gentian violet stains the nuclei of the WBCs), distilled water

3.3. Equipment: WBC pipette [It possesses a stem and a bulb, the latter acts as mixing chamber and contains a white bead. The bead acts as stirrer and indicates whether the bulb is dry or not (Fig. 4)].

3.4. Glassware/Plastic ware: Microscope slides, blotting paper, hemocytometer (for details see the description in the standard Medical Laboratory Handbook), capillary puncture.

4. Principle

The various cells in the blood are counted microscopically with the help of a counting chamber called a Hemocytometer, a device invented by the 19th century French anatomist Louis-Charles Malassez to perform blood cell counts. Hemocytometer consists of a thick glass microscope slide with a grid of perpendicular lines etched in the middle (Fig. 2 and 3). The modified version of Hemocytometer is known as Neubauer counting chamber. A Neubauer counting chamber with double rulings to distinguish RBC counting cells is called the Improved Neubauer counting chamber. Some Improved Neubauer counting chambers are metalized (reflective background) to provide better contrast while viewing the counting area.

The four squares (1 mm × 1 mm) placed at the corners of a hemocytometer are used for white cell counting. Since their concentration is lower than red blood cells a larger area is required to perform the cell count. Whole blood is diluted in 1:20 dilution (0.5 mark to 11 mark in the WBC pipette) with an acid reagent (Turk's Fluid) which hemolyzes the red blood cells and mildly stains the WBCs to make the counting easier. EDTA is used as the anticoagulant while collecting blood for counting.

5. Procedure

- i. Take out blood with the help of a Lancet puncture at tip of a finger. The wound should allow for free flow of blood without much squeezing to prevent dilution with tissue fluid.
- ii. Carefully suck the blood into WBC pipette up to the 0.5 mark without air bubble.
- iii. Carefully suck Turk's fluid up to the 11 mark without air bubble.
- iv. Hold the pipette horizontal and mix properly by rotating the pipette with fingers. Keep the pipette aside.
- v. Place the hemocytometer cover glass on the hemocytometer properly.
- vi. Place the Hemocytometer slide under low power of the microscope and focus it properly.
- vii. Discard few drops of the diluted blood from the pipette and charge the counting chamber with blood such that the blood does not flow into the moat.
- viii. Wait for few minutes so that the cells will settle down.
- ix. Count all types of leucocytes in four corner squares ("L" in Fig. 5) of counting chamber by moving the slide only in longitudinal and then upward direction.
- x. Count at least 1000 cells (approximately 250 cells in each square "L").

xi. Note that, only, cells falling in the square and touching any two adjacent sides of the square (i.e. either right-hand side boundary and bottom boundary OR Left-hand side boundary and upper boundary) should be counted.

a) Following formula is applied to calculate the Total Count of WBCs:

b) Concentration (Cells /mL) =

$$\frac{\text{Number of cells}}{\text{Volume (in mL)}}$$

c) Area of a single WBC square: $1 \times 1 = 1 \text{ mm}^2$

d) Area of 4 WBC square: 4 mm^2

e) Depth of counting chamber: 0.1 mm^2

f) Volume of single WBC square: $1 \times 0.1 \text{ mm}^3 = 0.1 \text{ mm}^3$

g) Volume of 4 counting squares: $4 \times 0.1 = 0.4 \text{ mm}^3$

h) Let total number of cells counted in 0.4 mm^3 of diluted blood: X cells

i) Therefore, total number of cells in 1 mm^3 of diluted blood: $X/0.4 \text{ mm}^3$ cells

j) Since the dilution is 10 times, the total number of cells in 1 mm^3 of undiluted blood = $(X \times 10)/0.4 \text{ mm}^3$ cells

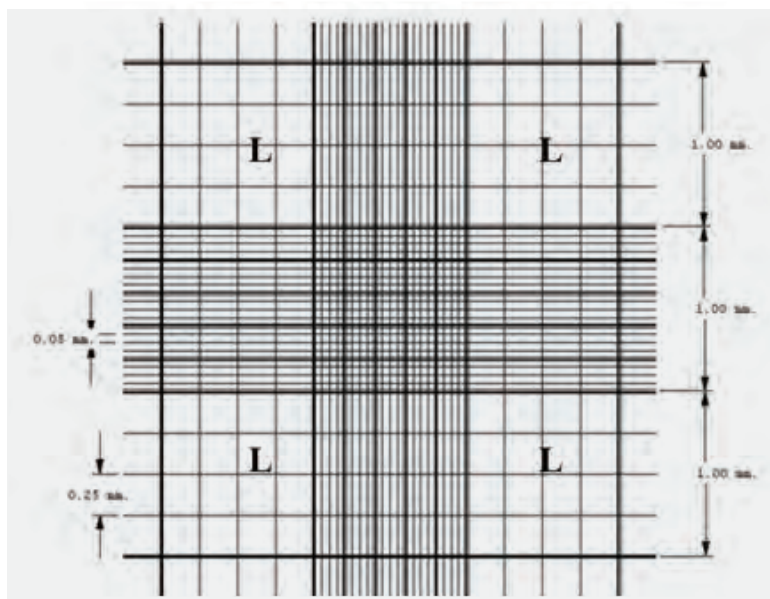


Fig. 5: Improved Neubauer Counting chamber with double ruling

(Source: ABCAM)

6. Result

The number of WBCs in 1 mm^3 of blood is calculated and the result can be presented by converting 1 mm^3 to 1 cm^3 which is also same as 1mL.

7. Precautions

- i. The tip of finger should be sterilized with medical spirit and dried before wounding.
- ii. Wound should be made with a lancet pin / lancet blade to get free flow of blood.
- iii. Clean the wound area with medical spirit to stop bleeding.
- iv. Pipetting of the blood should be done carefully to avoid air bubbles.
- v. Dilution should be done carefully till the specified mark mentioned.
- vi. Always wear gloves while drawing and handling blood.
- vii. Heparin or sodium citrate anticoagulant blood should not be used.
- viii. Blood should be used within 6 hrs of drawing.
- ix. Blood should not be refrigerated.

9. Extension Activity

Carry out Total RBC count using the same sample of blood.

Red blood cells being small are counted in the central big square area (1 mm × 1 mm) which is divided into 25 smaller squares (0.20 mm × 0.20 mm). The red cells are counted in the four small corner squares and the small central square. Due to high cell density in the blood, it is necessary to dilute it so much, as to get about 200 cells per count. The dilution is usually made in the ratio of 1:200 (blood: isotonic solution) using an isotonic diluent.

- a) Use the RBC pipette (Red bead) for collecting and diluting the blood.
- b) Collect fresh blood upto the 0.5 mark on the RBC pipette.
- c) Use normal saline (0.9% NaCl) as diluent.
- d) Dilute 200 times (0.5 mark to 101 mark) with the diluent.
- e) Charge the hemocytometer and count in the central RBC counting area.
- f) Observe and count under the 40 X objective.
- g) Use appropriate area and dilution factors to arrive at the total RBC count.

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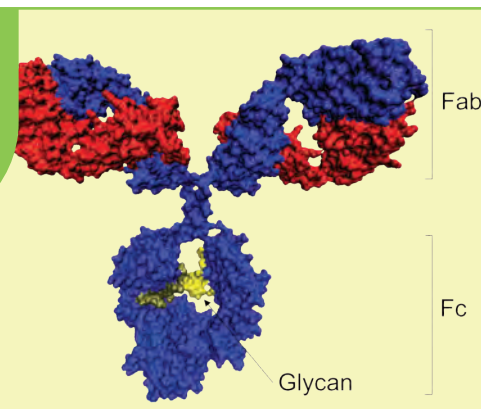
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PURIFICATION OF IMMUNOGLOBULIN (IgG)



1. Aim

To purify IgG from human serum using protein A Sepharose column chromatography.

2. Introduction

Immunoglobulins, also known as antibodies, are glycoprotein molecules produced by plasma cells. They act as critical part of the immune response by specifically recognizing and binding to particular antigens, such as proteins of bacteria or viruses and aiding in their destruction. There are five major types of antibodies; IgA, IgG, IgD, IgM and IgE. Of these, IgG antibodies contribute 75% to 80% of all the antibodies in the body and are found in all body fluids. They are the only type of antibody that can cross the placenta in pregnant women to help the foetus fight infections. IgG can be purified from human serum by affinity chromatography.

Affinity chromatography helps to purify proteins based on the specific biological interactions between a molecule (ligand) and the protein of interest. Immunoglobulin G (IgG) has a very strong affinity for protein A (ligand). Protein A is obtained from the cell wall of *Staphylococcus aureus* and binds to the Fc region of antibody molecule. When serum is passed through an affinity column having protein A as ligand all the serum proteins except IgG percolate down the column. The bound IgG is then eluted by breaking the electrostatic interactions between IgG and the ligand. In this experiment, protein A Sepharose gravity flow column (purchased from vendors) is used. Protein A Sepharose Columns are prepared by covalently coupling recombinant protein A to 6% cross-linked Sepharose beads. Sepharose in agarose in beads form. The coupling technique is optimized to give a higher binding capacity for IgG and minimum leaching of recombinant protein A. The IgG binding capacity of protein A Sepharose Column is around ≥ 16 mg human or rabbit IgG per mL of wet beads

Staphylococcus aureus is an invasive pathogen that causes skin and soft tissue infections (SSTI), bacteremia, sepsis and endocarditis. Protein A (SpA), is a surface molecule of *S. aureus*, which binds the Fc γ domain of immunoglobulin (Ig). The immunoglobulin (Ig)-binding protein is present on the bacterial surface and is freely secreted into the extracellular environment. Protein A consists of a single polypeptide chain of 42 kDa.



Fig. 1: Representative image of protein A Sepharose Gravity flow Columns

(Source: GE Healthcare Life science)

3. Materials Required

- 3.1. **Biological Material:** Serum sample.
- 3.2. **Chemicals/Reagents:** Protein A Sepharose–gravity-flow column (available with several vendors), phosphate buffered saline, 0.1 M Glycine-HCl (pH 2.8), 0.1 N NaOH.
- 3.3. **Equipment:** Column Chromatography apparatus, spectrophotometer.
- 3.4. **Glassware/Plastic ware:** Tubes, cuvette.

4. Procedure

- i. Set up the affinity column vertically using a clamp and stand.
- ii. Connect a cock to the column tip for adjusting the flow rate (an intravenous cannula cock will also suffice).
- iii. Keep a 10 mL test tube below, on a stand, for collecting the eluent.
- iv. Add gently twice the bed volume, PBS to the column to wash the protein A Sepharose column.
- v. Adjust the flow rate to one drop per ten seconds. Collect the eluent.
- vi. Set up a stand with a series of Eppendorf tubes marked serially from 1 to 30.
- vii. Add another volume of PBS (twice the bed volume) before the column is fully drained (when the earlier solution reaches about 1 mm above the bedding surface).
- viii. Load 300 μ L of serum sample on to the column.
- ix. Add twice the bed volume, PBS to elute the unbound protein.
- x. Repeat this at least twice.
- xi. When the PBS level reaches about 1 mm above the sepharose bedding, add twice the bed volume, glycine HCl buffer to the column.
- xii. Collect the eluting bound IgG into sequentially numbered Eppendorf tube (1 mL fractions).
- xiii. Add fresh volumes of glycine-HCl buffer each time the level of buffer reaches 1 mm above the sepharose bedding.
- xiv. Collect up to thirty, 1 mL fractions into 1 – 30, serially numbered eppendorf tubes.
- xv. Add 0.1 N NaOH to each of the fraction to adjust the pH to near neutral (Find the volume of NaOH required for neutralizing the 1st fraction and then add the same volume to all other fractions).
- xvi. Record the absorbance (optical density) of each of the collected fraction, at 280 nm.
- xvii. Pool together all fractions showing maximum absorbance.
- xviii. Wash the column thrice with double the bed volume, PBS.
- xix. Store the column at 4 °C for future use.

5. Observations

- i. Plot a graph of absorbance against fraction number to depict the elution profile.
- ii. Fractions showing high absorbance will have the maximum amount of protein.

6. Result

Human IgG can be successfully purified through the protein A Sepharose column. The second peak shown in (Fig. 2) is the IgG fraction. It can be confirmed with Immunodiffusion.

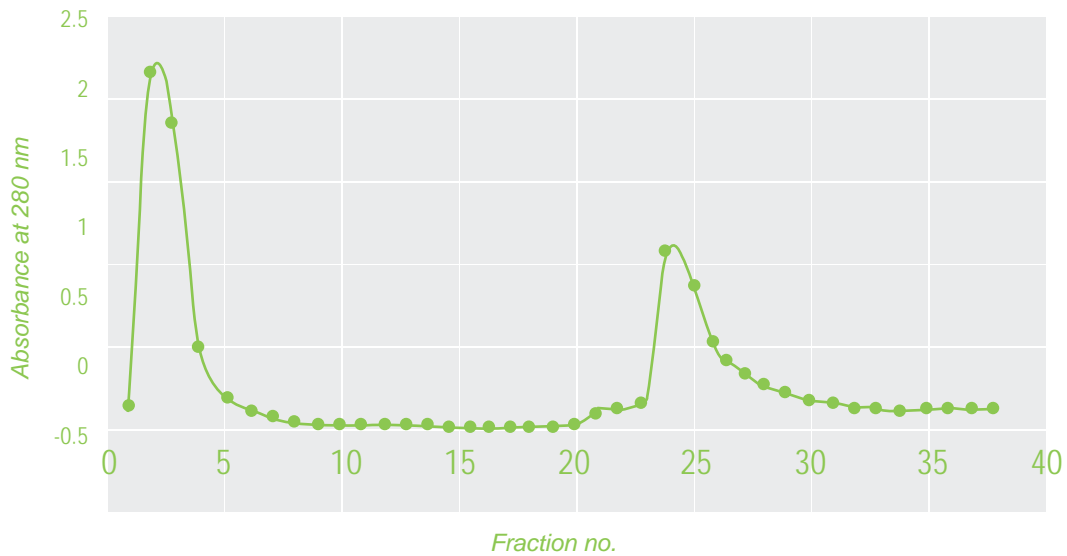


Fig. 2: Graph depicting peak of purified IgG

7. Precautions

- Add the eluent gently by the sides of the column to avoid disturbing the bedding.
- Load the serum samples slowly on the column.
- Load the column with fresh volume of eluent, as the eluent drops to 1mm level above the sepharose bedding
- Maintain uniform flow rate while collecting fractions.
- Neutralize the eluted IgG fractions immediately after collection.

8. Extension Activity

The pooled fractions with maximum absorbance at 280 nm, can be evaluated further in double immunodiffusion using anti IgG for confirming fractions containing IgG. The pooled fraction reacted positively with anti IgG in double immunodiffusion and immuno-electrophoresis (Fig. 3).

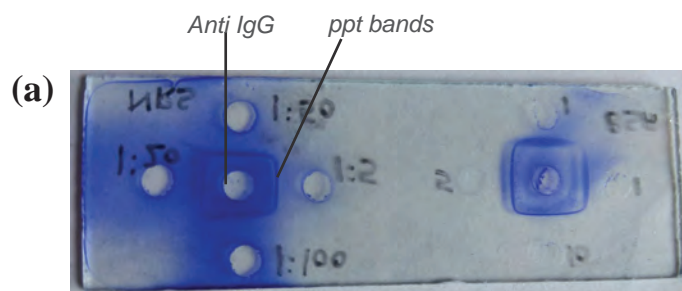
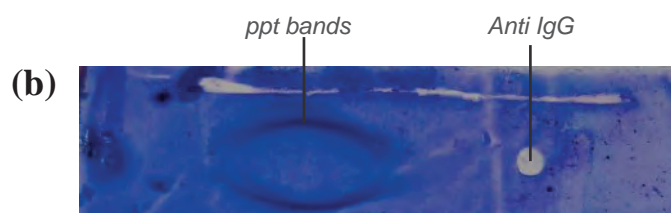


Fig. 3: (a) Doubleimmunodiffusion,



(b) Immuno-electrophoresis, showing IgG fraction

Run the pooled fractions (purified IgG) further on SDS PAGE to determine the molecular weight using a standard protein ladder. In SDS PAGE, two bands corresponding to heavy and light chains of IgG molecule were observed (Fig. 4).

9. Discussion

Protein A derived from cell walls of *Staphylococcus aureus* has a strong affinity for Fc region of IgG. Hence a very good yield of IgG is obtained using protein A sepharose column. Presence of IgG can be confirmed by double immunediffusion. The structure of IgG is analyzed in SDS-PAGE where in depending on the molecular weight of heavy and light chains (50,000 and 25,000 respectively) two specific bands can be observed.

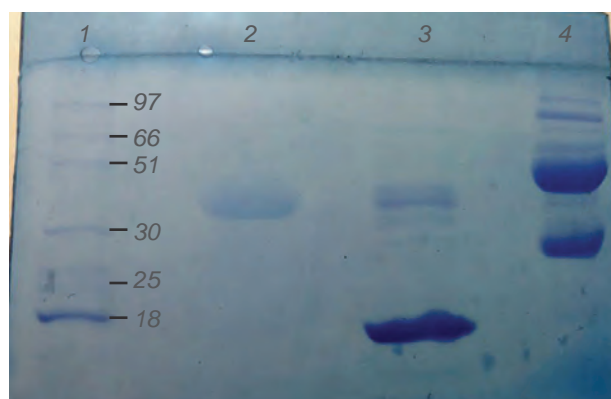


Fig. 4: SDS PAGE analysis of proteins: IgG fraction run on SDS PAGE

Lane 1: Mol. Wt. marker Lane 2: pepsin,
Lane 3: Lysozyme Lane 4: IgG

Suggested Reading(s)

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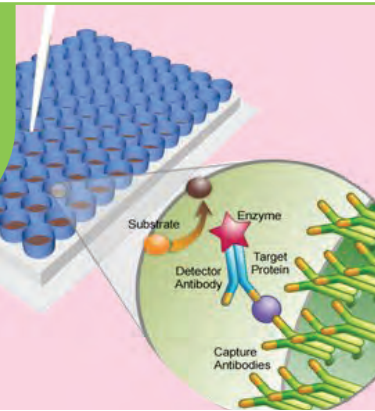
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Source

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ESTIMATION OF CYTOKINE LEVELS IN SERUM



1. Aim

To estimate cytokine levels using sandwich ELISA technique in serum samples from patients suffering Lumbar disc-degeneration.

2. Introduction

Cytokines are produced by a broad range of cells including immune cells like macrophages, B lymphocytes and T lymphocytes as well as other cell types like endothelial cells, fibroblasts and various stromal cells. A given cytokine may be produced by more than one type of cell. They act through receptors and are especially important in the immune response. Cytokines modulate the balance between humoral and cell based immune responses. They regulate maturation, growth and responsiveness of particular cell populations. Cytokines are important in health and diseases, specifically in host response to infection, immune responses, inflammation, trauma, cancer etc. Several diseases result from over-expression or under-expression of cytokines. Hence, presence of cytokines in blood samples can be associated with pathogenesis. Sandwich ELISA technique can be used to estimate cytokine levels in serum.

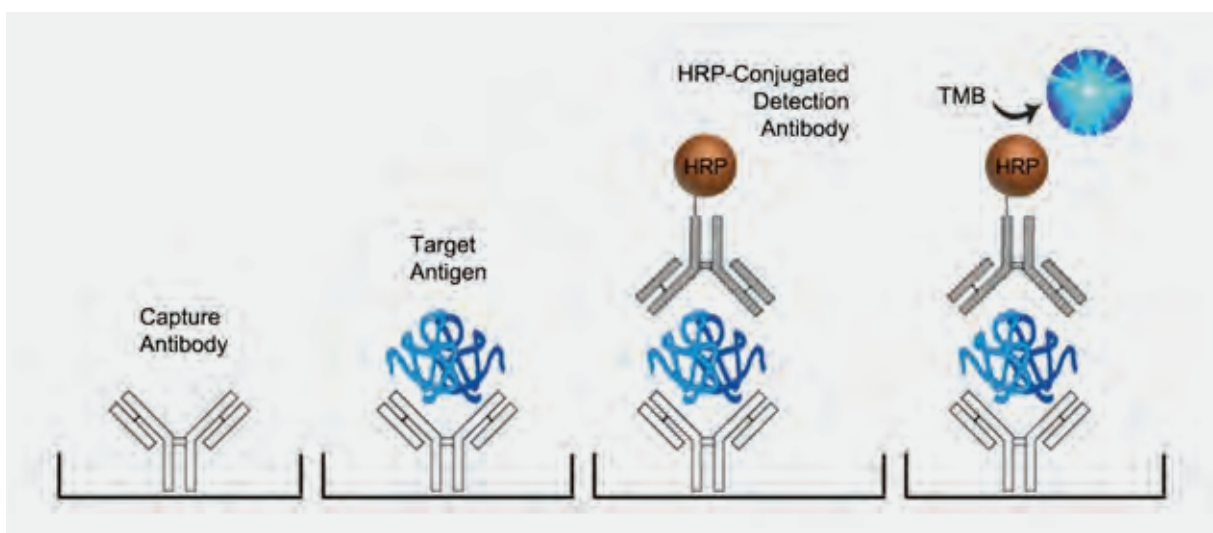


Fig. 1: An overview of Sandwich ELISA

(Source: LifeSpan BioSciences, Inc.)

3. Materials Required

3.1. Biological Material: Serum sample from patients having Lumbar disc-degeneration

3.2. Chemicals/Reagents: Wash buffer, blocking buffer, coating buffer, standard antigens, capture antibodies, detection antibodies, substrate solution, stop solution

3.3. Equipment: ELISA reader

3.4. Glassware/Plastic ware: ELISA plates, tips, pipettes.

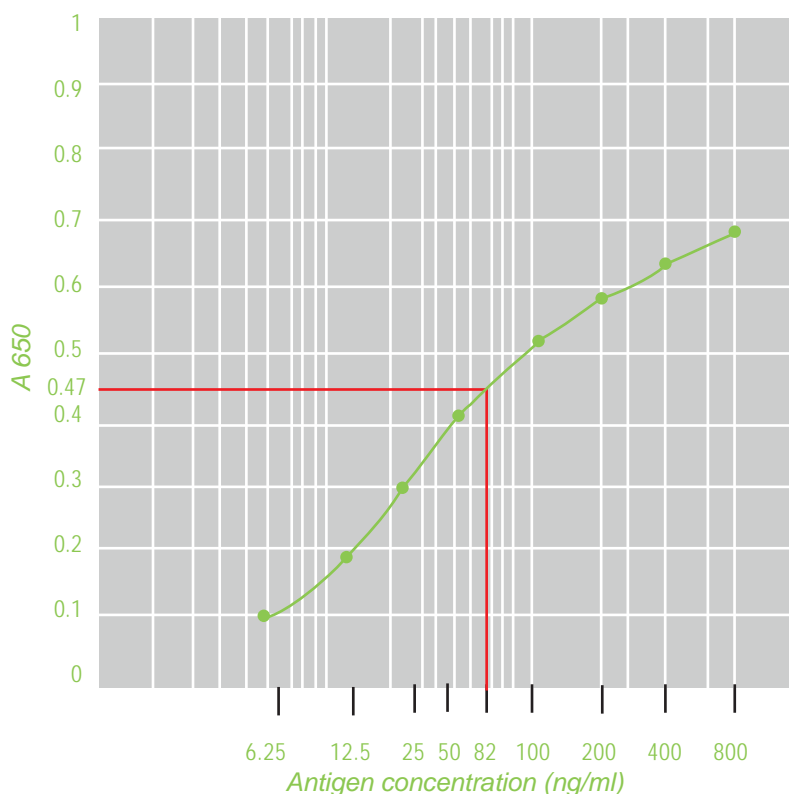
4. Procedure

- i. Coat ELISA plates with 100 μL of capture antibody in coating buffer, to each well and incubate overnight at 4 $^{\circ}\text{C}$.
- ii. Aspirate coating buffer from wells and wash 3 times with wash buffer (15 minutes each time).
- iii. Add 200 μL of blocking buffer to each well and incubate at 37 $^{\circ}\text{C}$ for 1 hr.
- iv. Aspirate blocking buffer from wells and wash 3 times with wash buffer (15 minutes each time).
- v. Add 100 μL of standard antigens to each well and serum samples to the appropriate wells and incubate overnight at 4 $^{\circ}\text{C}$.
- vi. Aspirate solutions from wells and wash 3 times with wash buffer (15 minutes each time).
- vii. Add 100 μL of detection antibody in each well and incubate at 37 $^{\circ}\text{C}$ for 1 hr.
- viii. Aspirate solutions from wells and wash 3 times with wash buffer (15 minutes each time).
- ix. Add 100 μL of substrate solution to each well and incubate the plate at room temperature for 15 minutes.
- x. Add 50 μL of stop solution to each well.
- xi. Read the plate at 450 nm and analyze the data.

5 Observations

5.1. Observation Table:

Antigen concentration (ng/mL)	O.D at 450 nm
800	0.638
400	0.596
200	0.535
100	0.485
50	0.396
25	0.278
12.5	0.167
6.25	0.0987
T (Test Sample)	0.47



Plot a semi-log graph with absorbance against concentration of antigen

Fig. 1: Graph depicting the ELISA results
Test sample concentration = 82 ng/mL (from graph)

6. Result

It is possible to detect significant level of cytokine (IL-1) in serum samples obtained from patients having Lumbar disc-degeneration.

7. Discussion

ELISA is a sensitive technique for detecting cytokines. The enzyme linked secondary antibody acts specifically on the substrate to produce colour. The standard graph is used to detect the amount of cytokine in the serum samples.

8. Precautions

- i. Pipetting should be done carefully.
- ii. Washing can be increased if background is observed.
- iii. Colour development should be done in dark.

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CELL VIABILITY STUDY BY TRYPAN BLUE DYE



1. Aim

To estimate cell viability using Trypan blue dye exclusion test.

2. Introduction

Viable cell count refers to the number of living cells in a given volume or unit for a given sample. Dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that can, by active transport, exclude certain dyes, such as trypan blue, eosin, or propidium, whereas dead cells do not. In this test, a cell suspension is simply mixed with the dye and then visually examined under microscope to determine whether cells take up or exclude dye. In the protocol described below, a viable cell will show a clear cytoplasm whereas a nonviable cell will show a blue cytoplasm. Main application of this technique is in cell enumerations during cell culture studies where number of live cells should be known before starting any *in vitro* procedures.

3. Materials Required

- 3.1. Biological Material:** Cell suspension at $2-6 \times 10^6$ cells/mL (macrophages from peritoneal cavity of mouse/rat).
- 3.2. Chemicals/Reagents:** Trypan Blue 0.4% (w/v) in distilled water, PBS.
- 3.3. Equipment:** Neubauer counting chamber (Haemocytometer).
- 3.4. Glassware/Plastic ware:** 5 mL glass or plastic centrifuge tubes, 10 mL syringe with needle, pasteur pipette, micro pipettes.

4. Procedure

- i. Inject 10 mL of PBS into the peritoneal cavity of an anaesthetised mouse/rat (intraperitoneal injection).
- ii. Give gentle massage to the abdomen for 2-3 minutes.
- iii. Carefully give incision to the abdominal wall without draining the abdominal fluid.
- iv. Aspirate the peritoneal fluid with the help of Pasteur pipette.
- v. Euthanize the rat/mice suitably.
- vi. Collect peritoneal fluid aspirate into a clean, graduated centrifuge tube.
- vii. Centrifuge the tube (1000 rpm for 5 minutes) to sediment the peritoneal cells.
- viii. Drain the supernatant and gently add fresh 5 mL of PBS.
- ix. Aspirate the cells and agitate the cell sediment gently with a pasteur pipette to wash cell suspension in PBS.
- x. Centrifuge the suspension at 1000 rpm for 5 minutes and resuspend the cells in PBS.
- xi. Repeat the washing procedure thrice.

- xii. Finally, drain the supernatant and suspend the cell pellet in 5 mL of fresh ice cold PBS (Adjust the volume of suspension depending upon the size of pellet).
- xiii. Transfer 50 μL of cell suspension into an Eppendorf tube and add 50 μL of trypan blue (1:1 dilution).
- xiv. Using a micropipette, pipette out about 100 μL of Trypan Blue-treated cell suspension and charge the hemocytometer. If using a glass hemocytometer, then gently fill both chambers underneath the coverslip. Allow the cell suspension to spread the entire counting area, without flooding the moats. If using a disposable hemocytometer, then pipette the cell suspension into the well of the counting chamber, allowing capillary action to draw it inside.
- xv. Using a microscope, focus on the grid lines of the hemocytometer with a 10X objective.
- xvi. Count the live, unstained cells (live cells do not take up Trypan Blue) in one set of 16 squares (marked “L” in Fig. 1). Count the cells which are set within a square or on the right-hand or bottom boundary line.
- xvii. Count the dead, stained cells using the same guidelines.
- xviii. Move the hemocytometer to the next set of 16 corner squares and carry on counting until all 4 sets of 16 smaller squares at the four corners are counted.

4.1. Estimation of viability:

To calculate the number of viable and dead cells/mL:

- i. Take the average cell count from each of the sets of 16 corner squares.
- ii. Multiply by 10,000 (10^4).
- iii. Multiply by 5 to correct for the 1:5 dilution from the Trypan Blue addition.
- iv. The final value is the number of viable cells/mL in the original cell suspension.

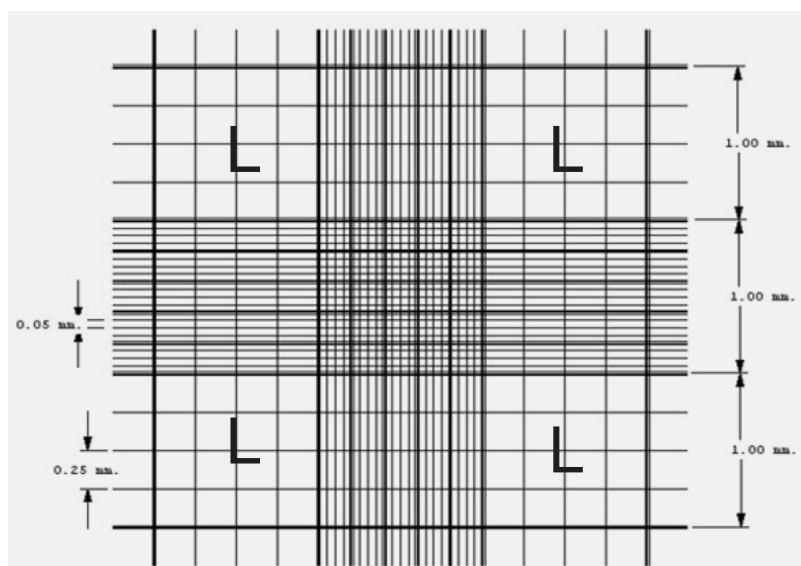


Fig. 1: Improved Neubauer Counting chamber with double ruling

(Source: ABCAM)

5. Precautions

- i. Cell suspension and trypan blue mixture should not be kept for more than 1-2 minutes because longer incubation with the dye may be toxic to viable cells and will result in overestimating the number of dead cells.
- ii. The cell mixture should flow under the coverslip by capillary action and spread to the entire counting area.
- iii. Complete the count quickly since cells will start dying inside the counting chamber leading to overestimation of dead cells.

Suggested Reading(s)

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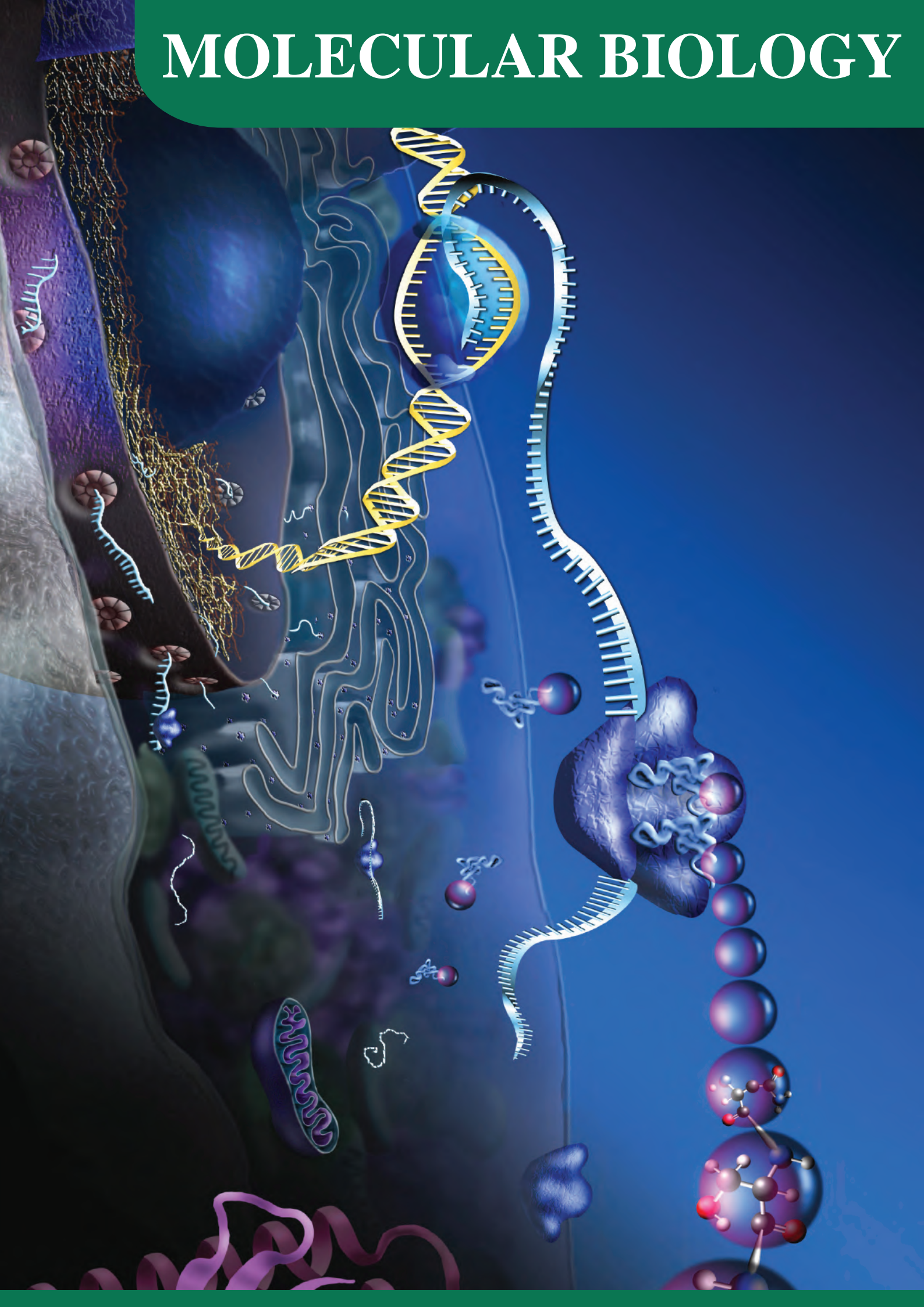
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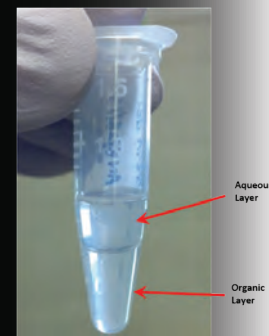
MOLECULAR BIOLOGY



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ISOLATION OF GENOMIC DNA FROM BACTERIAL CELL



1. Aim

To isolate genomic DNA from bacterial Cell.

2. Introduction

The chromosome of most bacteria is a single super coiled, double stranded, circular DNA molecule. The *E. coli* chromosome is well studied, as the total length of the circular chromosomal DNA is about 1300 μm whereas the rod-shaped bacterium has a diameter and length of about 1 and 3 μm , respectively. A complex packaging mechanism is necessary in order to ensure that the entire DNA is folded within the bacterial cell in a manner, which will not inhibit transcription nor allow entanglement of the two daughter strands to occur during replication. For the isolation of DNA from bacterial cell, cell envelope must be removed. In bacteria, there are two distinct classes of cell envelopes known as Gram- positive and Gram-negative. Gram-positive cell envelopes consist of plasma membrane and peptidoglycan layer (cell wall) whereas the architecture of Gram-negative cell envelope is more complex, consisting of plasma membrane, periplasmic space, peptidoglycan and outer membrane. *E. coli* is an example of Gram-negative bacterium. Therefore its cell wall is more complicated than that of Gram-positive cells. In order to isolate chromosomal DNA from *E. coli*, the cell envelope structure needs to be weakened and ruptured. Treatment of cell with various reagents such as trypsin (which hydrolyze proteins), detergents (which remove lipids) and lysozyme (muramidase- break down bacterial cell wall) causes dissolution of peptidoglycan layer. The isolation of *E. coli* chromosomal DNA is based on the following principle:

2.1. Principle:

The isolation of bacterial genomic DNA is one of the primary requirements in the areas of bacterial genetics, molecular biology and biochemistry. Purified DNA is required in many applications such as studying structure and chemistry of DNA, examining DNA-protein interactions, carrying out DNA hybridizations, sequencing, PCR and gene cloning. The isolation of DNA from bacteria is a relatively simple process. The common step in all the procedures is that a cell is first broken and then the DNA is separated from other intrusive compound such as proteins, RNA, lipid and carbohydrates. Purity of the DNA is essential as slight contaminants can inhibit further experiments like restriction digestion, Polymerase chain reaction, sequencing etc.

2.2. Major five steps of DNA extraction are as per following:

i. Homogenization or disruption of cells:

The cell must first be lysed (broken open) to release the nucleus in eukaryotes or nucleoid in prokaryotes. Cells are disrupted by grinding, tissue homogenization or treatment with lysozyme. Along with lysozyme, addition of the ionic detergent dissolves the cell membrane and denatures many proteins. In the extraction medium, high salt concentration (0.15 M NaCl) is also used which helps to prevent strand separation of the DNA.

ii. Inhibition of DNAase:

At this stage, the DNA must be protected from the enzymes, which may degrade it. Many of the nucleases present in cells can digest nucleic acids. When the cell is disrupted, the nucleases can cause extensive hydrolysis. Chelating agents (viz EDTA) are added to remove metal ions, which are essentially required for nuclease activity.

EDTA forms complexes (chelates) with several metal ions. Divalent metal contains, such as Mg^{+2} are required cofactors by the majority of DNases. The DNA being extracted is protected from DNases degradation since the complexed Mg^{+2} cannot be utilized by enzyme.

iii. Dissociation of nucleoprotein complexes:

DNA-protein interactions are interfered through SDS, phenol or broad spectrum proteolytic enzymes such as proteinase or proteinase K. These enzymes proteins which are freely available in solution or bound to the DNA. Alkaline pH and high concentration of salts improve the efficiency of the process.

iv. Removal of intrusive compound:

Contaminating molecules especially proteins are removed by treatment with phenol-chloroform-isoamyl alcohol or phenol-chloroform. Proteins can also be removed by salting out process using sodium acetate. RNase is also added in the solution to degrade high molecular weight RNA. RNases and proteases are exceptionally stable enzymes may also remain active in the presence of denaturing detergents such as sarkosyl and at high temperature.

v. Precipitation of DNA:

In the presence of salts, DNA and RNA precipitate from solutions containing high percentages of isopropanol or ethanol. The DNA in the aqueous phase is precipitated with chilled (0 °C) ethanol. The precipitate is usually redissolved in buffer and treated with phenol or organic solvent to remove the remaining traces of protein, followed by reprecipitation with chilled ethanol.

3. Materials Required

3.1. Biological Sample: Overnight grown fresh culture of *E. coli* DH5 α strain.

3.2. Chemicals/Reagents: Saline –EDTA (0.15M NaCl, 0.1M EDTA adjusted to pH 8.0), lysozyme solution (10mg/mL), 10% sodium dodecyl sulphate (SDS), 10 mM Tris buffer (pH 8.0), saturated phenol:chloroform:isoamyl alcohol (25:24:1 Mixture), TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0), isopropanol, dehydrated ethanol, and DNase free RNase solution (10 mg/mL).

[NOTE: Media and solution must be used after sterilization is done by autoclaving at 15 lbs for 15 min].

3.3. Equipment: Electrophoresis unit, UV transilluminator, centrifuge, incubator, pH meter, and weighing Balance.

3.4. Glassware/Plastic ware: Conical flasks, petri plates, centrifuge tubes, micropipettes and tips.

[NOTE: All glassware and plastic ware must be used after sterilization.]

4. Procedure

The following protocol was used for mini preparation of *E. coli* chromosomal DNA:

- i. Pick a single colony of *E. coli* (DH5 α strain) from a freshly grown plate and transfer it into 20 mL of LB broth in a 250 mL of flask. Incubate the culture for 16-20 hrs. at 37 °C with vigorous shaking (200-250 cycles/minute in a rotary shaker).
- ii. Take the above grown stationary phase culture (1.5-2.0 mL) in sterile microfuge tube. Centrifuge it at 5,000 rpm for 5 min. Decant the media from the cell pellets, stand the tubes in an inverted position for one minute to allow the last traces of media to drain away. Resuspend the cell pellet in to 1 mL 10 mM Tris-Cl by inverting the tube. and repeat the centrifugation step to wash the pellet.
- iii. Resuspend the cell pellet in 0.8 mL saline EDTA buffer thoroughly.
- iv. Add 50 μ L freshly prepared lysozyme solution and mix properly. Incubate at 37 °C for 20 min.
- v. Add 0.2 mL 10% SDS, mix well by inversion and incubate in water bath at 60 °C for 15 min.
- vi. Add 0.5 mL equilibrated phenol into the tube and mix the content by inverting the tube. Centrifuge it at 10,000 rpm for 15 min.
[NOTE: After centrifugation upper phase must appear clear, if not -then repeat the step]
- vii. Carefully transfer the upper aqueous phase into a fresh microfuge tube and add equal volume of phenol: chloro form: isoamyl alcohol (25:24:1). Mix the content by inverting the tube. Centrifuge it at 10,000 rpm for 15 min.
- viii. Carefully transfer the upper aqueous phase to a sterile microfuge tube and add equal volume of chilled isopropanol or double volume of absolute ethanol from sidewall of the tube. Invert the tubes gently for 1-2 times.
- ix. Incubate the tubes for 30 min at -20 °C. Centrifuge it at 10,000 rpm for 15 min. Decant the supernatant and invert the tube on sterile filter paper.
- x. Add 100 μ L of 70% ethanol to the DNA pellet. Rotate the tube horizontally several times and centrifuge the tube at 10,000 rpm for 5 min in a microfuge. Carefully remove the supernatant by inverting and allow the DNA pellet to dry in air for 15 min.
- xi. Dissolve the DNA pellet in 50 μ L of 1X TE buffer by gently tapping the tube. Store at 4 °C for further use.
- xii. Prepare agarose gel of 0.7% in TAE buffer.
- xiii. Load 10-15 μ L of isolated DNA sample, after addition of gel loading dye (1X) and run the gel at 100 V for 30-40 min. DNA marker should also be loaded in a marked lane to confirm the size of DNA.
- xiv. Visualize the DNA sample under UV light and record the observation.

5. Observation

The gel was observed under UV light to find out the presence or the absence of DNA bands. DNA can be seen as fluorescent band under UV light.

6. Result

In Fig. 1, after electrophoresis, one band of bacterial genomic DNA was observed in each sample close to the well which indicates that the band is of high molecular weight and thus it's a genomic DNA.

Absence of Smear on gel also confirms that shearing of DNA has not taken place.

7. Conclusion

The bacterial genomic DNA is intact as well as of high concentration, hence can be used for future applications.

8. Precautions

- Use caution when operating the centrifuge.
- The laboratory working area should be very clean throughout all the procedural step of the experiment.
- DNase free plastic wares and reagents should be used. Use only clean, dry and autoclaved tubes and tips.
- Since ethanol is highly flammable take precaution while handling it.
- The phenol chloroform extraction should be repeated depending on the source of DNA to obtain pure DNA.
- Take precautions in while working with ethidium bromide (it is carcinogenic). Wear gloves while handling ethidium bromide stained agarose gels.
- Examine the reagents for precipitation. If any reagent forms a precipitate (other than enzymes), lukewarm at 55-65 °C until the precipitate gets dissolved and then allow to cool down to room temperature (15-25 °C) before use.

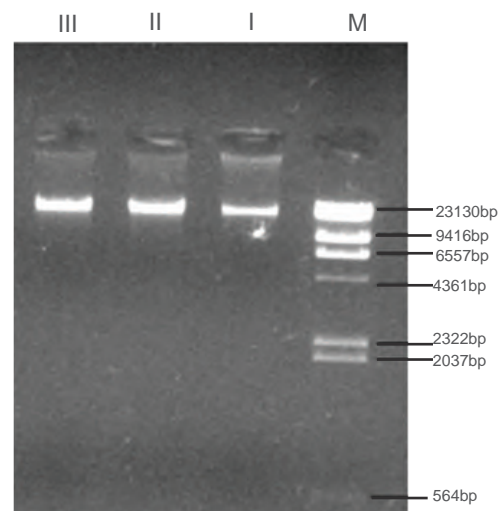


Fig. 1: DNA isolated from Bacterial Cell

Lane I-III: DNA Samples
Lane M: DNA Marker

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ISOLATION OF GENOMIC DNA FROM PLANT CELL

1. Aim

To isolate total plant genomic DNA by CTAB method.

2. Introduction

DNA isolation is an essential technique in molecular biology. Isolation of high-molecular weight DNA has become very important with the increasing demand for DNA fingerprinting, restriction fragment length polymorphism (RFLP), construction of genomic or sequencing libraries and PCR analysis in research laboratories and industries. DNA isolation is also the first step in the study of specific DNA sequences within a complex DNA population, and in the analysis of genome structure and gene expression.

In prokaryotic cells, DNA is localized in nucleoid that is not separated from the rest of the cell sap by a membrane. In eukaryotic cells, the bulk of DNA is localized in the nucleus, an organelle that is separated from the cytoplasm by a membrane. The purpose of DNA isolation is to separate DNA from all the other components of cell, resulting in a homogeneous DNA preparation that represents the entire genetic information contained within the cell. There is no difficulty in separating DNA from small molecules since the molecular weight of DNA is very large. The two main problems in isolating DNA from plants are the presence of DNase that degrade the DNA and the presence of other macromolecules (polysaccharides, polyphenols and proteins) that co-precipitate with DNA during isolation procedure. Three major types of techniques or combination of them are employed in isolation of nucleic acids: differential solubility, adsorption methods and density gradient centrifugation. Selection of method depends on the type of DNA being isolated and its application. Major goal of nucleic acid isolation is removal of proteins, which is accomplished due to their different chemical properties. The most commonly used basic plant DNA extraction protocols are those of Dellaporta *et al.* 1983 and Saghai Maroof *et al.*, 1984 along with many others that are modifications of the components of these protocols to suit a particular tissue type or downscaling them for miniprep.

2.1. Principle:

Good quality DNA is a prerequisite for all experiments of DNA manipulation. All plant DNA extraction protocols comprise of the basic steps of disruption of the cell wall, cell membrane and nuclear membrane to release the DNA into solution followed by precipitation of DNA, while ensuring removal of the contaminating biomolecules such as the proteins, polysaccharides, lipids, phenols and other secondary metabolites. This is brought about by disruption of the tissue in a mortar and pestle aided by liquid nitrogen and the various components of the homogenization or extraction buffer followed by precipitation and purification. Since the plant DNA can be extracted from various types of tissues such as seedlings, leaves, cotyledons, seeds, endosperm, tissue culture callus, roots etc., the tissue type along with the concentration of DNA finally required, determine the methodology of DNA extraction to be followed by the experimenter.

2.2 Components:

The role of various components of DNA extraction protocol is as follows:

a) The extraction buffer:- Cell Lysis:

This includes a detergent such as Cetyl Trimethyl Ammonium Bromide (CTAB) or SDS which disrupts the membranes, a reducing agent such as β -mercaptoethanol which helps in denaturing proteins by breaking the disulphide bonds between the cysteine residues and for removing the tannins and polyphenols present in the crude extract, a chelating agent such as EDTA which chelates the magnesium ions required for DNase activity, a buffer which is almost always Tris at pH 8 and a salt such as sodium chloride which aids in precipitation by neutralizing the negative charges on the DNA so that the molecules can come together. These denaturing conditions efficiently solubilized the nucleic acid and generally do not adversely affect them and lyse the cell by destabilizing the cell wall.

b) Phenol chloroform extraction:- Protein Precipitation:

Nucleic acid solutions commonly contain undesirable contaminants that are mainly contributed by proteins and polysaccharides. A classic method of removing these contaminants is phenol – chloroform extraction in which the nucleic acid solution is extracted by successively washing with a volume of phenol (pH 8.0); a volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) and chloroform: isoamyl alcohol (24:1). Centrifugation is performed alternately and the upper aqueous phase is transferred to a new tube while avoiding the interphase. The contaminants are denatured and accumulated in the organic phase or in the marginal layer between the two phases and the nucleic acids are preserved in the aqueous phase. Another way of removing proteins is by using the enzyme proteinase K which however again is denatured by phenol via phenol chloroform extraction.

c) Precipitation of nucleic acids:

Alcohol precipitation is the most commonly used method for nucleic acid precipitation. This requires diluting the nucleic acid with a monovalent salt, adding alcohol to it and mixing gently. The nucleic acids get precipitated spontaneously and can be pelleted by centrifugation. The salts and alcohol remnants are removed by washing with 70% alcohol. The most commonly used salts include sodium acetate pH 5.2 (final volume 0.3M), sodium chloride (final concentration 0.2M), ammonium acetate (2-2.5M), lithium chloride (0.8M) and potassium chloride. Ethanol (twice the volume) or isopropanol (two thirds volume) are the standard alcohols used for nucleic acid precipitation.

d) Resuspending DNA:

The nucleic acid pellet can be resuspended in either sterile distilled water or TE (10 mM Tris: 1mM EDTA)

e) Purification of DNA:

The DNA is purified by incubating the nucleic acid solution with RNaseA (10 mg/mL) at 37°C and reprecipitation following phenol: chloroform extraction to remove the RNase.

3. Materials Required

3.1.Plant Material: Leaves.

3.2. Chemicals/Reagents: Saturated phenol pH 8.0, chloroform: isoamylalcohol (in 24:1 ratio) mix, TE Buffer = Tris: EDTA (10mM : 1mM) pH 8.0, 70% ethanol,

- i. RNase A (10 mg/mL):
 - a. Dissolve RNase A in 10 mMTris-Cl, pH 7.5, 15 mMNaCl. Heat at 100 °C in water bath for 2-5 mins.
 - b. Cool to room temperature. Store as aliquots at -20 °C.
- ii. Extraction (CTAB) Buffer:

Reagent	Amount to add (for 10 mL)	Final concentration
10% Cetyltrimethyl ammonium bromide(CTAB)	3 mL	3%
5 M NaCl	2.8 mL	28%
0.5 M EDTA (pH 8.0)	0.4 mL	4%
1 M Tris-Cl (pH 8.0)	1 mL	10%
Polyvinylpyrrolidone (PVP) (MW 40 kDa)	0.3 g	3%
β-Mercaptoethanol	0.02 mL	0.2%
H2O	2.48 mL	24.8%

3.3. Equipment: Water bath, micropipettes, centrifuge, electrophoresis chamber and UV trans illuminator.

3.4. Glassware/Plastic ware: Sterile tips, sterile microcentrifuge tubes, stands for tubes and flask, mortar and pestle.

4. Procedure

- i. Grind 1.0 g tissue in liquid N₂
- ii. Transfer the powder in 50 mL centrifuge tube containing 10 mL of 2% CTAB buffer and add 7 μL of β-mercaptoethanol. Keep at 60 °C for about half an hour.
- iii. Perform chloroform: isoamyl alcohol (24:1) extraction once or twice and centrifuge at 6000 rpm for 15 min at room temperature.
- iv. Pipette out upper aqueous phase in a fresh centrifuge tube and add half volume of 5 M NaCl and 1/10th volume of 3 M Sodium acetate.
- v. Precipitate DNA with chilled iso propanol (5 mL).
- vi. Mark the centrifuge tubes with probable location of pellet formation.
- vii. Centrifuge for 3 min at 3000 rpm and continue for next 3 min at 5000 rpm.
- viii. Wash DNA pellet in chilled 70% ethanol.
- ix. After drying the pellet resuspend the DNA in 1X TE and transfer the suspension to microcentrifuge tube using wide bore pipette tip.
- x. Centrifuge again at 3000 rpm at 4 °C for 5min to remove any remaining debris.
- xi. Add 1 μLRNase (from 10 mg/mL stock) to 100 μL DNA suspension and incubate at 37° C for one hour.
- xii. Add MilliQ water (double distilled water) to make up the volume to 500 μL.

- xiii. Perform chloroform:isoamyl alcohol (24:1) using its equal volume (i.e. 500 μ L) extract twice and remove upper phase using wide bore tips.
- xiv. Precipitate DNA in 1/10th sodium acetate and 95% ethanol (add 5.0 M NaCl if necessary).
- xv. Centrifuge at 10,000 rpm for 10min at 4 °C and wash pellet thrice with 95% EtOH.
- xvi. Dry pellet and elute in 0.1X TE and quantify on gel.

5. Observation

The gel was observed under UV radiation for the presence or the absence of DNA bands. DNA marker also loaded in lane 1 to confirm size of DNA.

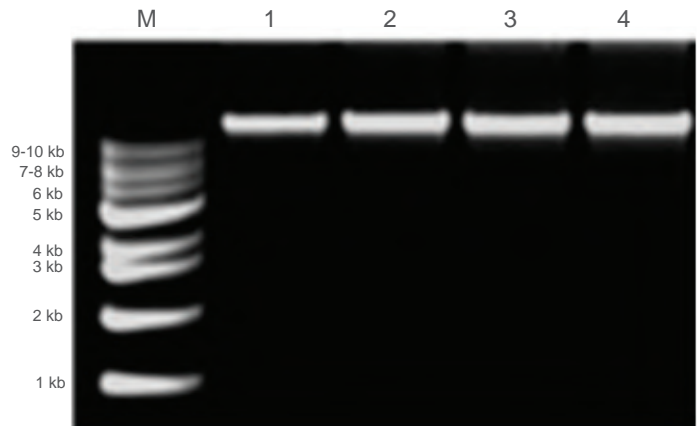
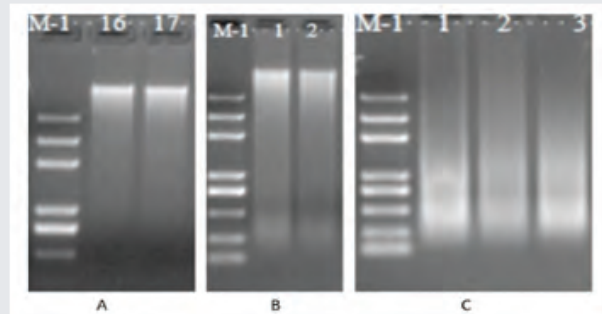


Fig. 1:DNA isolated from Plant Tissue

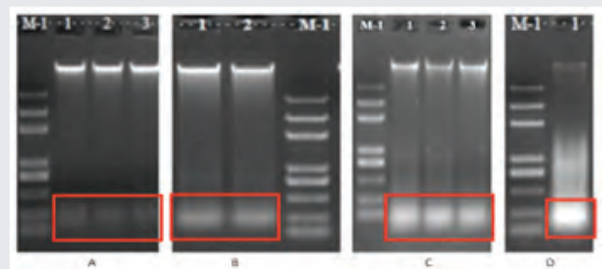
Lane 1-4: DNA Samples, Lane M: DNA Marker

5.1. Other possible observations with causes:

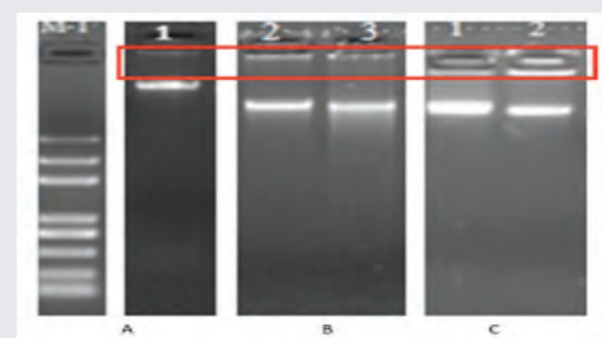
Band smearing:Trailing and smearing of DNA bands is most frequently observed when the DNA is denatured.



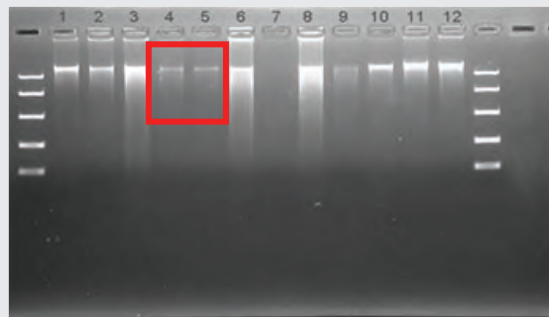
Bands beyond the loading Dye:When bands beyond the loading dye are observed it states the presence of RNA contamination in the sample.



Aqueous phase extraction: While separating and collecting the aqueous phase from the organic phase, it must be clear (not coloured), if it is not clear it indicates traces of protein. Hence, perform the PCI step twice or thrice.



Intactness and lightness: States that either the DNA extracted is in less concentration.



Presence Phenol and 70% ethanol: If trace of phenol during the PCI step and 70 % ethanol due to inefficient drying, are found in the DNA samples, it can denature the pure DNA.



6. Result

The bands at the top of the gel suggest that they are of high molecular weight (higher than all the DNA fragments of Marker DNA) hence they are genomic DNA. The intensity and the brightness state that it is in high concentration and the presence of straight band postulates the intactness of the genomic DNA.

7. Conclusion

The genomic DNA is intact as well as of high concentration, hence can be used for future applications.

8. Precautions

- i. Use caution when operating the centrifuge.
- ii. The laboratory working area should be very clean throughout all the procedural step of the experiment.
- iii. DNase free plastic wares and reagents should be used. Use only clean, dry and autoclaved tubes and tips.
- iv. Since ethanol is highly flammable take precaution while handling it.
- v. The phenol chloroform extraction should be repeated depending on the source of DNA to obtain pure DNA.
- vi. Take precautions in while working with ethidium bromide (it is carcinogenic) Wear gloves while handling ethidium bromide stained agarose gels.
- vii. Examine the reagents for precipitation. If any reagent forms a precipitate (other than enzymes), take warm at 55-65 °C until the precipitate get dissolves and then allow to cool down to room temperature (15-25 °C) before use.

Suggested Reading(s)

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ISOLATION OF GENOMIC DNA FROM WHOLE BLOOD



1. Aim

To isolate and purify genomic DNA from whole blood.

2. Introduction

Blood is a specialized body fluid composed of cells suspended in a liquid called blood plasma. Whole blood contains three types of cells:

- i. Red blood cells (RBCs)
- ii. White blood cells (WBCs)
- iii. Platelets

Red blood cells (RBCs) do not have any DNA, as they lose their nuclei during maturation. The white blood cell (WBC) component of the blood contains the DNA. The blood sample is treated with detergents, which break open the cell membrane to release the contents. Enzymes are then used to break down all the proteins, RNA, sugars and fats in the solution.

The DNA purification procedure comprises of the following steps:

- i. Lysis of RBC and WBC
- ii. Protein Precipitation
- iii. Precipitation of genomic DNA
- iv. Removal of residual contaminants
- v. Elution of pure genomic DNA

Genomic DNA purification from whole blood involves lysis of the red blood cells with RBC lysis buffer followed by the lysis of white blood cells and their nuclei with WBC lysis buffer. Impurities like cellular proteins are removed by precipitation and short washing steps while high molecular weight genomic DNA remains in the solution. High quality genomic DNA is then purified by isopropanol precipitation.

3. Materials Required

3.1. Biological Material: Blood Sample

3.2. Chemicals/Reagents: Agarose, autoclaved distilled water (A.D.W.), ethylene diaminetetra acetic acid (EDTA), glacial acetic acid, (hydroxymethyl) amino methane, trizma base (Tris), ethidium bromide (10 mg/mL), ethanol, isopropanol, RBC lysisbuffer, WBC lysis buffer, precipitation buffer, elution buffer, RNase A solution, 50X tris acetic acid – EDTA buffer (TAE), 6X gel loading buffer.

RBC Lysis Buffer:

Stock Solutions	Amount to add (for 100 mL)	Final concentration
3.2 M Sucrose	10 mL	0.32 M
50 mM HgCl ₂	10 mL	5 mM
1 M Tris-Cl (pH 8.0)	1 mL	10 mM
10 % Triton X 100	10 mL	1 %
Finally make up volume to 100 mL using autoclaved distilled water		

WBC Lysis Buffer:

Stock Solutions	Amount to add (for 100 mL)	Final concentration
1 M Tris-Cl (pH 8.0)	10 mL	100 mM
0.5 M EDTA (pH 8.0)	4 mL	20 mM
10 % SDS	30 mL	3%
10 % Triton X 100	50 mL	5%
Finally make up volume to 100 mL using autoclaved distilled water		

3.3. Equipment: Submarine/horizontal gel electrophoresis apparatus with power supply, centrifuge, magnetic stirrer, pH meter, bacterial/orbital shaker (incubator), micropipettes, vortex mixer (optional), hot plate/micro wave oven, autoclave, UV-trans illuminator.

3.4. Glassware/Plastic ware: Conical flask, measuring cylinder, beaker ,microcentrifuge tubes (1.5mL), culture flasks/tubes, 15 mL and 50 mL centrifuge tubes, micro tips, disposable gloves, gel scoop.

4. Procedure**4.1. Take the blood in 2 mL collection tube.****4.2. RBC Lysis:**

- i. Take 300 μ L of whole blood, already kept in a 2.0 mL collection tube.
- ii. Add 900 μ L of RBC lysis buffer and mixed well by inverting the tube 6-8 times. Incubate at room temperature for 5 minutes. Mix the tube contents intermittently by inverting 2-3 times during incubation.
- iii. Centrifuge at 15,000 rpm for 1 min at room temperature. Discard the supernatant carefully without disturbing the white pellet such that very small amount (15 μ L) of residue liquid remained back in the tube.

NOTE: If some red blood cells or cell debris are observed along with the white blood cell pellet, then re-suspend the white blood cell pellet in 600 μ L of RBC lysis buffer. Incubate at room temperature for 2 minutes. Pellet down the white blood cells by repeating centrifugation.

- iv. Vortex the tube vigorously so as to re-suspend the white blood cells completely.

4.3. WBC Lysis

- i. Add 300 μL WBC lysis buffer to the re-suspended white blood cells and gently pipette to lyse the cells. Solution will become viscous. If any cell clumps are still present, incubate the solution at 37 °C until the clumps get dissolve.
- ii. Add 1.5 μL of RNase A solution. Invert the tube 20-25 times to ensure thorough mixing of enzyme and incubate for 10 minutes at 37 °C.
- iii. Cool the sample to room temperature before further processing.

4.4. Precipitation of Proteins and Cell Debris:

- i. Add 100 μL of precipitation buffer to the cell lysate. Mix by vortexing for 30 seconds; incubate on ice for 5 minutes, as some protein clumps may be visible after vortexing.
- ii. Centrifuge at 15,000 rpm for 3 minutes at room temperature.

[NOTE: Protein will precipitate and form a compact, dark, brown pellet. If the pellet is not compact then, incubate on ice for 5 minutes and repeat the centrifugation step.]

4.5. Precipitation of DNA:

- i. Transfer the above supernatant to a new 2.0 mL collection tube. Add 300 μL of 100% isopropanol and mix by inverting the tube gently till the DNA in white fibrous form is visible (30-40 times).
- ii. Centrifuge at 15,000 rpm for 1 minute at room temperature. Small white pellet of DNA will be visible. Discard the supernatant.
- iii. Remove the residual supernatant by carefully inverting the tube on a clean tissue paper without disturbing the pellet. Add 300 μL of 70% ethanol to the DNA pellet and wash by inverting the tube 6-8 times.
- iv. Centrifuge at 15,000 rpm for 2 minutes at room temperature. Discard the supernatant carefully. The pellet may be very loose at this point, so discard the supernatant carefully without disturbing the pellet. Repeat the wash step for one more time.
- v. Invert the tube on a clean tissue paper and air dry the pellet for 10-15 minutes.
- vi. Resuspend the pellet in 30 μL of 10 mM Tris-Cl buffer (pH 8.0).

6. Observation and Results

Agarose gel electrophoresis was performed after the completion of the DNA isolation procedure. DNA bands were observed and captured UV Trans-illuminator.

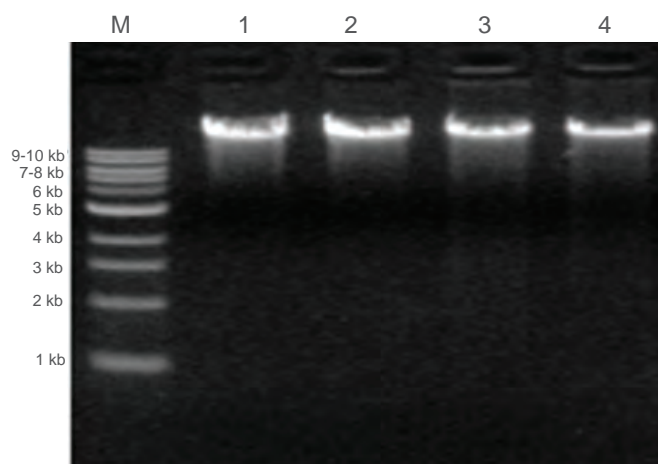


Fig. 1: Expected profile of isolated DNA run on agarose gel after electrophoresis

Lane M: 1 Kb DNA ladder; Lane 1-4: gDNA samples isolated from Blood

6. Precautions

- i. Preheat heating block or water bath to 65 °C and 37 °C.
- ii. Thoroughly mix the reagent. Examine the reagents for precipitation. If any reagent forms a precipitate (other than enzymes), Luke warm the solution at 55-65°C until the precipitate get completely dissolves and allow cooling down to room temperature (15-25 °C) before use.
- iii. Use only clean, dry and steriletubes and tips for the procedure.
- iv. Ensure that the blood is collected under sterile conditions in an anticoagulant coated tube (e.g. EDTA).

Suggested Reading(s)

- i. Sambrook, J., & Green, R. M. (2012). *Molecular cloning a laboratory Manual*. (4th ed.). New York: Cold Spring Harbor Laboratory Press.
- ii. Brown, T. A. (2010). *Gene cloning and DNA analysis: An introduction*. (6th ed.). Wiley Blackwell, John Wiley & Sons, Ltd., Publication.

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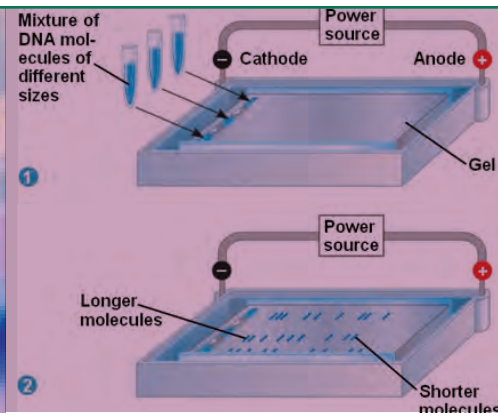
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AGAROSE GEL ELECTROPHORESIS



1. Aim

To perform agarose gel electrophoresis of isolated DNA.

2. Introduction

Electrophoresis is the process of separating ionically charged molecules based on their charge and/or size. This method utilizes Agarose, a purified linear galactan hydrocolloid isolated from agar or agar bearing marine algae, for gel preparation. When agarose is heated with water or standard buffer to boiling point and then allowed to cool, it forms a gel by hydrogen bonding. During the process of gelling, pores are formed and the pore size depends upon the concentration of agarose. Low concentration of agarose results in large pores allowing high molecular weight DNA to move through. Conversely, high concentration results in smaller pores which are suitable for low molecular weight DNA. The principle behind this experiment is that the rate of electrophoretic movement of DNA depends upon its size. In general, bigger DNA molecules move slowly compared to small size DNA molecules. Circular plasmid move slowly than linear plasmid. When electric charge is applied, DNA being negatively charged, moves towards the anode, while passing through the pores of the gel (Fig. 1). The larger DNA molecules move slowly through the gel, while smaller ones moves faster and hence gets separated faster into bands of different size at different positions on the gel, which can be further evaluated.

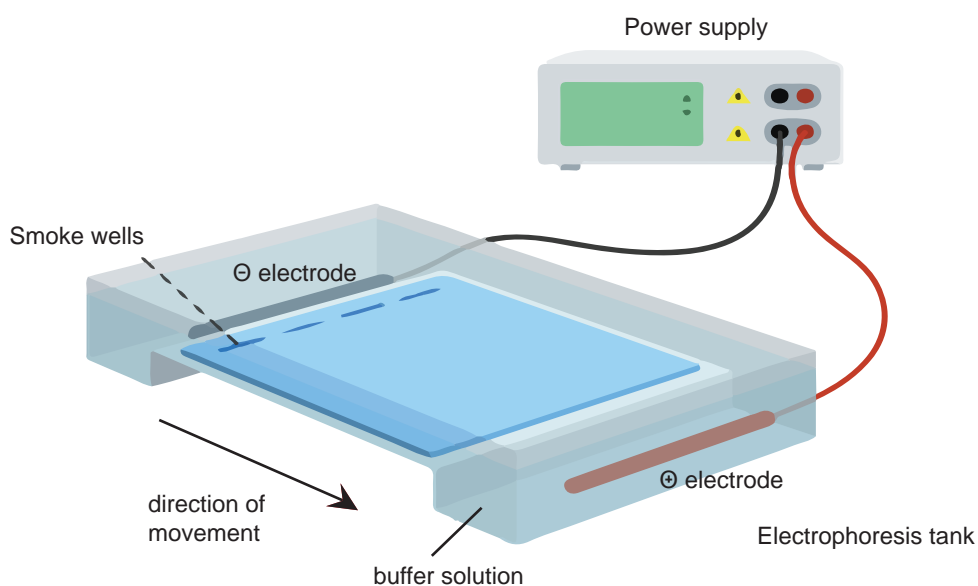


Fig. 1: Illustration of DNA electrophoresis equipment used to separate DNA fragments.

(<https://www.yourgenome.org/facts/what-is-gel-electrophoresis>)

Usually, TAE/TBE buffer in Mili-Q water is used to prepare the gel as DNase and RNase in normal water can denature the nucleotide molecules. A loading dye is mixed with the DNA samples. It generally contains a dye to assess how "fast" the gel is running and a reagent to render the samples denser than the running buffer (so that the samples sink in the well). When a DNA–ethidium bromide complex is illuminated with UV- β rays (280-320 nm), part of it is absorbed and rest is irradiated back in the range of 590-620 nm, as a result of which DNA-ethidium bromide complex appears as orange colour band.

3. Materials Required

3.1 Biological Material: DNA sample.

3.2 Chemicals/Reagents: TAE buffer -(Tris, EDTA, glacial acetic acid, NaOH:-stock solution – 50X, working solution – 1X), agarose, bromophenolblue dye/ 6X gel loading dye, ethidium bromide, 100 kb/1 kb DNA size marker and distilled water.

3.3 Equipment: Submarine gel electrophoretic system with power supply unit, UV transilluminator with face shield.

3.4 Glassware/Plasticware: Micropipettes (Accupipette T-20 or Gilson-P-20), glass tray, tissue paper, parafilm, cello tape etc.

4. Preparation of Reagents

- i. **50X TAE (stock solution):** Prepare 50 X TAE solutions by dissolving 242 gm Tris, 100 mL 0.5 M EDTA (pH 8.0) and 57.1 mL Glacial Acetic acid, and adjust the volume to 1000 mL by adding double distilled water. As EDTA does not dissolve easily, it is to be put in a magnetic stirrer for about 5 hours (NaOH can be added to balance pH to accelerate dissolving). The buffer thus prepared is to be autoclaved.
- ii. **1X TAE (working solution):** A working solution is prepared by 1:49 dilution (20 mL stock solution of 50X TAE + 980 mL double distilled water).
- iii. **Agarose Gel:** 0.8% Agarose gel is prepared by dissolving 800 mg Agarose in 100 mL 1X TAE buffer. Boil the solution to dissolve Agarose. When the solution cools down a bit, 2 μ L ethidium bromide is added to it and casted in an electrophoretic casting plate and an electrophoretic comb is placed at an end of the gel in a way that the legs of the comb remain inside the liquefied gel. It is then allowed to solidify.
- iv. **DNA sample for loading:** 12 μ L DNA sample is mixed with 2 μ L (approx.) 6X Gel Loading dye and loaded in the gel.

5. Procedure

Placing the gel and loading the sample:

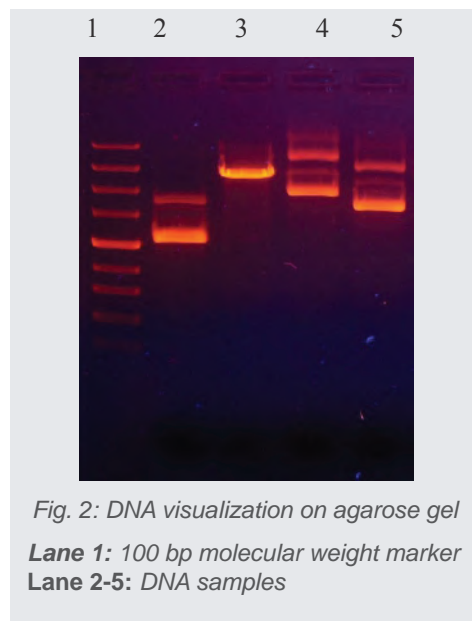
- i. Remove the comb slowly, after the gel gets solidified, leaving behind fine wells in the gel. Place the solidified agarose gel, along with the casting tray, place inside the electrophoresis chamber keeping the wells towards the cathode.
- ii. Fill the electrophoretic tank with 1X TAE buffer to such an extent that the gel remains submerged.
- iii. Load the DNA samples along with molecular size markers into the wells with the help of T-20 micropipette at the cathode end.
- iv. Run the gel till the time the tracking dye covers more than $\frac{3}{4}$ distance in the gel.

5. Observation and Result

A DNA-ethidium bromide complex is seen as a dark orange coloured band separated on agarose gel according to confirmation and size of DNA fragments (Fig. 2).

6. Precautions

- i. Generally 0.8% agarose gel is used.
- ii. For low molecular weight DNA (i.e. ≤ 1.5 kb, like amplified product of PCR) gel strength should be 1.3-1.5%.
- iii. Care should be taken not to switch the leads on the power source, as it will result in the sample running backwards.
- iv. Gel should be carefully transferred from the chamber/tray on to the transilluminator to avoid dropping the gel or breaking in between.
- v. Ethidium bromide is a strong carcinogen. Chemically treat it as per standard protocol for bio hazardous chemicals before disposal.
- vi. Wear hand gloves during the entire course of operation.
- vii. Care should be taken not to run the gel for too long as it can exhaust the buffering capacity of the solution.



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- i. Johansson, B. G. (1972). Agarose Gel Electrophoresis. *Scandinavian Journal of Clinical and Laboratory Investigation*, vol: 29, 7-19.
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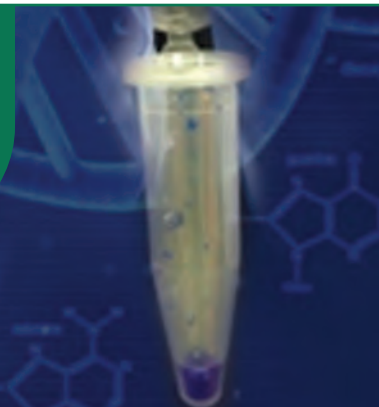
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SPECTROPHOTOMETRIC ESTIMATION OF ISOLATED DNA



1. Aim

To estimate the quantity and purity of the DNA sample by using UV-VIS Spectroscopy.

2. Introduction

After isolation of DNA, quantification and analysis of quality are necessary to ascertain the suitability of DNA sample for further analysis. This is important for many applications including digestion of DNA by restriction enzymes or PCR amplification of target DNA. The most commonly used methodologies for quantifying the amount of nucleic acid in a preparation are: (i) gel electrophoresis and (ii) spectrophotometric analysis.

Analysis of UV absorption by the nucleotides provides a simple and accurate estimation of the nucleic acids concentration in a sample. Purines and pyrimidines in nucleic acid show absorption maxima around 260 nm (e.g. dATP: 259 nm; dCTP: 272 nm; dTTP: 247 nm) if the DNA sample is pure without significant contamination from proteins or organic solvents. The ratio of OD_{260}/OD_{280} should be determined to assess the purity of the sample. This method is however limited by the quantity of DNA and the purity of the preparation. Accurate analysis of the DNA preparation may be impeded by the presence of impurities in the sample or if the amount of DNA is too little. In the estimation of total genomic DNA, for example, the presence of RNA, sheared DNA etc. could interfere with the accurate estimation of total high molecular weight genomic DNA. While calculating the ratio for purity of DNA:

- A ratio between 1.8-2.0 denotes that the absorption in the UV range is due to nucleic acids.
- A ratio lower than 1.8 indicates the presence of proteins and/or other UV absorbers.
- A ratio higher than 1.8 indicates the presence of RNA

2.1. Principle:

Absorption spectroscopy has long been the method of choice to measure the amount of DNA or RNA in a solution. Nucleic acids absorb UV light due to presence of conjugated double bonds of the constituent purines and pyrimidines bases. These have absorbance maxima at wavelengths of 260 nm. The ratio between the reading at 260 nm and 280 nm ($OD_{260} : OD_{280}$) provides an estimate of the purity of the nucleic acid. Pure preparation of DNA and RNA have ($OD_{260} : OD_{280}$) value 1.8 and 2.0 respectively.

If there is significant contamination with protein or phenol, $OD_{260} : OD_{280}$ value will be less than the value given above, and accurate quantification of nucleic acid will not be possible.

Significance absorption at 230 nm indicates contamination by phenolate ion, thiocyanate and other organic compounds, whereas absorption at higher wavelength (320 nm and higher) is usually caused by light scattering and indicate the presence of particulate matter. Absorption at 280 nm indicates the presence of protein, because aromatic amino acids absorb strongly at 280 nm.

Thus, ratio of absorption at 260 nm and 280 nm has been used as a measure of purity of isolated nucleic acids. The reverse is not true because extinction coefficient of nucleic acids at 260 nm and 280 nm are so much greater than that of proteins. So significant contamination with protein will not greatly change the OD_{260} : OD_{280} of nucleic acids solution.

Based on the extinction coefficient, an O.D. of 1.0 at 260 nm corresponds to an approximately 50 μ g/mL of double stranded DNA and for single stranded DNA and RNA its 40 μ g/mL.

Spectrophotometric Conversions for Nucleic Acids:

$$1 A_{260} \text{ of ds DNA} = 50 \mu\text{g/mL}$$

$$1 A_{260} \text{ of ss oligonucleotides} = 33 \mu\text{g/mL}$$

$$1 A_{260} \text{ of ss RNA} = 40 \mu\text{g/mL}$$

3. Materials Required

3.1. Sample: DNA sample to be estimated

3.2. Chemicals/Reagents: TE buffer

- a. Measure 10 mL of 1M Tris-Cl buffer and 2 mL of 0.5 M EDTA solution.
- b. Mix the solutions with distilled H₂O and make up the volume to 1000 mL using a graduated measuring cylinder.

3.3. Equipment: Spectrophotometer with all accessories.

3.4. Glassware/Plastic ware: Test tube.

4. Procedure

- i. To measure the concentration of DNA, make its appropriate dilution with TE buffer (either 1:50 or 1:100).
- ii. Standardize the spectrophotometer using TE buffer as blank.
- iii. Measure the absorbance of the sample at 260 and 280 nm
- iv. Calculate the DNA concentration of the sample using given formula:

$$\text{Concentration of DNA } (\mu\text{g/mL}) = OD_{260} \times 50 \times \text{dilution factor}$$

or

$$\text{Concentration of DNA } (\mu\text{g}/\mu\text{L}) = OD_{260} \times 50 \times \text{dilution factor}/1000$$

5. Observation

Readings of the DNA sample at 260 nm and 280 nm using UV-VIS Spectrophotometer are as follow;

$$A_{260} - 0.550 \text{ OD};$$

$$A_{280} - 0.324 \text{ OD}$$

6. Result

Assuming that the DNA pellet has been resuspended in 50 μ L buffer.

$$A_{260} - 0.550 \text{ OD}; A_{280} - 0.324 \text{ OD}$$

A. The quantity of the DNA present in the sample:

$$\text{Concentration of DNA } (\mu\text{g/mL}) = \frac{\text{OD}_{260} \times 50 \times \text{dilution factor [if required]}}{1 \text{ O.D}}$$

$$\text{Concentration of DNA } (\mu\text{g/mL}) = \frac{0.555 \times 50}{1.0}$$

$$\text{Concentration of DNA } (\mu\text{g/mL}) = 27.5 \mu\text{g/mL}$$

B. The Purity of DNA in given sample:

The A_{260}/A_{280} ratio is

$$\frac{0.550}{0.323} = 1.703$$

7. Conclusion

The quantity of DNA in the sample has been found to be 27.5 $\mu\text{g DNA/mL}$ and the A_{260}/A_{280} ratio is 1.7, hence it has some amount of protein contamination. The PCI treatment in DNA extraction should be repeated to remove the protein contamination.

8. Precautions

- i. Calibrate the UV –VIS Spectrophotometer prior to use for accurate measurement.
- ii. Switch on the spectrophotometer 15 min prior to take readings.

9. Trouble Shooting

Common problems encountered in Spectrophotometric estimation of DNA are described below, along with several possible causes:

- a) Protein Contamination: If phenol traces or protein contamination is present in the DNA sample, the A_{260}/A_{280} ratio will be less than 1.8.
- b) RNA Contamination: If RNA is present in the DNA sample, the A_{260}/A_{280} ratio may be greater than 1.8.
- c) Pure RNA: Pure RNA preparations will have an A_{260}/A_{280} ratio close to 2.0.
- d) Amount of Salt in the DNA Sample: Strong absorbance around 230 nm, in other words A_{260}/A_{280} ratio is less than 1.5; indicate organic compounds or chaotropic salts are present in the purified DNA.
- e) Non-nucleic acid contamination: A reading at 320 nm will indicate presence of other possible contaminants.

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PLASMID DNA ISOLATION BY ALKALINE LYSIS METHOD



1. Aim

To isolate of plasmid from bacterial cell by alkaline lysis method.

2. Introduction

Alkaline lysis is one of the most commonly used methods for lysing bacterial cells prior to plasmid isolation. Plasmid can be efficiently isolated using following stages.

2.1. Resuspension:

Harvested bacterial cells are resuspended in solution-I (Tris Cl-EDTA buffer). Glucose in this solution provides an isoosmotic condition to prevent physical shock. There suspended solution's pH is raised to basic level with Tris to help denature DNA. EDTA stabilizes the cell membrane by binding the divalent cations. RNase will destroy RNA from the cell contents when the membrane is lysed.

- Ensure that bacteria are resuspended completely leaving no cell clumps in order to maximize the number of cells exposed to lysis reagents.
- Buffer volumes for alkaline lysis in plasmid isolation procedure are optimized for particular culture volumes in LB medium. Do not use a culture volume larger than recommended in the protocol as this will lead to inefficient lysis and reduce the quality of plasmid preparation.
- For large scale isolation of low-copy plasmids, for which larger culture volumes are used, it may be beneficial to increase the lysis buffer volume in order to increase the efficiency of alkaline lysis and there by the DNA yield.

2.2. Lysis:

Cells are lysed with NaOH/SDS. Sodium dodecyl sulfate (SDS) solubilizes the phospholipid and protein components of the cell membrane, leading to lysis and release of cell contents. NaOH denatures the chromosomal and plasmid DNA, as well as proteins.

- Avoid vigorous stirring or vortexing of the lysate as this can shear the bacterial chromosome, leaving free chromosomal fragments in the supernatant which will co-purified with the plasmid DNA. The solution will be mixed gently but thoroughly by inverting the lysis vessel 4-6 times.
- Do not allow lysis to proceed for longer than 5 minutes. 5 minutes incubation allows maximum release of plasmid DNA from the cell, while minimizing the release of chromosomal DNA and reducing the exposure of the plasmid to denaturing conditions.

2.3. Neutralization:

The lysate is neutralized by the addition of acidic potassium acetate. The high salt concentration causes potassium dodecyl sulfate (KDS) to participate, and denatured proteins, chromosomal DNA, and cellular debris are coprecipitated in insoluble salt-detergent complexes. Plasmid DNA, being circular and covalently closed, renatures correctly and remains in solution.

- Precipitation is enhanced by using chilled neutralization buffer and incubating on ice.
- Mix the solution gently but thoroughly to ensure complete precipitation.

2.4. Clearing of lysates:

Precipitated debris is removed by either high speed centrifugation or filtration, producing cleared lysates.

2.5. Precipitation of the plasmid DNA:

Alcohol (ethanol or isopropanol) and a salt (such as ammonium acetate, lithium chloride, sodium chloride or sodium acetate) can be used to precipitate the plasmid DNA present in solution after centrifugation. DNA is negatively charged, so adding a salt masks the charges and allows DNA to precipitate. This will place your DNA in the pellet.

2.6. Resuspension of Plasmid DNA:

Precipitated plasmid DNA can be converted into the soluble form by rehydrating buffer (TE buffer).

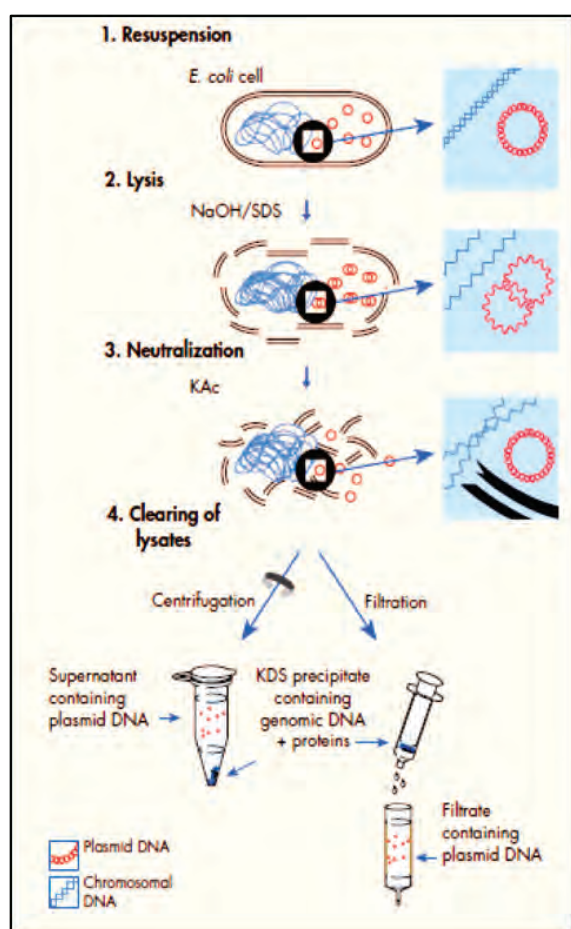


Fig. 1: Plasmid Isolation by Alkaline Lysis Method

3. Materials Required

3.1. Biological Sample: Plasmid bearing bacterial broth.

3.2. Chemicals/Reagents: Antibiotic for plasmid selection, 95-100% isopropanol, ethanol, TE (pH 8.0) buffer, agarose powder.

- Alkaline lysis solution I:** 50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0), de-ionized water.
- Alkaline lysis solution II:** 0.2 N NaOH, 1% (w/v) SDS, de-ionized water. (Freshly prepared)
- Alkaline lysis solution III:** 5 M potassium acetate (60 mL), glacial acetic acid (11.6 mL), de-ionized water. Store the solution at 4 °C till use.

3.3. Equipment: Microwave oven, agarose gel electrophoresis assembly, UV- transilluminator, gel- documentation system, micro centrifuge, water bath (37 °C), automatic micropipettes with tips.

3.4. Glassware/Plastic Ware: Micro centrifuge tubes.

4. Procedure

[NOTE: Streaking and inoculation should be done aseptically.]

DAY 1: Streak the given bacterial culture (containing plasmid) on LB Agar containing appropriate antibiotic. Incubate at 37 °C (Inverted) overnight.

DAY 2: Inoculate a single colony into 5 mL of LB medium containing appropriate antibiotic. Incubate at 37 °C with shaking for 8-16 hrs.

DAY 3: Follow the steps carefully (for harvesting and alkali lysis).

- i. Transfer 1.5 mL overnight grown culture into microcentrifuge tube and centrifuge for 5 minutes at 6000 rpm.
- ii. Drain the supernatant and gently tap the tube inverted on a paper towel to remove the excess medium.
- iii. Add 100 µL of alkaline lysis solution I to this suspension and mix the contents by finger flicking the tube. Gently vortex the pellet using vortex mixer so that the suspension disperses uniformly.
- iv. No visible clumps of bacteria should remain. Incubate at room temperature (RT) for 5min.
- v. Add 200 µL of solution II and gently mix the contents by inverting the tube 4-5 times. The cell suspension should look clear at this stage.
- vi. Add 150 µL of solution III and mix the contents by inverting the tube 4-5 times.
- vii. Centrifuge the tube containing lysate at 10,000 rpm for 15 min. (White precipitate is seen on the wall of the tube).
- viii. Gently transfer the supernatant into a fresh tube without disturbing the pellet. (The pellet is goeey and suddenly can slip into the tube).
- ix. Add 0.5 mL of isopropanol to this suspension and mix by inverting the tube. Allow to stand for ~10 min (The suspension should turn turbid slightly).
- x. Centrifuge at 10,000 rpm for 15 min.
- xi. Carefully drain off the supernatant and mark the pellet.
- xii. Add 200 µL of 70% ethanol to the opposite side of the wall from the pellet. Drain off the alcohol and keep the tube inverted on paper towel (DNA can be dried at 37 °C also).
- xiii. When the pellet turns transparent, add 50 µL of 1 X TE to the pellet and resuspend by finger flicking.
- xiv. Prepare an Agarose gel of 1% strength.
- xv. Load 10-15 µL of your DNA sample after addition of gel loading dye and run the gel at 100 V for 30- 40 minutes.
- xvi. Visualize with UV doc system and note the observation.

5. Expected Observations & Result

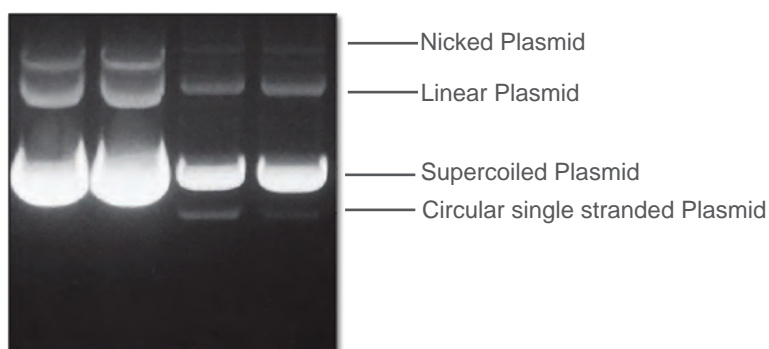


Fig. 2: Visualization of plasmid DNA on agarose gel

6. Conclusion & Interpretation

Alkaline lysis method of plasmid DNA isolation proves to be a useful method for isolating plasmid from bacterial sample. In agarose gel electrophoresis, various forms of plasmid DNA can be visualized i.e. nicked plasmid, linear plasmid, supercoiled plasmid and circular single stranded plasmid. Different forms of plasmids were found because of harsh chemical treatment during purification. Supercoiled form of plasmid represents the native form. Nicked plasmid was found to be slowest and circular single stranded plasmid was found to be fastest in electrophoresis due to their size and structure. RNA was also found which can be removed by giving RNase treatment to the sample.

7. Precaution

- i. Use gloves while handling corrosive chemicals.
- ii. Carefully maintain the temperature and incubation period.
- iii. DNA staining dye ethidium bromide (EtBr) must be handled with gloves because of its carcinogenic property.
- iv. Carefully dispose the agarose gel after analysis.

8. Trouble Shooting

- i. Maintaining temperature and incubation periods is very important.
- ii. Chemicals like Alkaline lysis solution- II (SDS- NaOH) must be prepared freshly

Suggested Reading(s)

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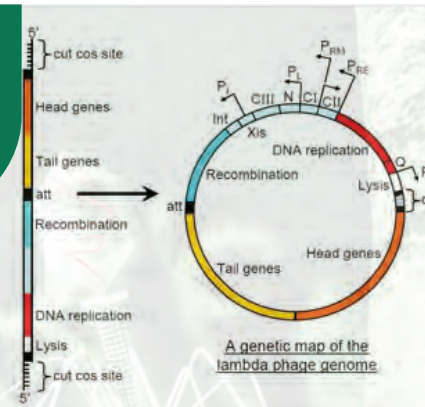
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RESTRICTION DIGESTION OF DNA



1. Aim

To perform restriction digestion of
a) Genomic DNA, b) Plasmid DNA.

2. Introduction

Restriction enzymes or restriction endonucleases are enzymes that cut double stranded DNA at specific palindrome sequences (sequences that are same when read from both directions) (Fig. 1a). The resulting fragments are called restriction fragments. There are three types of restriction enzymes viz: Type-I, Type-II and Type-III. Type-II enzymes are mainly used for recombinant DNA technology experiments. The nomenclature of these enzymes is based on the organisms from where they are isolated, their strain type and numbers of enzymes present in it. All enzymes are named by abbreviating together the first letter of the genus name and first two letters of species name followed by strain type if any and number of the enzyme. For example the name of the enzyme HindIII is derived from the bacterium *Haemophilus influenzae* strain D, 3rd enzyme. Similarly EcoRI derived from *Escherichia coli* RY13. Restriction enzymes after cutting can either produce blunt ends (Fig. 1c) or sticky ends (Fig. 1b). Blunt ends are produced if the cleavage points occur exactly on the axis of symmetry, i.e. the enzyme cuts both strands at the same site. Sticky ends are produced if the cleavage points do not fall on the axis of symmetry, i.e. the enzyme cuts both strands at different points

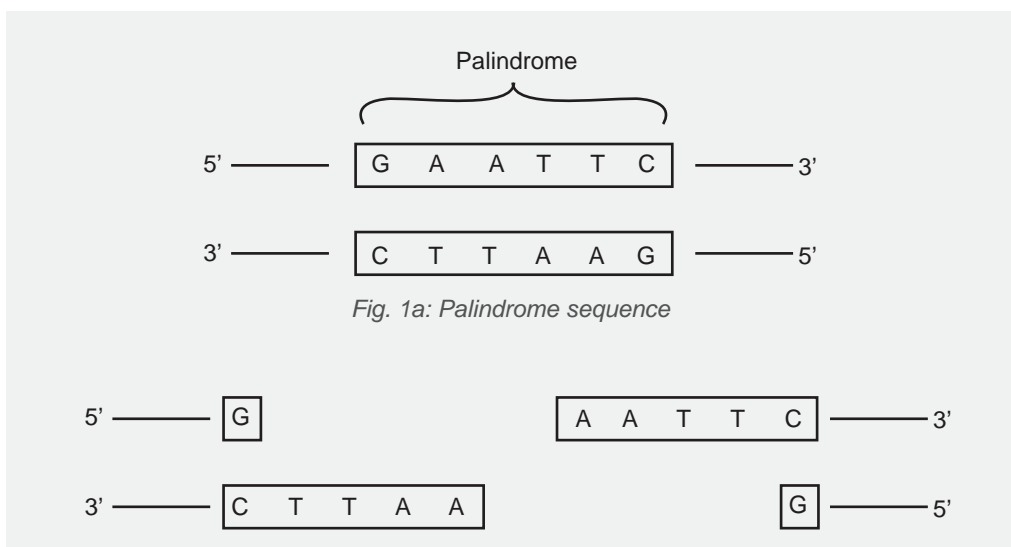


Fig. 1b: Sticky ends



Fig. 1c: Blunt ends

(http://mcat-review.org)

2.1. λ genomic DNA: It has molecular weight of 48.3 kb. When λ DNA is digested with enzyme Hind III, a hexanuclease having a restriction site $\overline{\text{A}\downarrow\text{AGCTT}}$ $\overline{\text{TTCGA}\uparrow\text{A}}$ it gives seven bands.

Molecular weight of each of these bands has been determined. Hence, Hind III digested λ DNA is used as a marker during electrophoresis to find out the molecular weight of genomic DNA.

2.2. Plasmid pUC18: It is a double stranded, circular, autonomous and extrachromosomal unit present in multiple copies within *E.coli* cell. It is 2686 bp in size and contains ampicillin resistance gene as selectable marker along with a HindIII fragment of lacZ gene (Fig. 2). The lac Z gene harbors many overlapping restriction sites and therefore allows ligation of foreign DNA fragment in any of these sites.

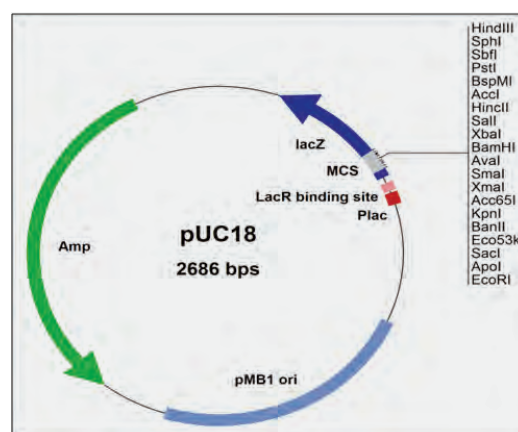


Fig. 2: Detail map of pUC18

(https://www.mobitec.com)

3. Materials Required

- 3.1. Biological Material:** Genomic DNA (λ DNA), plasmid DNA (pUC18).
- 3.2. Chemicals/Reagents:** Enzyme HindIII, DNAase free distilled water, BamHI, EcoRI, enzyme buffers.
- 3.3. Equipment:** Water Bath, centrifuge, electrophoretic apparatus.
- 3.4. Glassware/Plastic ware:** Micropipette, microtips, floaters, microcentrifuge tubes.

4. Procedure

- i. Thaw the enzyme buffer, distilled water, plasmid DNA and λ DNA, from $-20\text{ }^{\circ}\text{C}$ to $4\text{ }^{\circ}\text{C}$ by placing them in ice for about an hour.
- ii. Prepare the reaction mixture by adding components as given below-

Components	Volume	Components	Volume
λ DNA (0.3 $\mu\text{g}/\text{mL}$)	50 μL	Plasmid DNA (0.3 $\mu\text{g}/\text{mL}$)	20 μL
Buffer (10X)	15 μL	BamHI	5 μL
Hind III (10 units/ μL)	0.5 μL	EcoRI	5 μL
Distilled water	84.5 μL	4X Buffer	10 μL
TOTAL	150 μL	TOTAL	40 μL

- iii. Fix the tube in floater and place in a preset water bath at 37 °C for 3 hours.
- iv. After incubation, inactivate the enzyme by placing the digestion mixture in water bath at 65 °C for 2 min.
- v. Aliquot it into two tubes and store one aliquot at -20 °C. Use the other for electrophoresis to visualize the result of restriction digestion.

5. Observations

- a. Restriction digestion of genomic DNA:
 - i. Seven prominent bands were observed.
 - ii. The bands (Kilo Base in size) in the descending order are of 22.13 kb, 9.41 kb, 6.54 kb, 4.36 kb, 2.32 kb, 2.03 kb and 0.57 kb (Fig. 3).
- b. Digested products of pUC 18 with EcoRI and BamHI on electrophoresis is shown as two fragments on 1% agarose gel. One fragment was that of linearized plasmid and the other was fragment released by restriction enzymes. Uncut plasmid showed one band on the gel.

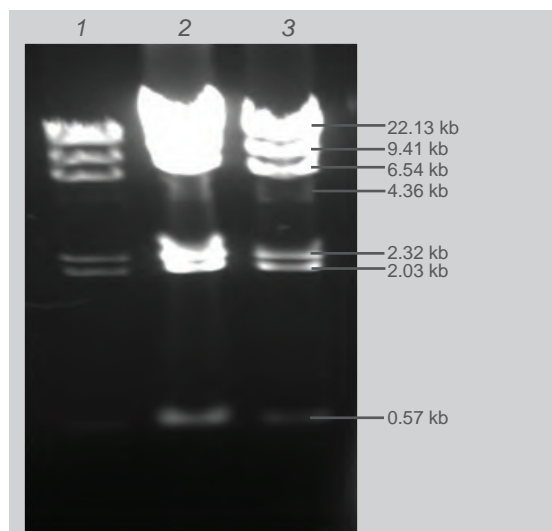


Fig. 3: λ DNA Hind III digest showing seven bands.
Lane 1: Known λ DNA Hind III digest;
Lanes 2 & 3: Experimental samples

6. Precautions

- i. All the plastic ware and glassware should be sterile.
- ii. Quantify DNA must be quantified before digesting it with enzymes.
- iii. Pipetting should be carried out carefully.
- iv. Temperature of the water baths should be checked before incubation.
- v. Mix the contents thoroughly before incubation.
- vi. Wear gloves while preparing gel and handling ethidium bromide
- vii. Optimum concentration of enzyme, high concentration may lead to star activity.

[NOTE: Some restriction enzymes may cleave sequences other than their defined recognition sequence under sub-optimal reaction conditions. In general, these conditions include high salt concentration, presence of impurities, or excessive enzyme compared to substrate DNA. This altered specificity is called star activity. The result is typically cleavage at non-canonical recognition sites, or sometimes complete loss of specificity.]

- viii. All component of reaction must be stored in ice until microfuge tubes are finally incubated at 37 °C.

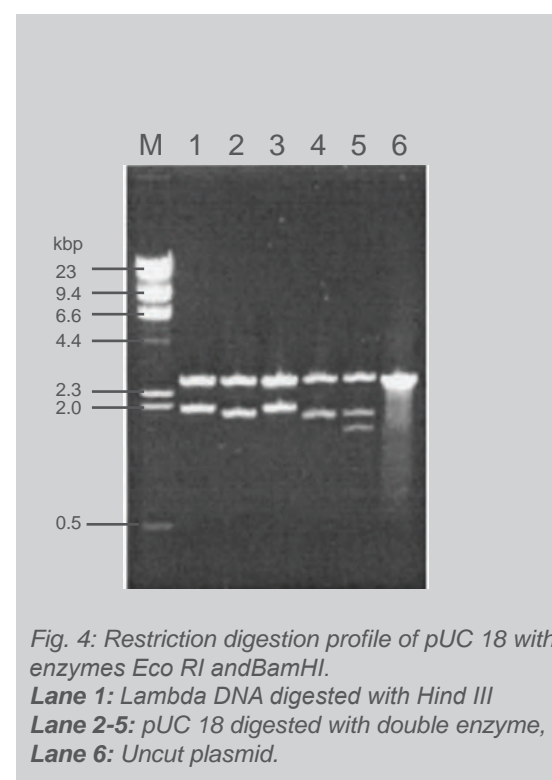


Fig. 4: Restriction digestion profile of pUC 18 with enzymes EcoRI and BamHI.
Lane 1: Lambda DNA digested with Hind III
Lane 2-5: pUC 18 digested with double enzyme,
Lane 6: Uncut plasmid.

Suggested Reading(s)

- i. Sambrook, J., & Green, R. M. (2012). *Molecular cloning a laboratory Manual*. (4th ed.). New York: Cold Spring Harbor Laboratory Press.
- ii. Brown, T. A. (2010). *Gene cloning and DNA analysis: An introduction*. (6th ed.). Wiley Blackwell, John Wiley & Sons, Ltd., Publication.

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LIGATION OF DNA INTO LINEARIZED PLASMID

1. Aim

To ligate the DNA insert to linearized plasmid.

2. Introduction

The term recombinant DNA encapsulates the concept of recombining fragments of DNA from different sources into a new and hopefully useful DNA molecule. Joining linear DNA fragments together with covalent bonds is called ligation. More specifically, DNA ligation involves creating a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another (Fig. 1). The enzyme used to ligate DNA fragments is T4 DNA ligase, which originates from the T4 bacteriophage. This enzyme will ligate DNA fragments having overhanging, cohesive ends that are annealed together, as in the BamHI example below - this is equivalent to repairing "nicks" in duplex DNA. T4 DNA ligase will also ligate fragments with blunt ends, although higher concentrations of the enzyme are usually recommended for this purpose.

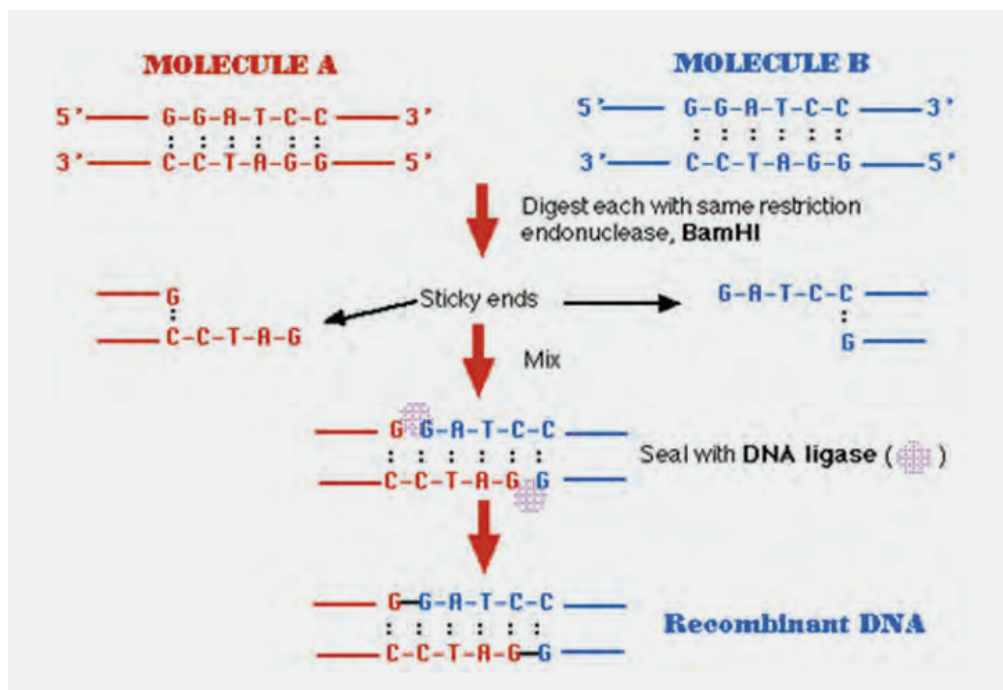


Fig. 1: DNA Ligation

(<http://www.biology-pages.info>)

3. Materials Required

- 3.1. Biological materials:** Fragments of DNA that have either blunt or compatible cohesive ("sticky") ends.
- 3.2. Chemicals/Reagents:** T4 DNA Ligase, 10X buffer for ligase, sterile water.
- 3.3. Equipment:** Water bath, micropipettes.
- 3.4. Glassware/Plastic ware:** Sterile tips, sterile microcentrifuge tubes, stands for tubes.

4. Procedure

For best results in ligation reactions, 3-4 fold molar excess of the insert fragment over the vector fragment is taken. For example: if vector DNA is 3000 bp and the insert fragment is 1000 bp then molar ratio of the insert/vector is 1:3. Thus equal amounts i.e. nanograms of Vector and Insert will be in a ratio of 1:3. One could add excess of the Insert fragment to the ligation reaction to increase the ratio to 1:4 or 1:6.

- i. Aliquot the following reagents using a clean micro tip for each reagent.

Components	Volume
Vector DNA	40 μ L
Insert DNA	20 μ L

- ii. Next add the ligation reagents.

Components	Volume
4X Ligation Buffer	10 μ L
T4 DNA Ligase	10 μ L

(The volume of the above reagents will vary as per the size of insert DNA and vector DNA being used)

- iii. Incubate the tubes at 16 °C overnight or at room temperature for 4 hours.
- iv. In order to screen for successful DNA ligation run the reactions on agarose gel, else store the ligation reactions at 4 °C until required.

5. Observation

In order to check results, products of ligation reaction are run on agarose gel along with unligated vector DNA.

6. Results

As can be seen in the gel pictures (Fig. 2), ligation of insert with vector resulted in increase of size of fragment in lane 2 as compared to linearised vector in lane 1. In lane 2, other bands correspond to unligated vector and insert fragment and non-specific ligation.



Fig. 2: Gel profile showing ligation results.

Lane M: Molecular weight marker(37-100 kb) ;
Lane 1: Linearised vector;
Lane 2: Ligated product

7. Precautions

- i. Insert and vector must be added in correct ratio.
- ii. All reactants must be properly mixed and spin down at bottom of microfuge tube before incubation.
- iii. Success of ligation must be checked on gel by taking unligated vector DNA as control.

Suggested Reading(s)

- i. Sambrook, J., & Green, R. M. (2012). *Molecular cloning a laboratory Manual*. (4th ed.). New York: Cold Spring Harbor Laboratory Press.
- ii. Brown, T. A. (2010). *Gene cloning and DNA analysis: An introduction*. (6th ed.). Wiley Blackwell, John Wiley & Sons, Ltd., Publication.

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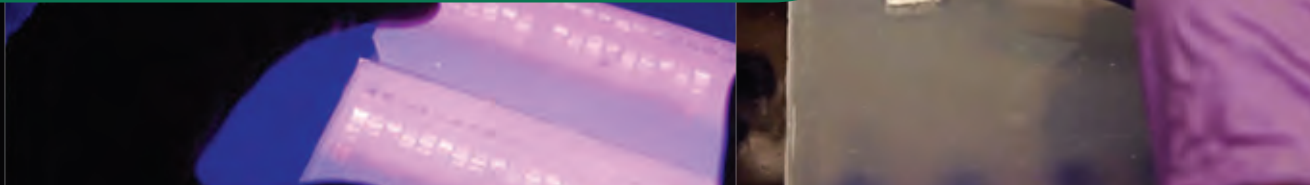
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Title picture: <https://wallup.net>, <http://www.cell.com>

ELUTION OF DNA BAND FROM AGAROSE GEL



1. Aim

To extract specific bands of DNA from agarose gel.

2. Introduction

DNA isolation is a critical step in molecular biology. It is necessary to obtain a specific DNA fragment from the extracted DNA in molecular biology techniques. After isolating plasmids it may contain some chromosomal DNA contamination it will interrupt the further processing of cloning. So it is better to recover the plasmid DNA by eluting it from agarose gel (extraction). The first step in extracting DNA is identifying the DNA band which is to extract, by illuminating under UV light. The desired band is then carefully cut by a scalpel blade. There are several methods for extracting DNA from the agarose gels. Recovery of DNA from agarose gels by electrophoresis onto DEAE-cellulose membrane is one of the rapid and effective methods. Electroelution is also a good method for DNA recovery especially for larger DNA fragments. Several kit based methods are also used in laboratories. In electro elution, the gel fragment of desired DNA band is placed into a dialysis bag with buffer. The bag is then placed into a gel box containing buffer and subjected to an electric current. The DNA extracted is precipitated from the solution. In another recovery method using DEAE cellulose membrane, the gel piece is slide into the slit of DEAE cellulose paper which will bind the DNA. Then an electric current is applied in order to move the band in the paper. DNA is washed off from the paper and is precipitated with ethanol. Freeze-thaw method of extraction is a commonly used advantageous DNA recovering method which will supports the common laboratory facilities. It is very simple and easy to perform method where the yield of DNA is also good.

Most of the molecular biology laboratories use low melting point agarose for the separation of DNA from agarose. Low melting point agarose melts at a lower temperature than standard agarose and this temperature does not denature double stranded DNA. It is better to extract DNA fragments in a TAE buffered gel than TBE buffered gel because borate present in the buffer interferes with purification methods.

3. Materials Required

3.1. Chemicals/Reagents: Elution buffer [10 mM Tris-Cl Buffer (pH 8.0) and 1 mM EDTA(pH 8.0)], agarose, TE buffer, n-butanol, 70% ethanol, 95% ethanol.

3.2. Equipment: UV transilluminator, dry bath incubator, centrifuge, cyclomixer, -20 °C freezer, -70 °C freezer.

3.3. Miscellaneous: Scalpel blade, micro pipettes, micro pipette tips, microcentrifuge tubes, cryo-box.

4. Procedure

- i. Visualize the low melting point agarose gel with DNA bands under a UV transilluminator and locate the desired DNA band to cut.
- ii. Carefully cut around the desired DNA band using a scalpel blade.
- iii. Transfer the gel piece into a microfuge tube.
- iv. Add elution buffer into the microfuge tube until the level of buffer is just above the level of gel slice.
- v. Heat the gel slice at 65 °C until it melts.
- vi. Freeze the melted gel with DNA by placing in a -70 °C freezer for 10 min.
- vii. After freezing, centrifuge for 10 min and transfer the supernatant into a new microcentrifuge tube.
- viii. Again add half amount of elution buffer that you added in the previous step into the pellet.
- ix. Heat at 65 °C until the agarose melts.
- x. Freeze the melted gel with DNA by placing in a -70 °C freezer for 10 min.
- xi. Centrifuge the tube again for 10 min and transfer (pool) the supernatant into the previous tube with supernatant.
- xii. Discard the tube with pellet.
- xiii. Add an equal volume of n-Butanol to the supernatant and mix the contents well.
- xiv. Vortex the tube for 15 min in order to remove the ethidium bromide.
- xv. Discard the upper phase of butanol and repeat the process by adding n-butanol again for one or more times.
- xvi. Add double volume of 95% ethanol and mix thoroughly.
- xvii. Keep for precipitation in -70 °C freezer for 30 min to overnight.
- xviii. After precipitation, centrifuge for 15 min.
- xix. Discard the supernatant into a waste beaker and add 200 µL of 70% ethanol to the pellet.
- xx. Centrifuge for 5min and discard the supernatant again.
- xxi. Allow the pellets to dry well.
- xxii. Suspend the pellets in 20 µL of TE buffer. (If you want to confirm the recovered DNA, run (1 µL) it on a gel.
- xxiii. The recovered DNA can be now used for further process of cloning otherwise can be stored at -20 °C freezer.

5. Result

The extracted DNA can be detected or estimated by taking optical density measurements at 280 nm in a nanospectrophotometer.

6. Precautions

- i. One must wear gloves in order to avoid contact with EtBr, which is a mutagen.
- ii. Wearing eye protection glasses are must while visualizing agarose gel and cutting the DNA band under UV transilluminator.

Suggested Reading(s)

- i. Xia, H., Tan, Z., Qiao, J., & Liang, C. (2011). Recovery of DNA from agarose gel by trap method. *African Journal of Biotechnology*, 10(50): 10280-10286.

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Source

Title picture: <http://genelon.in>, <https://www.neb.com>

BACTERIAL TRANSFORMATION

1. Aim

To prepare the competent cells and carry out transformation.

2. Introduction

Transformations are procedures for inducing microbes to take up exogenous DNA from their environment. Transformation is the most frequently used procedure for introducing recombinant DNA molecules into competent bacteria. These competent cells allow DNA to enter through pores or channels in the cell membrane and in the case of plasmids, permit subsequent plasmid replication.

Some species of bacteria naturally take up DNA at a certain stage of growth called competence. Some species however are not naturally competent at any stage of growth. Competence can be artificially induced by treating them with CaCl_2 prior to addition of DNA. The Ca^{+2} ions destabilize the cell membrane and a Ca-Phosphate-DNA complex is formed which adheres to the cell surface and is resistant to DNases. The DNA is taken up during a heat shock step when the cells are exposed briefly to a temperature of 42 °C. Immediate chilling on ice ensures closure of pores. Selection for cells containing transformed DNA is greatly enhanced by the selection marker carried by the DNA. The plasmid pUC series and pBR 322 have ampicillin resistance factor which enables only the transformed cells to grow on LB Ampicillin plates.

The pUC18 plasmids also have the gene for β -galactosidase (*lac Z* from *Escherichia coli*). If these plasmids are transformed into a *lac Z* strain of *Escherichia coli*, they will make them Lac⁺. Although neither the host nor the plasmid encoded fragments are themselves active, they can associate to form an enzymatically active protein. This type of complementation is known as alpha complementation.

Lac⁺ bacteria that result from alpha complementation can be recognized, as they form blue colonies in presence of X-gal (as β -galactosidase cleaves this chromogenic substrate) and IPTG (that acts as an inducer for the expression of the enzyme).

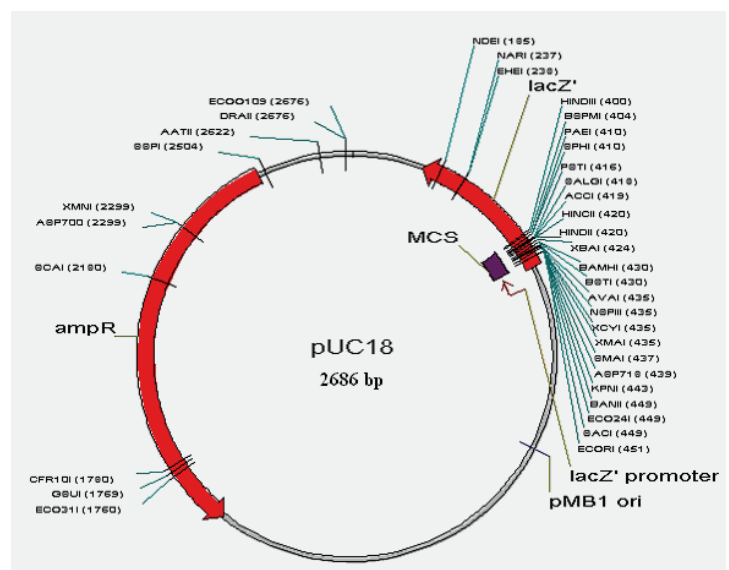


Fig. 1: pUC18 plasmid
(<https://www.atcc.org>)

Any plasmid which has a DNA fragment cloned into it (*lac Z* genes site) will not have a functional *lac Z* gene and thus will produce white colonies which are unable to cleave X-gal.

3. Materials Required

3.1. Biological Material: pUC18 plasmid DNA (50 ng/ μ L or 0.05 μ g/ μ L)

3.2. Chemicals/Reagents: 0.1 M CaCl_2 , LB broth, LB ampicillin plates: luria broth, ampicillin, X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside), agar-agar, IPTG (isopropyl-beta-D-thio galacto pyranoside).

3.3. Equipment: Automatic micropipettes with tips, water bath, incubator, ice box with ice.

3.4. Glassware/Plastic ware: Conical flask, measuring cylinder, beaker, microfuge tubes (1.5 mL), microtips.

4. Reagent Preparation

- i. CaCl_2 solution (0.1 M):
10.8 g CaCl_2 was dissolve into 100 mL distilled water. Final volume was made up to the 1000 mL.
(Solution was filtered using 0.2-0.45 μ m filter disc and store at 4 °C.)
- ii. IPTG (Isopropyl β -D-1-thiogalactopyranoside):
Stock solution- 2% IPTG (20 mg/mL)
Working solution- 40 μ L/mL
(Filter IPTG solution using 0.2-0.45 μ m filter disc and store at 4 °C.)
- iii. X-gal solution (5-Bromo-4-Chloro-3-Indolyl β -D-Galactopyranoside):
Stock solution- 2 %
Working solution- 40 μ L/20 mL of media
Filter X-gal solution using 0.2-0.45 μ m filter disc and store at 4 °C.
- iv. Ampicillin solution:
Stock solution- 100 mg/mL in distilled water
Working solution- 100 μ L/100 mL
Store at 4 °C.

5. Procedure

5.1. Preparation of Competent cells:

[NOTE: This procedure allows the preparation of cells that can be used immediately]

- i. Pick a single colony of DH5 α strain of *E.coli* bacteria and incubate in the evening into 5 mL LB medium.
Incubate the tube overnight at 37 °C for about 16 to 18 hrs.
- ii. On the third day, begin the preparation of competent cells as outlined.
- iii. Transfer the 5ml overnight saturated culture into 100 mL LB medium and incubate at 37 °C. Grow until the O.D. (A_{600}) reaches 0.23- 0.26 [Note: It takes around 2-3 hrs to reach the required O.D.].

[NOTE: The experiment should be done strictly under aseptic conditions]

- iv. Once the O.D. is reached, quickly chill the culture flask on ice and leave it on ice in refrigerator for 10-20 min
(from this point all work must be done at 4 °C)

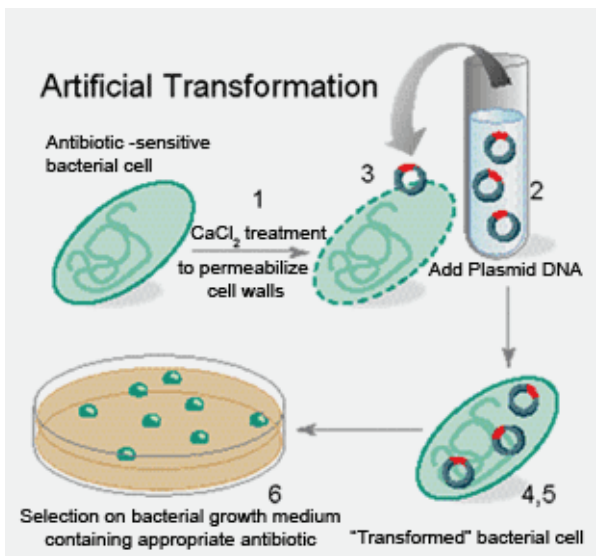


Fig. 2: Transformation of Competent cells

<https://www.slideshare.net/richapandey/26/ppt-of-bacterial-genetic-system>

- v. Transfer the culture aseptically into sterile centrifuge tubes and spin down at 6000 rpm for 8 min (if you have a refrigerated centrifuge spin at 4 °C).
- vi. Discard the supernatant and to the cell pellet add 15 mL of 0.1 M CaCl_2 . Suspend the cell pellet gently in CaCl_2 and this should be done by keeping the tubes in ice bucket. Keep on ice for 30 min. Centrifuge at 6000 rpm for 8 min.
- vii. Discard the supernatant and resuspend the pellet gently in 0.5 mL CaCl_2 (0.1M).
- viii. Aseptically transfer 100 μL of the above competent cells made into microfuge vials. All transferring should be done on ice.

[NOTE: While resuspending, keep your centrifuge tube on ice and 0.1 M CaCl_2 should be ice cold].

5.2. Transformation Procedure:

- i. Add 5 μL of pUC18 plasmid DNA to 100 μL of competent cells.



Fig. 3: Add 5 μL of plasmid to each of the competent cells

- ii. Incubate on ice 20 min.



Fig. 4: Set for 20 min on ice

- iii. Place tubes in 42 °C water bath for 120 seconds. Quickly remove the vial after heat shock and chill the vial on ice for 20 min.



Fig. 5: Cells are then heat shocked to 42 °C

- iv. Incubate at room temperature for 5 min.



Fig. 6: Cells are then incubated for 5 mins

- v. Add 1 mL of LB and incubate broth at 37 °C for 1 hour to allow bacteria to recover and express the antibiotic resistance.



Fig. 7: Broth medium is added to the cells that have been heat shocked

- vi. Add 100 μL to selective LB+Ampicillin + X- gal + IPTG plates and spread with glass spreader.



Fig. 8: "Transformed" cells are placed on media plates

- vii. If transforming for β- Galactosidase assay, incubate agar plates at 37 °C or room temperature for 30-48 hours.



Fig. 9: Spread to evenly distribute

- viii. Observe the blue color colonies on LB + Ampicillin + X- gal + IPTG plate and count the number of transformants.

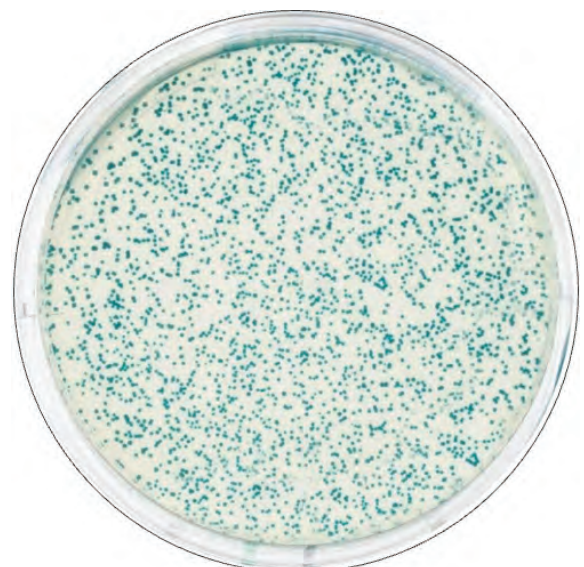


Fig. 10: Plates are incubated at 37°C overnight

6. Observations & Result

Fig. 11: Tranformants on LB + Ampicillin + X-gal + IPTG Plate

Transformation efficiency = colonies on plate/ng of DNA plated × 1000 ng/μg



6.1. Interpretation:

Transformation of competent *E. coli* cells with pUC18 Plasmid DNA has led to synthesis of functional β -Galactosidase in cells, which degrade X-gal into blue color compound. Only the transformants will be able to grow on LB + Ampicillin + X-gal + IPTG plates due to the Ampicillin resistance conferred by pUC18 plasmid. So, the blue color colonies on the selective medium indicate successful transformation of *E. coli*.

7. Precautions

- i. Maintain complete aseptic conditions.
- ii. X-gal and IPTG must be prepared in appropriate solvents and stored in dark vials as they are photosensitive.
- iii. Thermo sensitive chemicals like Antibiotics must be filter sterilized before addition to medium.
- iv. Carefully maintain the temperature and incubation period.
- v. Do timely observations of transformants.

8. Troubleshooting

- i. Maintaining cold temperature and incubation periods is very important.
- ii. Photosensitive chemicals must be stored in dark and heat labile chemicals like Antibiotics must be filter sterilized.

Suggested Reading(s)

- i. Sambrook, J., & Green, R. M. (2012). *Molecular cloning a laboratory Manual*. (4th ed.). New York: Cold Spring Harbor Laboratory Press.
- ii. Maniatis, T., Fritsch, E. F., Sambrook, J., & Engel, J. (1985). *Molecular Cloning—A laboratory manual*. New York: Cold Spring Harbor Laboratory.

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Source

Title picture: <http://slideplayer.com/7055283/24/images/3/Blue%2Fwhite+screening%28Bacterial+colonies+transformed+with+pUC18%29.jpg>

POLYMERASE CHAIN REACTION



1. Aim

To amplify 16S rRNA gene from *E. coli* by Polymerase Chain Reaction (PCR).

2. Introduction

The polymerase chain reaction, which was devised by Kary Mullis (1983), has revolutionized DNA technology. For his work, Mullis received the Japan Prize and the Nobel Prize for chemistry, both in 1993.

The polymerase chain reaction is a test tube system for DNA replication that allows a "target" DNA sequence to be selectively amplified, or enriched, several million-fold in just a few hours. Within a dividing cell, DNA replication involves a series of enzyme-mediated reactions, whose end result is a faithful copy of the entire genome. Within a test tube, PCR uses just one indispensable enzyme - DNA polymerase - to amplify a specific fraction of the genome. During cellular DNA replication, enzymes first unwind and denature the DNA double helix into single strands. Then, RNA polymerase synthesizes a short stretch of RNA complementary to one of the DNA strands at the start site of replication. This DNA/RNA heteroduplex act as a "priming site" for the attachment of the DNA polymerase, which then produces the complementary DNA strand. During PCR, high temperature is used to separate the DNA molecules into single strands, and synthetic sequences of single-stranded DNA (20-30 nucleotides) serve as primers. Two different primer sequences are used to bracket the target region to be amplified. One primer is complementary to one DNA strand at the beginning of the target region; a second primer is complementary to a sequence on the opposite DNA strand at the end of the target region.

2.1. PCR reaction components and their roles:

- a) **PCR Buffer:** Contains-100 mM Tris-HCL (pH 8.3 at 25 °C), 50 mM KCl, 15 mM MgCl₂, 1 mg/mL gelatin, 0.1% Tween 20, 0.1% NP-40.

Tris-HCl: Maintains the pH values between 6.8-8.3 (variation due to temperature)during the PCR reaction. Taq Polymerase has higher fidelity at the lower pH values that occurs at the higher temperature of PCR.

KCl: Assists in primer template annealing.

Magnesium: One of the most critical components of PCR mix. Taq Polymerase is dependent upon the presence of Mg⁺² for activity. Its concentration greatly affects the specificity and efficiency of the PCR. It requires 1.2-1.3 mM free Mg⁺² ion for its activity.

- b) **Nucleotides (dNTPs):** Are supplied as stock solution of 100-300 mM. Must be diluted so as to have a final concentration of 50-200 μM in the PCR mix.

- c) **Primers:** Primers are short oligonucleotides (20-30 bp long) sequences complementary to flanking ends of the DNA segment or gene to be amplified. A primer should be specific, contain equal number of each nucleotide, avoid runs of three or more G or C and should not form secondary structures or primer dimers.
Primer concentration for PCR should be between 5-50 (20) pmoles. Working stock 10-100 pmoles/ μ L
- d) **Template DNA:** Contains the sequence to be amplified. At least 3×10^5 templates molecules is required for PCR
- e) **DNA polymerase:** In routine PCR, DNA dependent DNA polymerases are used. DNA polymerases are compared on basis of Fidelity and Efficiency of synthesis. DNA polymerases used in PCR are: Taq DNA Polymerase (from *Thermusaquaticus*), Pfu polymerase, Deepvent polymerase etc.

2.2. Programming PCR:

PCR is an iterative process, consisting of 3 elements; denaturation of the template by heat, annealing of the oligonucleotide primers to the single stranded target sequence(s), and extension of the annealed primers by a thermo-stable DNA polymerase.

- a) **Denaturation:** It is achieved by heating the reaction to a temperature of 94–96 °C (or 98 °C if extremely thermo-stable polymerases are used), which is held for 1–9 minutes (during initial denaturation) or 30 sec during every regular cycle. It results in melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.
- b) **Annealing:** This is carried out at lower temperatures (35-68 °C). It allows for hybridization of the primer to the template strand. The annealing temperature depends on T_m of primers as discussed earlier.
- c) **Extension:** The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand.

3. Materials Required

3.1. Chemicals/Reagents: PCR buffer (containing 1.5 mM $MgCl_2$), dNTPs, primers, template DNA, Taq polymerase, MiliQ water.

3.2. Equipment: Thermal cycler, micropipettes.

3.3. Glassware/Plastic ware: PCR tubes, and microtips.

4. Procedure

- i. Prepare the following 50 μ L reaction in a 0.5 mL PCR tube on ice:

PCR System (for 50 μ L volume):

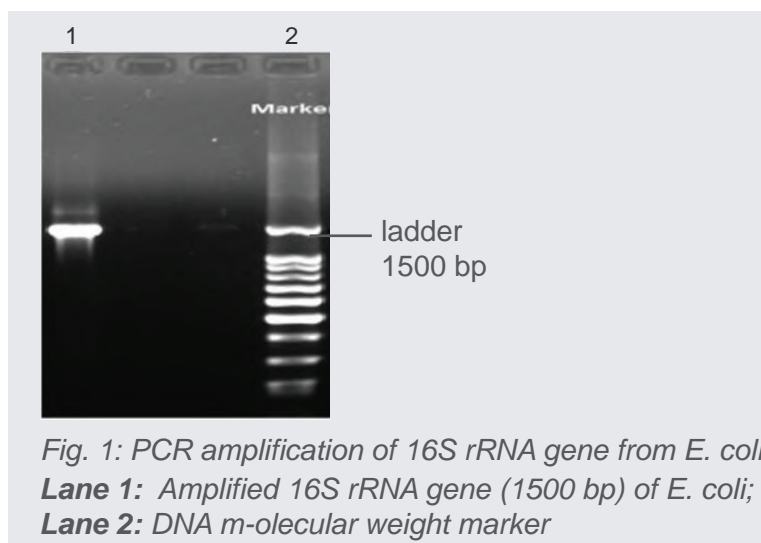
S.No	Component	Stock	Concentration required	Volume to be taken
1	PCR Buffer	10X	1X	5 μ L
2	dNTPs	10 mM (each)	50 μ M (each)	1 μ L (0.25 μ L each)
3	Primer (Forward)	10 mM	20 pmoles	2.5 μ L
4	Primer (Reverse)	10 mM	20 pmoles	2.5 μ L
5	Template DNA	Variable	1ng-1 μ g	5.0 μ L
6	DNA polymerase	3 units/ μ L or 5 units/ μ L		0.5 μ L
7	MiliQ water			33.5 μ L

- i. Gently mix the reaction and spin down in microcentrifuge. If the thermocycler does not have a heated cover, add one drop of mineral oil to the reaction tube to prevent evaporation.
- ii. Set the cycling conditions for PCR in the thermocycler. Routine cycling conditions for a PCR using Taq Polymerase are provided for reference.

Cycle step	Temperature	Time	Cycles
Initial Denaturation	95 °C	30 seconds	1
Denaturation	95 °C	15-30 seconds	30
Annealing	45-68 °C (Depends on T _m of primers)	45 seconds	
Extension	72 °C	Depending upon size of DNA to be amplified (1 min per kb to be amplified)	
Final Extension	72 °C	5 minutes	
Hold	4 °C	∞	1

- iii. After the PCR cycle is over, load the products on to an agarose gel and document it.

5. Observation



6. Result

Agarose gel electrophoresis of PCR product confirms, specific amplification of given gene of expected 1500 bp size

7. Precaution

- When setting up a PCR experiment, it is important to be prepared. Wear gloves to avoid contaminating the reaction mixture or reagents. Include a negative control, and if possible a positive control.
- Arrange all reagents needed for the PCR experiment in a freshly filled ice bucket, and let them thaw completely before setting up a reaction. Keep the reagents on ice throughout the experiment.
- Organize laboratory equipment on the workbench and also keep your record book handy with table showing reagents to be added. After adding each reagent, strike it out in the record book. This will ensure that one has added all the reagents.
- Dispenser microtips should be changed every time we change the reagent.

8. Troubleshooting

Observation (Trouble)	Possible Cause	Solution
SEQUENCE ERRORS	Low fidelity polymerase	<ul style="list-style-type: none"> Choose a higher fidelity polymerase
	Suboptimal reaction conditions	<ul style="list-style-type: none"> Reduce number of cycles Decrease extension time Decrease Mg⁺⁺ concentration in the reaction
	Unbalanced nucleotide concentrations	<ul style="list-style-type: none"> Prepare fresh deoxynucleotide mixes
	Template DNA has been damaged	<ul style="list-style-type: none"> Start with a fresh template Try repairing DNA template with the PreCR® Repair Mix (NEB #M0309) Limit UV exposure time when analyzing or excising PCR product from the gel

	Desired sequence may be toxic to host	<ul style="list-style-type: none"> • Clone into a non-expression vector • Use a low-copy number cloning vector
INCORRECT PRODUCT SIZE	Incorrect annealing temperature	<ul style="list-style-type: none"> • Recalculate primer T_m
	Mis priming	<ul style="list-style-type: none"> • Verify that primers have no additional complementary regions within the template DNA
	Improper Mg ⁺⁺ concentration	<ul style="list-style-type: none"> • Adjust Mg⁺⁺ concentration in 0.2–1 mM increments
	Nuclease contamination	<ul style="list-style-type: none"> • Repeat reactions using fresh solutions
NO PRODUCT	Incorrect annealing temperature	<ul style="list-style-type: none"> • Recalculate primer T_m values using the NEB T_m calculator • Test an annealing temperature gradient, starting at 5°C below the lower T_m of the primer pair
	Poor primer design	<ul style="list-style-type: none"> • Check specific product literature for recommended primer design • Verify that primers are non-complementary, both internally and to each other • Increase length of primer
	Poor primer specificity	<ul style="list-style-type: none"> • Verify that oligos are complementary to proper target sequence
	Insufficient primer concentration	<ul style="list-style-type: none"> • Primer concentration can range from 0.05–1 μM in the reaction. Please see specific product literature for ideal conditions
	Missing reaction component	<ul style="list-style-type: none"> • Repeat reaction setup
	Suboptimal reaction conditions	<ul style="list-style-type: none"> • Optimize Mg⁺⁺ concentration by testing 0.2–1 mM increments • Thoroughly mix Mg⁺⁺ solution and buffer prior to adding to the reaction • Optimize annealing temperature by testing an annealing temperature gradient, starting at 5°C below the lower T_m of the primer pair
	Poor template quality	<ul style="list-style-type: none"> • Analyze DNA via gel electrophoresis before and after incubation with Mg⁺⁺ • Check 260/280 ratio of DNA template
	Presence of inhibitor in reaction	<ul style="list-style-type: none"> • Further purify starting template by alcohol precipitation, drop dialysis or commercial clean up kit • Decrease sample volume
	Insufficient number of cycles	<ul style="list-style-type: none"> • Rerun the reaction with more cycles
	Incorrect thermocycler programming	<ul style="list-style-type: none"> • Check program, verify times and temperatures
Inconsistent block temperature	<ul style="list-style-type: none"> • Test calibration of heating block 	

	Contamination of reaction tubes or solutions	<ul style="list-style-type: none"> Autoclave empty reaction tubes prior to use to eliminate biological inhibitors Prepare fresh solutions or use new reagents and new tubes
	Complex template	<ul style="list-style-type: none"> Use Q5 High-Fidelity DNA Polymerases () For GC-rich templates, High-Fidelity DNA Polymerases. Include the appropriate GC enhancer. For longer templates, use LongAmp® TaqDNA Polymerase or Q5 high-Fidelity DNA polymerase or Q5 Hot-Start High-Fidelity DNA Polymerase
MULTIPLE OR NON-SPECIFIC PRODUCTS	Premature replication	<ul style="list-style-type: none"> Use a hot start polymerase, such as OneTaq Hot Start DNA Polymerase Set up reactions on ice using chilled components and add samples to thermocycler preheated to the denaturation temperature
	Primer annealing temperature too low	<ul style="list-style-type: none"> Increase annealing temperature
	Incorrect Mg ⁺⁺ concentration	<ul style="list-style-type: none"> Adjust Mg⁺⁺ in 0.2–1 mM increments
	Poor primer design (Non-specific priming by both or one primer)	<ul style="list-style-type: none"> Check specific product literature for recommended primer design Verify that primers are non-complementary, both internally and to each other Increase length of primer Avoid GC-rich 3' ends
	Excess primer	<ul style="list-style-type: none"> Primer concentration can range from 0.05–1 μM in the reaction. Please see specific product literature for ideal conditions.
	Contamination with exogenous DNA	<ul style="list-style-type: none"> Use positive displacement pipettes or non-aerosol tips Set-up dedicated work area and pipettor for reaction setup Wear gloves during reaction setup
	Incorrect template concentration	<ul style="list-style-type: none"> For low complexity templates (i.e. plasmid, lambda, BAC DNA), use 1 pg–10 ng of DNA per 50 μl reaction For higher complexity templates (i.e. genomic DNA), use 1 ng–1 μg of DNA per 50 μL reaction
BANDS IN NEGATIVE CONTROL		Contamination of solutions with template DNA

Suggested Reading(s)

- i. Dieffenbach, C. W., & Dveksler, G. S. (2003). *PCR primer: A laboratory manual*. Cold Spring Harbor Laboratory Press.
- ii. Innis, M. A., Gelfand, D. H., Sninsky, J. J., & White, T. J. (2012). *PCR Protocols: A Guide to methods and applications*. Academic Press.
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SODIUM DODECYL SULFATE -POLYACRYLAMIDE GEL ELECTROPHORESIS

1. Aim

To analyse proteins using sodium dodecyl sulfate polyacrylamide gel electrophoresis.

2. Introduction

Polyacrylamide gel electrophoresis (PAGE) is widely used technique to separate biological macromolecules, usually proteins or nucleic acids, on the basis of their electrophoretic mobility i.e. a function of the length, conformation and charge of the molecule. Molecules may be run in their native state, preserving the molecules higher-order structure, or a chemical denaturant may be added to remove this structure and turn the molecule into an unstructured linear chain whose mobility depends only on its length and mass-to-charge ratio. Sodium dodecyl sulphate (SDS) an anionic detergent is used to denature proteins and to impart a negative charge to the linearized proteins. This procedure is called SDS-PAGE.

When a protein mixture is heated to 100 °C in presence of SDS, the detergent wraps around the polypeptide backbone. It binds to polypeptides in a constant weight ratio of 1.4 g SDS/g of polypeptide. In this process, the intrinsic charges of polypeptides become negligible when compared to the negative charges contributed by SDS. Thus polypeptides after treatment become rod-like structures possessing a uniform charge density that is same net negative charge per unit weight. The electrophoretic mobilities of these proteins are a linear function of the logarithms of their molecular weights. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in mass-charge ratio, as each protein has an isoelectric point and molecular weight particular to its primary structure. This is known as native PAGE.

Following reagents are added for preparation of gel:-

- a) Acrylamide, when dissolved in water, slow, spontaneous auto polymerization takes place, joining molecules together by head on tail fashion to form long single-chain polymers. The presence of a free radical-generating system greatly accelerates polymerization.
- b) Bisacrylamide (N,N'-Methylenebisacrylamide) is the most frequently used cross linking agent for polyacrylamide gels. Chemically it can be thought of as two acrylamide molecules coupled head to head at their non-reactive ends. Bisacrylamide can crosslink two polyacrylamide chains to one another, thereby resulting in a gel. SDS is a strong detergent agent used to denature native proteins to unfolded, individual polypeptides.
- c) Ammonium per sulfate (APS) is a source of free radicals and is often used as an initiator for gel formation. An alternative source of free radicals is riboflavin, which generated free radicals in a photochemical reaction.

- d) TEMED (N, N, N', N'-tetramethylethylenediamine) stabilizes free radicals and improves polymerization. The rate of polymerization and the properties of the resulting gel depend on the concentrations of free radicals. Increasing the amount of free radicals result in decrease in the average polymer chain length, an increase in gel turbidity and a decrease in gel elasticity. Decreasing the amount shows the reverse effect. The lowest catalytic concentrations that allow polymerisation in a reasonable period of time should be used. APS and TEMED are typically used at approximately equimolar concentrations in the range of 1 to 10 mM.

3. Materials Required

3.1. Chemicals/Reagents: Acrylamide/Bisacrylamide, 10% (w/w) SDS, 1.5 M Tris-HCl (pH- 8.8), 0.5 M Tris-HCl (pH- 6.8), sample buffer (5X), 5X electrode (running) buffer, 10% APS, TEMED, staining solution, de-staining solution.

3.2. Equipment: Electrophoretic unit, power bank, water bath, rocker.

3.3. Glassware/Plastic ware: Micropipette, microtips, microcentrifuge tubes.

4. Reagents Preparation

i. Acrylamide/Bis (30% T, 2.67% C):

Acrylamide (29.2g/100 mL)

N'N'-bis-methylene-acrylamide (0.8 g / 100 mL)

Make 100 mL with deionized water. Filter and store at 4 °C in the dark.

ii. Sample Buffer (5X):

0.5 M Tris-HCl, pH 6.8-1.0 mL

Glycerol- 0.8 mL

10% (w/v) SDS- 1.6 mL

0.5%(w/v) bromophenol blue - 0.2 mL

β-Mercaptoethanol- 0.4 mL

Deionized Water- 4.0 mL

iii. 5X Electrode (Running) Buffer:

Tris base- 1.5 g

Glycine- 7.2 g

SDS- 0.5 g

Deionized Water- 100 mL

iv. Staining Solution:

0.1% (w/v) Coomassie Brilliant Blue

20% (v/v) Methanol

10% (v/v) Acetic acid

v. De-staining solution:

5 parts of methanol

1 part of acetic acid

4 parts of water

5. Procedure

Assemble the SDS-PAGE apparatus.

- i. Prepare 10% resolving gel and 4% stacking gel were prepared as per the table given below

Components	For 10% resolving gel Volume	For 4% stacking gel Volume
30% Monomer	3.3 mL	1.3 mL
Tris-Cl Buffer (pH 8.8)	2.5 mL	-
Tris-Cl Buffer (pH 6.8)	-	2.5 mL
Water	4.04 mL	6.04 mL
10% SDS	100 μ L	100 μ L
10% APS	50 μ L	50 μ L
TEMED	10 μ L	10 μ L
Total		10 mL

- ii. Pour the resolving gel mixture in between the glass plates of the apparatus, leaving a space of 1cm for the stacking gel. Fill this space with water to overlay the gel and obtain a uniform surface. Leave the gel to polymerize for 45 minutes.
- iii. After the polymerization of the resolving gel, pour off the overlaying layer of water, and over the resolving gel pour the stacking gel. Insert a comb of 0.75 mm thickness into the stacking gel, taking care to avoid trapping air bubbles. Leave the stacking gel undisturbed for 40 minutes to polymerize.
- iv. Mix the sample and the gel loading buffer in the ratio of 4:1 as the gel loading buffer is of 5X concentration and the required concentration is 1X. Mix 5 μ L of the gel with 20 μ L of sample in a micro centrifuge tube and boil at 90-100 $^{\circ}$ C to allow SDS to bind to proteins for 5 minutes.
- v. Perform the electrophoresis at 150 V for 45 minutes to 1hour.
- vi. After electrophoresis, immerse the gel in the staining solution for 30-40 minutes, followed by de-staining overnight.
- vii. The gel was then observed for the bands developed for the proteins in the sample mixture.

The following table provides the resolving gel percentage to be used for different protein size:

Protein Size (kD)	Resolving gel (%)
300-200 kD	4-6%
<200-100 kD	7-8%
30-100 kD	10%
<30 kD	12-15%

6. Observation and Result

The proteins will be observed on polyacrylamide gel as distinct blue bands.

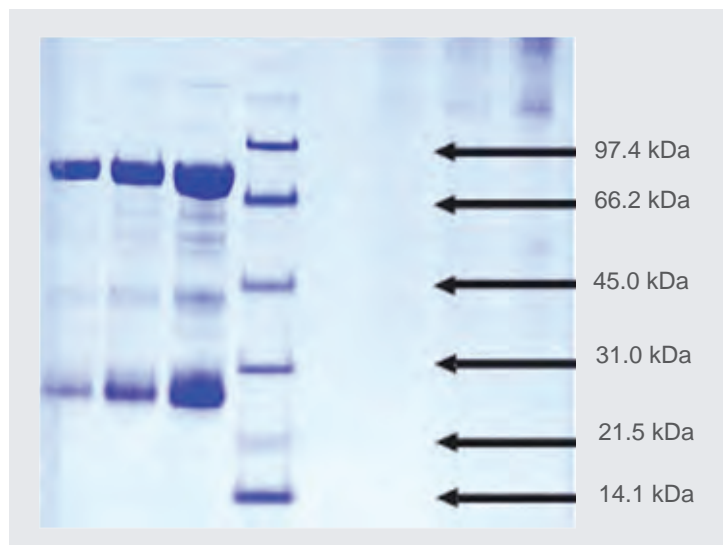


Fig. 1: Stained protein bands as observed on gel after SDS-PAGE

7. Precautions

- i. Do not allow the gel to dry after polymerization
- ii. Take out the comb carefully, making sure the well are not disturbed.
- iii. Keep a track of the gel while running.
- iv. Do not inhale SDS, as it is a neurotoxin and harmful when ingested and irritating for eyes
- v. β -Mercaptoethanol is a harmful and poisonous material. Avoid contact with eyes
- vi. Acrylamide is a potent cumulative neurotoxin: wear gloves at all times.

Suggested Reading(s)

- i. Sambrook, J., & Green, R. M. (2012). *Molecular cloning a laboratory Manual*. (4th ed.). New York: Cold Spring Harbor Laboratory Press.
- ii. Brown, T. A. (2010). *Gene cloning and DNA analysis: An introduction*. (6th ed.). Wiley Blackwell, John Wiley & Sons, Ltd., Publication.

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Title picture: <https://vdsstream.wikispaces.com>

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