JIGYAASA

A Multidisciplinary
Research Initiative of KC College, Mumbai

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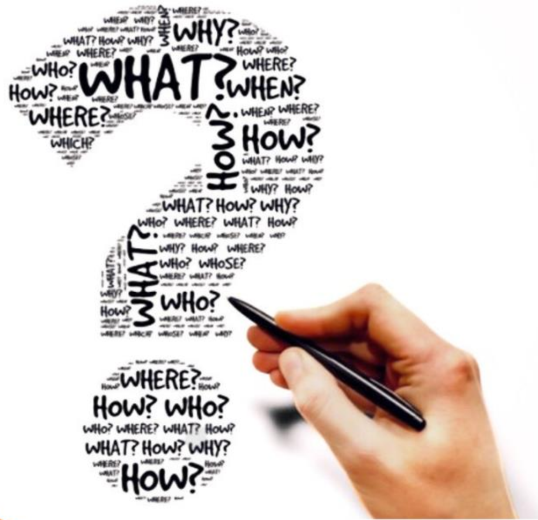

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Dr. Shalini R Sinha

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KISHINCHAND CHELLARAM COLLEGE

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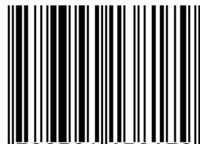

SHAILJA PRAKASHAN
57 P-Kunj Vihar-II, Yashoda Nagar, Kanpur-208011
Mob. : 8299046238, 9451022125
E-mail : shailjaprakashan@gmail.com

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JIGYAASA

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Shailja Prakashan
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Mobile No. 8299046238/9451022125
Email ID: shailjaprakashan@gmail.com

SHAILJA PRAKASHAN
57 P-Kunj Vihar-II, Yashoda Nagar, Kanpur-208011
Mob. : 8299046238, 9451022125
E-mail : shailjaprakashan@gmail.com

HSNC UNIVERSITY, MUMBAI
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EDITORIAL

K.C. College, believes in 'All inclusive' teaching learning process that involves experiential learning more than learning from a prescribed syllabus. The mantra of Innovation was always embraced by KC's pragmatic leadership, that found newer avenues to nurture the young minds of students for a 'Lab to Life' experience. The two major research platforms at KC College, the Science Honors Program-SHP and the Certificate Programme for Commerce and Arts-CPCA, thus continued their journey of curiosity under the flagship of the new HSNC Cluster University, Mumbai, which was launched in this year, 2020-21.

The faculty and students aspiring towards conducting small research projects were adequately supported by the leaders at the newly established University, even under the severely limiting constraints of the growing pandemic. The blended classrooms opened up more challenging yet possible avenues of conducting research in an online mode using internet facilities. Though the human exposure and actual hands-on activities were reduced, the availability of hybrid online mode offered more opportunities to connect with research groups at the National and International levels.

Thus, the fruits of accomplishment in bringing out this current Jigyaasa Volume IV, are sweeter. Like the previous volumes, this current research volume is a compendium of selected research papers based on research projects of the students enrolled in the above-mentioned undergraduate research programs. The chapters include interesting research papers from different fields such as Biotechnology, Computer Science, Life Sciences, Microbiology, Chemistry, Physics, Statistics, Social Sciences, Commerce and Humanities.

We are sure that the readers would like to dwell upon the ideas outlined and analyzed in chapters included in Jigyaasa Volume IV. They include varying range of topics such as Isolation of plastic degrading microorganisms, Insilco studies of Protein function of SMIM23, Creating Bioinformatic learning aid, Detection of Hazardous metals in food products, study of Mechanisms used by Retailers against Online shopping, Realtime monitoring thermometer, Binge watching and its links to sleep, depression, anxiety and stress, Contemporary relevance of Kautilya's Arthashastra and many more. These articles bear testimony to the efforts taken by the research guides and students, at the same time serving as a stepping stone for the future batches of students

who might be inspired to carry forward certain interesting possibilities of their predecessors and discover yet another life lesson.

As was once said by the noted author, Albert Camus, "In the depth of Winter, I finally learnt that within me there lay an invincible Summer". After going through these well written, research based articles, one would understand the importance of continuing with the true spirit of enquiry, even during the testing times of Covid -19 pandemic. Existence of such compendiums indicate gainful engagement and the confidence generated in students who are ready to publish their research work to the outside world through this compendium. The support of the HSNC University is highly appreciated in helping the staff and students to build bridges between the challenges and opportunities.

By -
Dr. Sagarika V. Damle (Convener SHP)
Dr. Shalini R. Sinha (Vice Principal and Coordinator CPCA)

FOREWORD

This year 2020-2021 is a landmark year since the HSNC Cluster University, Mumbai, was launched in this year in June 2020. This new Cluster University is guided by the New Education Policy 2020 and aims at becoming a leading educational centre, pursuing the path of administrative and academic excellence. This pandemic year was challenging for all but the fear, restrictions and constraints were not allowed to debilitate or hamper progress and growth.

We, at K.C College, continued striving to meet our goals and trying our best to fulfil the responsibility of being a Constituent College of the HSNC Cluster University, since K.C will be integral in the growth and evolution of the HSNC Cluster University, Mumbai and will lead from the front in achieving the objectives of this University. Amongst all the other goals, producing quality research is a prime objective of the HSNC University. K.C College already has a strong research culture with its innovative research-based programmes like Certificate Programme for Commerce and Arts (CPCA) and Jigyaasa Science Honors Program (SHP), and with the formation of the HSNC University, greater impetus has been added to these programmes.

Jigyaasa Vol IV is the culmination of the efforts of a few young researchers with their teacher mentors, who have taken to research like duck to water, only because of the learning achieved from interactive online sessions, knowledge of committed resource persons and capability of their teacher guides who mentored students, never once looking at the clock. Under such challenging circumstances, it gives me great joy to bring the research amalgamation of the two programmes Science Honors Program (SHP) and Certificate Programme for Commerce & Arts (CPCA), in this fourth volume of Jigyaasa.

These two programmes nurture the young minds, offering them a variety of platforms for holistic development, such as strengthening communication skills, developing confidence through presentations, awareness towards the environment and social responsibilities along with their journey of learning structured research. This compendium, Jigyaasa Volume IV, showcases research in the modern era of these budding researchers from various disciplines such as Biotechnology, Chemistry, Life Sciences, Microbiology, Physics, Statistics, Social Sciences, and Humanities. The NEP emphasizes upon breaking the barriers across the subjects and encourages interdisciplinary research.

Whereas K.C College, with its CPA and SHP programmes, has started building bridges across disciplines, since long ago. This volume of Jigyaasa displays commitment, devotion and sincerity and shows that even severe challenges can be overcome.

I congratulate all the contributors to this volume and wish for them great recognition and a research spirit of innovation and scientific enquiry that will lead them forward in life.

Dr. Hemlata K. Bagla
Principal, K. C. College,
Sr. Dean, Sciences &
Director, Niranjan Hiranandani
School of Management and Real Estate,
HSNC University, Mumbai

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SECTION 1 – LIFE SCIENCES

Chapter 1 – Creating Bioinformatics Learning Aids

Tasneem Nawab, Akanksha Kulkarni, Dr. Sagarika Damle*, Dr. Aashu Vajpai*

Department of Life Sciences, Kishinchand Chellaram College, Churchgate, Mumbai-400020.

Email: visanika@gmail.com, aashu.vajpai@kccollege.edu.in

ABSTRACT:

Bioinformatics is a multidisciplinary field which is introduced to a learner at an undergraduate level. It is an amalgamation of Biological Sciences, Computer Science, and Mathematics. A plethora of online resources for Bioinformatics is available for the learners across the world, ranging from various databases and web applications to data analysis software tools. With so much information available, searching for relevant information in bioinformatics seems like a daunting task for the beginners. The goal of this project was to create a few simple online resources to create awareness for the new entrant regarding Bioinformatics. The need for such resource was assessed by carrying out an extensive review of bioinformatics syllabi of universities and colleges throughout India and a review of resources available (Books and videos) for beginners in bioinformatics. It was followed by a survey of pre-knowledge of the subject among the target population in order to build a suitable learning aid that can be used by undergraduate students as well as Junior college and secondary level school students. These videos and e-books are user-friendly and will serve as resources, gave encouraging results. This project also served as capacity building in the subject of bioinformatics and use of technology in generating online resources.

KEYWORDS: Bioinformatics, free, course, for beginners, survey, undergraduates, learning aid, video, e-book.

INTRODUCTION:

The emergence of bioinformatics has ushered in a new era for scientific research in life sciences and its related fields. According to Bayat, Bioinformatics is defined as the application of tools of computation and analysis to the capture and interpretation of biological data. It is an interdisciplinary field, which harnesses computer science, mathematics,

physics, and biology [1]. The growth of web-based, collaborative and publicly accessible research data provides an important opportunity to transform the ways in which science is taught [2]. Due to major advances in experimental techniques over the last few decades, novel approaches and computational analyses have been seen in the fields of biology, biotechnology and medicine [2]. The growing disparity between the rapidly evolving world of scientific research and conventional methods of science education act as a barrier to realizing the full potential of publicly accessible data [3].

The rapid growth and cross disciplinary nature of bioinformatics poses a major challenge: while there are many biologists and computer scientists, there are very few computer-literate biologists or biology-literate computer scientists [4]. Key computational ideas need to be better communicated to and absorbed by biology undergraduates in a manner that excites them about bioinformatics, and encourages them to creatively employ bioinformatics ideas and methods in the future [5]. Another challenge faced in bioinformatics education is that, while undergraduate bioinformatics programs at top universities include a mixture of biological and computational courses, these programs are not ideally suited for biology students5.Thus, the introduction of a bioinformatics course to the undergraduate biology curriculum can bridge the gap by providing a quantitative, interdisciplinary coursework for bioscience students [6]. Data from a recent study revealed that, across India, 20% of universities had a bioinformatics course [7]. While there are many research papers and articles which assess the state of bioinformatics studies in high school and colleges across the world, there is a lack of such research pertaining to Indian high school education. One more important thing to note is that while bioinformatics is included (to a limited extent) in Indian high school syllabi, there is a severe lack of learning aids which are free and suitable for beginners. The goal of the present research was to design a learning aid which is open-source, free, beginner friendly, and specifically catered to an Indian undergraduate syllabus.

MATERIALS AND METHODS:

Research Design

- Syllabi of various universities and colleges was studied
- Review of existing bioinformatics learning resources

- Testing of pre-knowledge of sample population
- Testing post knowledge after using the learning aid by the sample population

The study for the effectiveness of the learning aids used a within-subject design (pre-filled questionnaire and post-filled questionnaire) with the percentage of correct answers as the dependable variable.

Sampling Procedure for Survey

One hundred and eleven high school biology students (12th grade), and first- year undergraduate life science students were selected by random sampling process for this study. The age range was 16-19 years.

Materials

The materials used were:

- Laptop/Personal Computer with a stable internet connection
- Google forms software
- Syllabi of High schools and Universities obtained from the web, Animaker software
- Youtube (as a platform for the learning aids)
- Word Processors (Pages and Microsoft Word)

Procedure

The experiment consisted of three phases.

<u>Phase 1:</u>

1) A review of the syllabi of high schools, State Universities and some autonomous colleges was carried out.

This was done in order to assess the degree of inclusion of bioinformatics in various Science syllabi. It also gave an idea about the topics which were to be covered in the learning aids.

2) Existing bioinformatics learning resources were reviewed. This included both online and offline resources.
3) A questionnaire was prepared to survey the existing knowledge of bioinformatics in the target audience.

Phase 2:

The second phase consisted of the creation of the learning aids, based on the data obtained from the first phase.

1) Data Handling and Data Analysis using Google forms.
2) Video animation using an online video animation software (Animaker)
3) Voiceover modulation using the text to speech feature (Animaker Voice)
4) Creation of a Youtube channel and the upload of the finished videos/learning aids
5) Development of the E-book: To serve as a supplementary guide to the videos, an E-book was also designed. The E-book also functions as a repository of existing resources (Youtube channels, tutorials, useful links and resources) for learning bioinformatics.

Phase 3:

In the third phase, the effectiveness of the learning aids was checked. For that purpose, two copies of a questionnaire were generated, one being the “entrance survey” and the other being the “exit survey”. First, the participants were asked to fill out the entrance survey, after which they were instructed to go through the learning aids. Lastly, they filled out the exit survey. Following this, the data from the two surveys was compared.

Data Analysis

Data analysis consisted of two parts: One for the first phase, and the other for the third phase.

For the first phase, the raw data collected was first converted to tabular formats. Next, this processed data was used to plot graphs and pie charts. For the third phase, the charts for the pre- filled and post-filled questionnaires were compared. The change in the percentage of correct answers represented the effectiveness of the learning aids.

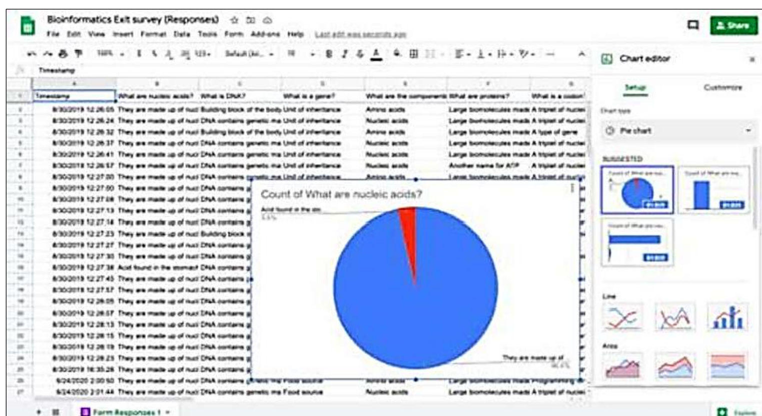


Fig. 1: A screenshot of data analysis carried out using Google Forms

OBSERVATIONS AND RESULTS:

Our goal was to design a learning aid which is open source, free, beginner friendly and specifically catered to an Indian syllabus for bioinformatics. In order to do this, we studied the syllabi of various high schools, universities and colleges in India.

Here is the list of the High School boards, Universities and autonomous colleges whose syllabi were studied (full list provided in supplementary data):

1) University Of Mumbai
2) CBSE Board (Central Board Of Secondary Education)
3) ISC Board (Indian School Certificate)
4) International Baccalaureate
5) Cambridge International
6) Maharashtra Board HSC
7) Andhra Pradesh Intermediate
8) AHSEC (Assam Higher Secondary Education Council)
9) BSEB (Bihar School Examination Board)
10) BSEH (Board Of School Education Haryana)

11) CGBSE (Chhattisgarh Board Of Secondary Education)
12) GBSHSE (Goa Board of Secondary & Higher Secondary Education)
13) GSEB HSC(Gujarat Secondary & Higher Secondary Education Board)
14) HPBOSE (Himachal Pradesh Board of School Education)
15) JKBOSE (Jammu And Kashmir Board of School Education)

The syllabi were studied on the basis of a few parameters:

1) Which year of high school/university the syllabi belonged to
2) The board/university who designed the syllabi
3) The difficulty level of the bioinformatics content included in the syllabus;

Basic: The syllabus included the definition of bioinformatics, or had a brief review of the Human Genome Project and genomics.

Intermediate: Introduction to the concept Bioinformatics, database searching, etc.

Advanced: Mainly for the syllabi of bioinformatics degrees, or other degrees giving in-depth knowledge of the subject.

The survey of syllabi helped in knowing if bioinformatics was taught at the high school and undergraduate levels, and if yes, how much of it. (Tables provided in Supplementary data.

It was observed that out of 34 high school programs, 29 included Bioinformatics at either a basic (22) or an intermediate (7) level in their science syllabi.

The high school programs with an intermediate level of bioinformatics in their syllabi were:

1) ISC Board (Indian School Certificate)
2) IB DP (International Baccalaureate Diploma Program (equivalent to 11th and 12th grade education))
3) BSEH (Board of School Education Haryana)
4) JKBOSE (Jammu and Kashmir Board of School Education)

5) COHSEM (Council Of Higher Secondary Education, Manipur)

6) MBOSE (Meghalaya Board Of School Education)

7) DGE (Tamil Nadu Directorate Of Government Examination)

It was also observed that out of the 33 universities studied; only 5 offered undergraduate courses in bioinformatics, namely:

1) Kannur University

2) Panjab University

3) Kurukshetra University

4) Saurashtra University

5) Pondicherry University

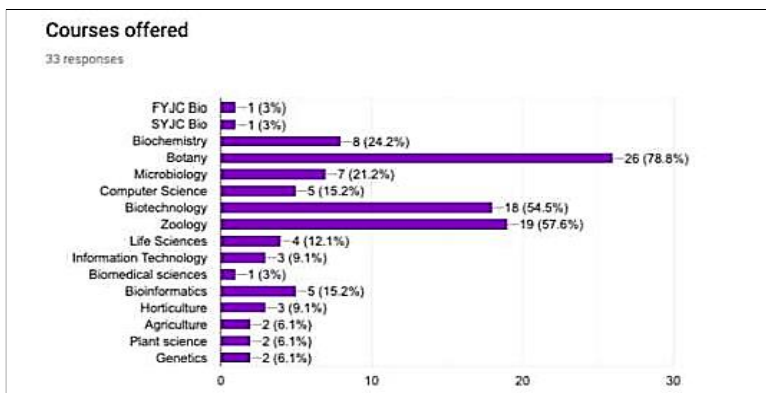


Fig. 2: Subjects offered by universities

Subsequently, the resources available were reviewed. It was observed that many of the offline resources were outdated; while the online ones were either too expensive or catered to post graduate students, skipping the basics. For the assessment of pre knowledge about the subject, a group of first year undergraduate life sciences students were asked to fill a questionnaire (Questionnaire provided in Supplementary data). The results showed incorrect answers as well as a lack of awareness. (Results provided in Supplementary data).

After the learning aids were created, their effectiveness was tested. One hundred and eleven undergraduates and high school students were asked

to fill the entrance survey, go through the learning aids and subsequently fill the exit survey. The following results were observed upon comparing the percentage of correct answers of the entrance and exit surveys: The percentages of correct answers had slightly increased (Full results provided in Supplementary data).

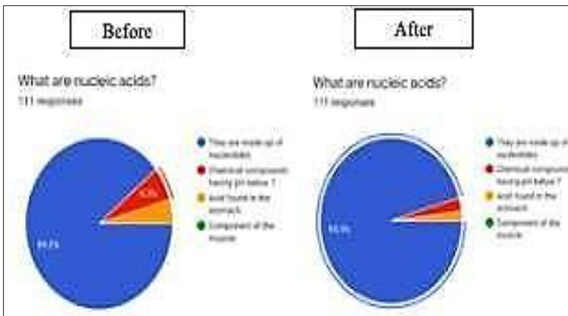


Fig. 3.1 and 3.2: Before and after percentages of correct answers the participants' awareness about the field had increased.

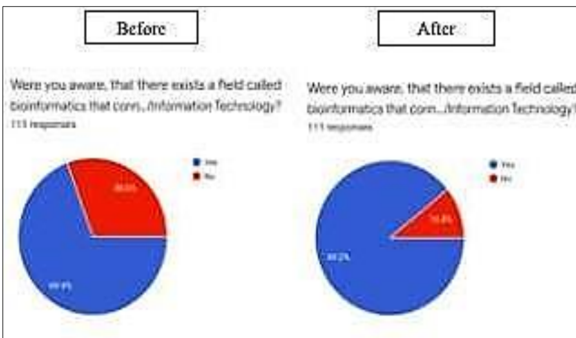
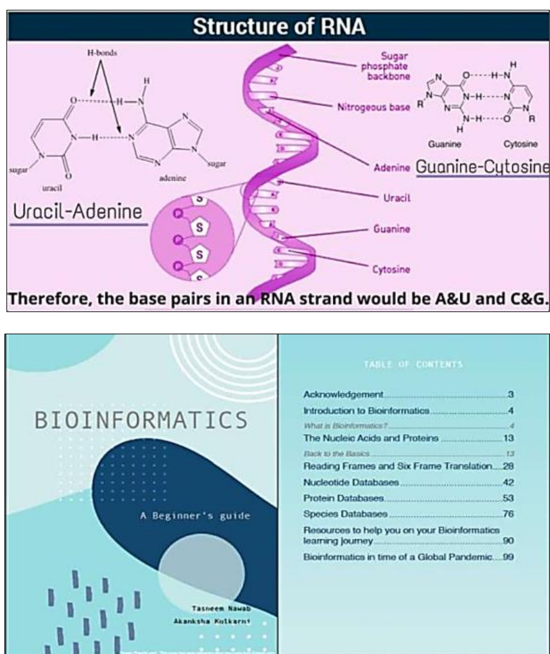


Fig 4.1 and 4.2: The graphs show an increase in awareness

The learning aids had generated curiosity and interest in the participants. (Charts provided in Supplementary data)



Figs. 5.1 and 5.2: Pictures of the finished videos and E-book

Results

The goal of the present article was to design a bioinformatics learning aid which is open-source, free, beginner friendly, and specifically catered to an Indian syllabus. In order to do this, we carried out our study in three stages. In the first stage, a review of Indian high school and University syllabi was carried out, in order to assess the inclusion of bioinformatics in them. Existing learning resources and aids (both online and offline) were also reviewed. Following this, the assessment of pre-knowledge of the subject was carried out amongst the target population. In the second stage, learning aids were designed based on the data obtained from the first stage. The third and final stage consisted of testing the effectiveness of the learning aids. Our findings revealed that many high schools and a few universities did include bioinformatics in their curriculum, but, in most cases, the inclusion was of a very basic level. In line with our hypothesis, there was a slight increase in student's knowledge after they were introduced to the learning aids. Our findings

for the University syllabi review converge with previous findings [7][8]. In addition, the inclusion of high school syllabi in our study, helps paint a better picture of the state of bioinformatics education in India. Through our high school syllabi survey, we found that every High school program which included bioinformatics in their syllabus, included it in the 12th grade syllabus (of subjects such as Biology, Zoology, Botany or Biotechnology). One more interesting finding was that, high school programs which offered more diverse subject choices for the 11th and 12th grade (for example, biotechnology), tended to include bioinformatics at an intermediate level in 12th grade Biology, as compared to High school programs which offered the three basic sciences (Chemistry, Physics and Biology), as the subject choices, and their basic inclusion of bioinformatics (through a brief summary of the Human Genome Project and genomics) in 12th grade Biology/Botany/Zoology. There was virtually no mention of bioinformatics in the Computer Science/Information Technology syllabi of the 11th or 12th grade in any high school program.

Discussion

Our findings point towards a new approach to deal with some challenges of conventional science education. These learning aids are catered to the current pre-knowledge level, making an unapproachable/difficult subject easier to comprehend. They would also help the teachers who might not have as much expertise in the field, as these learning aids are user-friendly and more up-to-date as compared to the prescribed textbooks. This same experiment can be applied to numerous other topics/subjects and can be used to make guides, wet lab tutorials, etc. Our study has two main limitations. First, the number of university syllabi surveyed was relatively low. However, there already exists a recent study with the review of Indian university syllabi [7], and our survey mainly focused on the available syllabi of State Universities, which are the backbone of Indian higher education [7]. Second, and the main limitation, was that the before and after survey for testing the effectiveness of the learning aids do not have a significant difference. This can be owed to a number of factors. Due to time constraints, participants did not have a chance to thoroughly go through the learning aids and pay attention.

CONCLUSION:

In the present article, we aimed to design a bioinformatics learning aid which is open- source, free, beginner friendly, and specifically catered

to an Indian syllabus. Even with the inclusion of Bioinformatics in high school and undergraduate syllabi, (as seen in our findings), many students struggle with finding the appropriate learning resources 9. Our study aimed to solve this problem, or to lessen it, by designing a learning aid specifically catered for Indian students from secondary, high school and undergraduate classes. Another goal of our project was to increase awareness about the subject and to generate interest in it. Our results suggest that we were successful in these goals. Future research may extend this work by designing learning aids not only for bioinformatics, but for any other subject or topic.

ACKNOWLEDGEMENTS:

We extend our sincere gratitude to DBT Star Scheme, HSNC University and the Department of Life Sciences (K.C. College).

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Chapter 2 – Extraction of Enzyme Asparaginase from Extremophiles

Bhakti Tari, Niharika Gupta, Dr. Shalini Rai*, Dr. Aashu Vajpai*
Department of Life Sciences, Kishinchand Chellaram College,
Churchgate, Mumbai-400020
Email: shalini.rai@kccollege.edu.in, ashvajpai23@gmail.com

ABSTRACT:

Extremophiles are organisms that possess the ability to survive in extreme conditions like highly saline area, extreme temperature range etc. This is achieved by production of several metabolites and enzymes. One such enzyme produced by extremophiles is an enzyme Asparaginase, that holds vast application in the Pharmaceutical and Food processing industry. This project aimed to identify such extremophiles from highly saline areas around the suburbs of Mumbai. After collection, the water samples were processed for isolation of extremophile species using microbial techniques. The isolates were further screened for the production of enzyme by inoculating in M9-Media. The isolate giving positive result were further preliminarily identified and then underwent the process of Media Optimization, combination of nitrogen source and carbon source were varied to obtain maximal growth. Further protein assay was performed using nesslerization techniques. Above study gave an insight that extremophiles can be explored as a source of Asparaginase in industries with further standardization of media with variable sources.

KEYWORDS: Extremophiles, Asparaginase, Nesslerization, M9-Media

INTRODUCTION:

Enzymes are proteins in nature. Being the integral part of cellular system, they play a major role in the pharmaceutical and food processing industries. One of such enzyme is L-Asparaginase. The amidohydrolase L- Asparaginase (E.C-3.5.1.1) converts L- Asparagine to L-Aspartate and ammonia. The optimum temperature for the reaction is 38.5°C and

the optimum pH for the reaction is 7.1. (Remya & Satyanarayana., 2020) In the pharmaceutical sector, since last four decades, it has been used for the treatment of leukemia. It is the most common therapeutic indications used for the treatment of acute lymphoblastic leukemia (mainly in children) (Verma et al., 2007). In recent years, some more applications of L-Asparaginase have been reported in the food processing industry for the production of acrylamide free food. (Pedreschi et al., 2008) In 1953, J G Kidd found that some active constituent in necrosis of lymphoma cells (Kidd, 1953) In 1963, Broome came up with the theory that the antitumor activities of guinea pig serum are found to possess traces of L- Asparaginase. (Broome, 1963) Furthermore, in 1963 a major advancement took place when it was reported that L- Asparaginase can be extracted from E.coli. (Mashburn and Wriston, 1963) The first clinical trials in patients with acute lymphoblastic leukemia were carried out with asparaginase preparations both from guinea pig serum and E.coli. (Roberts, J et al., 1966) Erwinia chrisanthemi showed maximum activity which could contribute for the production of enzyme. (Phillips, A W et al., 1971) Until now, approved L- Asparaginase for both applications are few, due to their lack of appropriate properties. The study conducted by (Ebrahiminezhad, Alireza et al., 2011) found that Halophilic bacteria might contain L-Asparaginase with novel immunological properties. L-Asparaginase is among the relevant enzymes that can be obtained from marine sources. (Izadpanah Qeshmi, Fatemeh et al., 2018).

MATERIAL & METHODS:

1. Sample Collection

The samples were collected from 6 locations from the Mumbai suburbs, that include:

1. Bhayandar Salt Pa
2. Girgaon Chowpatty
3. Tungareshwar Hot spring
4. Bhandup Pumping Station
5. Godrej Mangrooves
6. Mulund-Airoli Road

The collected sample was collected in a pre- sterilized container and was maintained.

2. Isolation of micro-organisms from the sample

The isolation of micro-organism from the collected sample was done referring to the techniques of (Erin R Sanders (2012)). In total seventeen colonies of bacteria were isolated from the collected sample which were labelled and maintained for further processing.

3. Screening for Production of L- Asparaginase Enzyme

After obtaining a pure culture of the isolated colonies they were further screened for the production of the desired enzyme by inoculating the bacteria in M9- Media. If there is enzyme activity taking place in the media, ammonia gets produce which increases the pH of the media, this brings change in pH which is indicated by phenol-red indicator. Thus, a colour change from yellow to pink is a positive indication for the screening of enzyme production. (Image1) & (Image 2) show the change in the colour of the media, suggesting the production of enzyme(Gulati R et al., (1997)), This is one of the test where change in colour was seen in only three colonies, further suggesting the elimination of remaining culture.

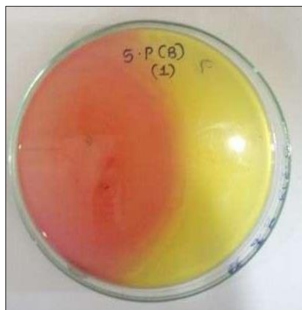

Fig 1: Screening for L-Asparaginase production (Qualitative)

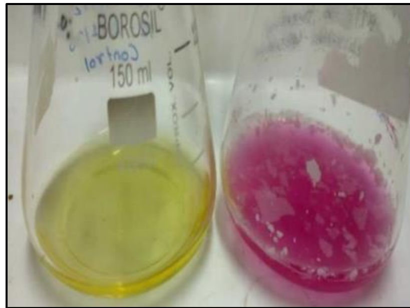

Fig 2: Screening for L-Asparaginase production (Quantitative)

After the quantitative analysis (Fig 2) the bacteria giving maximum absorbance was finalized for further analysis.

4. Identification of bacteria

The bacteria were identified on the basis of gram staining, colony characteristics, biochemical tests and the results were confirmed from a certified laboratory.

5. Media Optimization

Various carbon and nitrogen source were varied in the growth media, the carbon source such as beef, glucose, lactose and the nitrogen sources such as peptone, leucine and proline with asparagine as a substrate were varied and bacterial growth was observed by analyzing the colorimetric reading at 420nm, combination of 1 nitrogen and 2 carbon source was also varied and maximum growth was observed in media having 1 nitrogen source and 2 carbon source- peptone in combination with beef and glucose at an incubation time of 72 hours.

6. L-Asparaginase assay

The L-Asparaginase assay was performed according to the procedure described by (Peterson and Ciegler (1969)) and the calculations were performed according calculations performed by (Imada et al., (1967)). Enzyme activity was determined on the basis of liberation of ammonia calculated with reference to a standard curve of ammonium sulphate.

$$\text{Enzyme Activity (UI/ml)} = \frac{(\mu m \text{ of ammonia liberated})(\text{Initial vol of mix})}{(\text{Vol of mixture used}) \times (\text{incubation time})(1)}$$

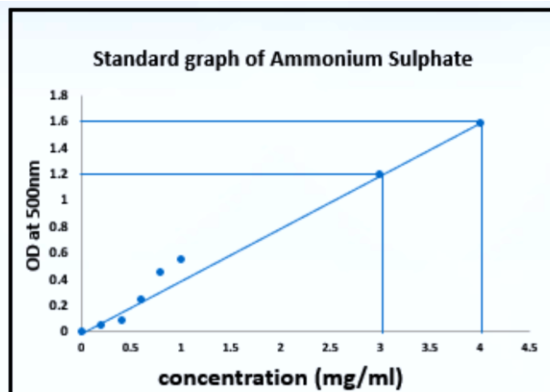

Fig 3: Standard ammonium sulphate graph used as reference to find samples concentration of ammonia

7. Preliminary Protein Purification using Salting out Method

A preliminary protein purification was done using the salting out method where the extracted enzyme was saturated with solid ammonium sulphate at 30%,70% and 90%. The obtained precipitate was further dialyzed in a dialysis membrane.

RESULTS AND DISCUSSION:

The isolation of bacteria was done following the Erin R Sanders (2012) technique, which gave seventeen pure bacterial colonies, further screening of these isolated bacteria was done for the production of L-Asparaginase enzyme using a rapid assay plate method as described by Gulati R et al., (1997). After which only three colonies were narrowed down,(Fig 1) a quantitative analysis was also done using the same technique (Fig 2) and a single bacterial colony was finalized for further analysis. Further the identification of the selected bacteria had to be done, which was preliminarily identified on the basis of gram nature, colony characteristics and biochemical test, the results were confirmed

from a certified laboratory and was found out to be pseudomonas. According to (Salzer,Wanda L et al.,2014) enzyme extracted from E.coli produces hypersensitivity reactions, a new bacterial source has to explored other than E.coli. Thus, the bacteria which has been isolated in our work can be explored for the production of enzyme. The Media optimization step is further performed to maximize the bacteria growth. A combination of 1 Nitrogen and 2 Carbon sources were finalized as they gave maximum growth. Highest growth was observed at 72 hour incubation period which was similar to that suggested by (V. Saranya et al., 2010; Benoit et al.,1990; Nam and Ryu.,1985). The bacteria growing in the selected broth with a composition of peptone, beef ,glucose and asparagine as a substrate at an incubation time of 72hours was further processed for assaying the L-Asparaginase activity described by (Imada et al., 1967; Peterson and Ciegler .,1969).(Fig 3) refers to the standard graph from which the concentration of ammonia was estimated. The enzyme Activity was further calculated to be 16 IU/ml after calculation, It is comparatively less, this could be because the enzyme activity gets influenced with pH and temperature (Ashraf A. El-Bessoumy et al., 2003). By studying all the responsible factors like concentration of components, pH and temperature better results can be achieved.

CONCLUSION:

The above study suggests us that bacteria isolated could be a pseudomonas species with a potential to produce the enzyme L Asparaginase. The sources as salt pans could be expored as a potential site for such bacteria in the city. Media optimization study suggests that by varying factors like concentration, pH and temperature the results can be improved with concentration-based study for two carbon and one nitrogen sources. Such bacteria can be used as an alternative source for enzyme production and can combat the problems associated with drug resistance in cancer treatment.

FUTURE PROSPECTS:

There is a need of concentration-based analysis of media optimization. The identification of bacteria by 16S r DNA gene sequencing is required.

Protein Purification of the obtained enzyme extract to remove all the impurities and compare it with a standard L-Asparaginase enzyme using chromatographic technique. Its applications are also to be tested in the food processing industry to reduce the amount of acrylamide in food.

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Chapter 3 – Comparative Study of Different Species of the *Curcuma* Family for their Properties and Synergistic Effects

Jasmine John, Vikas Gurudu, Dr. Tejashree Shanbhag*, Dr. Sagarika Damle*

Department of Life Sciences, Kishinchand Chellaram College, Churchgate, Mumbai-400020.

Email: tejashree.shanbhag@kccollege.edu.in, sagarika.damle@kccollege.edu.in

ABSTRACT:

Turmeric is one of the most commonly found condiment in Indian households. It has been a part of the traditional medicinal system since centuries and is now recognised for its medicinal properties in modern medicines. The aim of this research is to compare the total phenolic content, anti-microbial, antioxidant -amylase inhibitory properties of *Curcuma* species namely *C. aromatica, C. amada, C. longa* and *C. zedoaria*, and to investigate the probable synergistic effects of mixture of these extracts (1:1:1:1). The powdered plant material was subjected to Soxhlet extraction. The extracts thus obtained were used for anti-microbial analysis using Agar well diffusion method on four bacterial strains, *Propionibacterium acnes*, *Corynebacterium diphtheriae*, *Escherichia coli* and *Staphylococcus aureus*. α-amylase inhibitory effect of the plant extracts was determined in-vitro. The highest anti-diabetic effect was observed in *C.zedoaria*. The maximum anti- microbial effect was displayed by all four extracts on *C. diphteriae*. In the antimicrobial studies, the synergistic effect of these four extracts, were comparable to the effect produced by single plant extract. Therefore, further experimentation would be needed to establish the effectiveness of the mixture and provide leads in development of improved natural products with desired therapeutic effects.

KEYWORDS: curcuma, synergistic, α-amylase, anti-microbia

INTRODUCTION:

Turmeric or *Curcuma longa* is called as the golden spice of India. This spice gets its hue from curcumin. It is a perennial herb and belongs to the *Zingiberaceae* family which is also called as the ginger family. It was used in Indian and Chinese medicines since ages. In Ayurveda, it is known as Haridra. It is routinely used in cooking, cosmetics and dyeing in south Asian countries. Turmeric latte is gaining popularity worldwide due to its extensive health benefits. Other members of the *Zingiberaceae* family are *Curcuma aromatica*, *Curcuma zedoaria*, *Curcuma amada*. *Curcuma aromatica* is also known Perfumery [3]. Rhizomes of this family comprises of 1-6% of curcuminoids out of which 60-70% is curcumin, 20-27% is demethoxycurcumin and 10-15% is as wild turmeric. In Ayurveda, it's called Vana Haridra and has been profoundly used in curing skin ailments and snake bites. Traditionally, rhizomes of C.aromatica were used by the Khasi tribes to deliver babies. It is also known to have anti-microbial, anti-diabetic, radical scavenging, anti-fungal properties [1]. *Curcuma zedoaria* is also called as the white turmeric or Zedoary. It has odour similar to that of mango and can treat skin ailments and menstrual irregularities [2].

Ocimene, a component found in this plant is used in bisdemethoxycurcumin. While the bioactive component curcumin has a very low concentration in the available powdered form, it is furthermore reduced similarity to the original golden yellow colour of turmeric. The antibacterial effect was first studied in 1949. Curcumin has shown to have activity similar to the known TNF blockers like HUMIRA, REMICADE which makes it a probable candidate for its use against the Covid pandemic. ArtimiC, a medical spray which combines artemisinin and curcumin, is in studies convey curcumin as a poorly bioavailable, bio accessible compound. Studies also attributed curcumin to be the source of all medicinal properties, however recent studies on curcumin free extracts showed some effects too, throwing some light into the potency of other components present in the curcuma genus [4]. In our study we tried to compare various *Curcuma* species namely *Curcuma aromatica*, *Curcuma zedoaria*, *Curcuma amada* and *Curcuma longa* for

their phenolic content, amylase inhibitory, antioxidant and antibacterial effects. Although these plants belong to the same genus, their properties vary due to different proportion and composition of curcuminoids and volatile oils. Oleoresins of these plants were studied to look for differences in activities and explored for their synergistic effects. This paper contains preliminary investigations done for comparative study of different species of curcuma family for their phenolic content, amylase inhibition, antioxidant, antimicrobial and synergistic studies.

MATERIALS:

Biological samples: Fresh rhizomes of *Curcuma zedoaria*, *Curcuma aromatica*, *Curcuma amada*, powder of *Curcuma longa*

Chemicals: 99% Dimethyl sulfoxide, 99% Ethanol, sodium carbonate, Folin Ciocalteu reagent, gallic acid, starch, α-amylase, DNSA reagent, DPPH, ascorbic acid, agar, beef extract, peptone and distilled water.

Miscellaneous: Rotary evaporator, Soxhlet apparatus, incubator and water bath.

METHODS:

1. **Preparation of extract:** The collected rhizomes were sun dried for 2 weeks, later grinded using an electric mixer, sieved, packed in glass bottles and stored in refrigerator until further extraction. The powdered extracts were subjected to Soxhlet extraction using ethanol to obtain curcuminoids and essential oils. The ethanolic extracts, thus obtained were concentrated to a thick paste called as an oleoresin, using distillation and rotary evaporation.
2. **Confirmation of curcuminoids in the extract by TLC:** The solvent system used is chloroform: methanol [5]. Proportion of the solvents is changed for different *Curcuma* sps. For optimum separation of the curcuminoids. This difference is due to the composition of volatile components present in them [1].
3. **Estimation of total phenolic content:** Curcumin being a polyphenol can be quantitively estimated using Folin-Ciocalteu reagent. Gallic acid is used as the standard. Aliquots of gallic acid

solutions (2-10ug/ml) were used to prepare the standard curve. Briefly, 1ml of the of (0.05 mg/ml) was mixed with 4 ml of 7.5% sodium carbonate solution. Then, 5 ml of 10-fold diluted Folin-Ciocalteu reagent was added, and the final reaction mixture was incubated for 1 h in the dark. The intensity of the blue-coloured complex was measured at 700 nm.

4. **Estimation of amylase inhibition:** α-amylase hydrolyses starch to give reducing sugars. The property of the extracts to inhibit this activity in-vitro, is checked using DNSA reagent. In this assay, various concentrations of extracts (20-100ug/ml) and amylase solutions are mixed and then the substrate is added. This reaction mixture is incubated and later the DNSA reagent is added. A reference is made using without adding the extracts to check the hydrolysis of starch. The absorbances of the extracts and reference are colorimetrically estimated.
5. **Antioxidant property:** The antioxidant property of the extracts is checked using DPPH. DPPH is a stable free radical which is reduced by the extracts to give a pale yellow color. Ascorbic acid is used as a standard. Aliquots of ascorbic acid solution and extract in DMSO are incubated in the dark along with DPPH. The amount of decolorization depends upon the radical scavenging activity of the extracts. The decolorization is expressed as % inhibition of DPPH. The % inhibition of the extracts are calculated using the formula:

$$\% \ inhibition = \frac{Abs_R - Abs_E}{Abs_R} \times 100$$

Where, Abs_R is absorbance value of reference and Abs_E is the absorbance value of extract.

6. **Antimicrobial susceptibility and synergism testing:** Agar well diffusion method is used for antimicrobial susceptibility and synergism testing. In this method, wells are made on nutrient agar plates using a cork borer. The extract solutions are added in these wells and checked for activity against four bacteria namely *Staphylococcus aureus* - ATCC 6583, *Corynebacterium diphtheriae*, *Escherichia coli*, *Propionibacterium acne* - MTCC -

1951. For testing of synergism among the four species, the extracts were mixed in equal amounts (1:1:1:1)

RESULTS AND CONCLUSIONS:

1. Confirmation of curcumin in the extract by TLC:

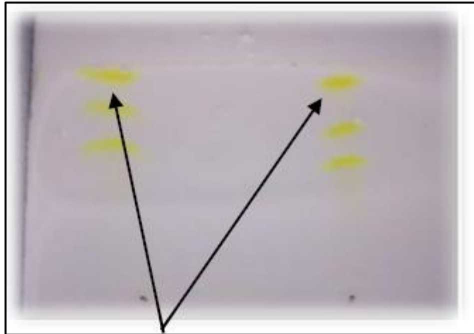

Fig 1: Curcumin bands seen in normal light

All the extracts showed three bands of curcuminoids namely, curcumin, bisdemethoxycurcumin and demethoxy curcumin. Since curcuminoids are UV fluorescent, the bands showed fluorescence under a UV transilluminator at 312nm. The bands obtained were comparable with the standard bands of curcuminoids.

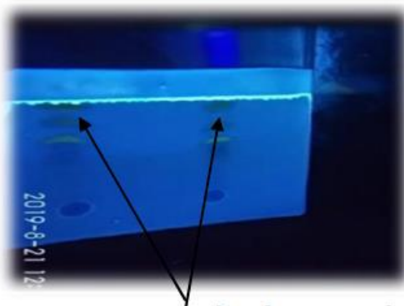

Fig 2: Curcumin bands seen under UV light

2. **Estimation of total phenolic content by Folin Ciocalteu method:** Gallic acid is used as a standard. the phenolic concentrations of the samples are calculated using the gallic acid equivalents obtained from the equation of the standard curve of gallic acid.

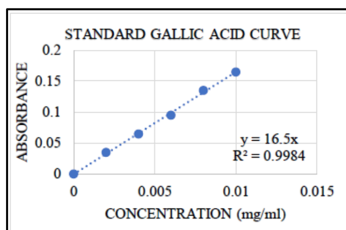

Fig 3: Standard Gallic acid curve

The phenolic concentrations of the extracts were in the range of 0.06 to 0.006mg/L with the highest phenolic concentration observed in *C.aromatica* and lowest in *C.amada*.

3. **Amylase inhibition assay using DNSA reagent:** Amylase breaks down starch to give reducing sugars. This ability of amylase is inhibited by the extracts and hence less amount of reducing sugars are present to react with the DNSA reagent. Thus, an increased inhibition will lead to decreased intensity of the DNSA reagent which can be colorimetrically quantified. The extracts show amylase inhibition upto 92%. Maximum amylase inhibition is seen with *C.amada* and *C.zedoaria*. Since the extracts show amylase inhibition, they can have a role in reducing blood sugar levels.

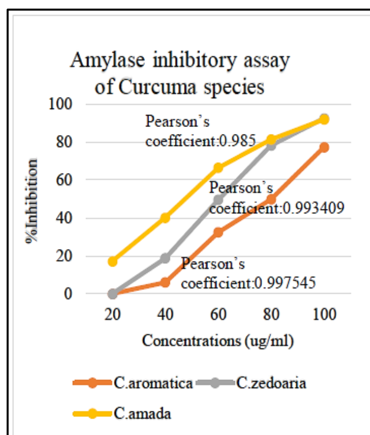

Fig 4: Amylase inhibitory assay

4. **Antioxidant activity using DPPH:** Antioxidants scavenge the free radicals in the body. This property is assessed using DPPH, a stable free radical which forms a purple-coloured solution with ethanol. The antioxidant property is visualised by the level of decolourization of DPPH. From the graph of antioxidant activity vs concentration, the IC50 values obtained for *C.zedoaria*, *C.aromatica*, *C.amada*, *C.longa* are 15.879 ug/ml, 22.071 ug/ml, 12.72 ug/ml, 11.32 ug/ml respectively, with ascorbic acid having an IC50 value of 10.310 ug/ml.

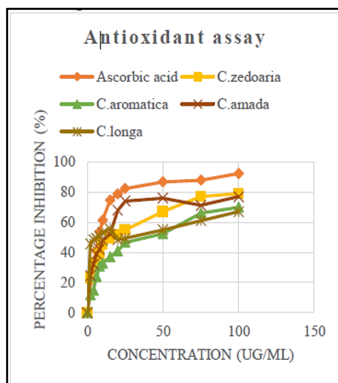

Fig 5: Antioxidant assay

5. **Anti-microbial susceptibility and synergism testing by agar well diffusion method:** The anti-microbial effect of the extracts are seen on the bacteria in the form of zones around the wells. The larger the diameter of these zones, the better is the action of that extract as an anti- microbial agent. The extracts showed inhibitions in the range of 12-24 mm. Maximum inhibition was seen with *C.aromatica* on all four bacteria. The synergistic effect of all four extracts were in the range of 15-20 mm which is comparable to the effect of individual extract.

Samples	Zone of Inhibition (mm)			
	C. diph	*E. coli*	*S. aureus*	*P. acnes*
C. aromatica	24	14	18	24
C. zedoaria	20	12	12	15
C. amada	18	12	13	15
C. longa	19	13	15	14
Mixture	21	15	15	16
Control	12	10	8	11

Table: Antimicrobial susceptibility and synergism testing

FUTURE PROSPECTS:

1. Since no significant increase was seen in the synergistic study of the extracts in equal proportions, different proportions can be studied for an increased effect.
2. Isolation and characterization of curcuminoids using LCMS.

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Chapter 4 – To Manufacture Paper from Crop Residue

Avani Newrekar, Manasi Prabhu, Dr. Tejashree Shanbhag*, Dr. Amruta Kothare*

Department of Life Sciences, Kishinchand Chellaram College, Churchgate, Mumbai-400020.

Email: tejashree.shanbhag@kccollege.edu.in

ABSTRACT:

Sugarcane bagasse, corn husk and pineapple skin are large parts of waste which are generally discarded by local vendors after their use thus adding to the problem of 'waste management'. India has been reported to generate 40 million metric tons of sugarcane bagasse which is directly discarded. The paper obtained by using sugarcane residues have been reported to contain high amounts of fibre, tensile strength and good folding endurance. The current research work focuses on the use of all the above waste materials in the manufacturing of a usable hand-made paper and is a 'first attempt' of making a paper using pineapple skin. The methodology includes treatment of waste, pulp making and molding, with minimal requirement of machineries and use of green chemistry. The paper prepared in-house shows better tensile strength and durability in comparison to the hand-made paper available in the market. Thus, this hand-made paper can hence be a potential substitute for regular paper and can open up new avenues for setting up a small-scale industry.

KEYWORDS: Waste Management, Fibre, Hand-Made Paper, Green Chemistry

INTRODUCTION:

Most of this crop residue is burnt or thrown into landfill to rot, in both ways contributing to air pollution and climate change by releasing greenhouse gases such as carbon dioxide and methane, which is 27 times more dangerous to the ozone than carbon dioxide [13]. Today we are living in an era where we follow the three R's; Reduce, Reuse, Recycle. Agriculture waste can be recycled and reused; Agro-industrial wastes are used for manufacturing of biofuels, animal feed, antibiotics etc. In order to achieve effective sustainable development each industry is contributing on its respective level. Waste of agricrop residue is used in food processing industry, sugar mills, beverage industry, etc. Pulp and Paper industry is flourishing. Paper is a network of cellulosic fibre [12]

and plant-based material has network of cellulosic fibres. Paper is an integral to daily life and is consumed on a large-scale by people across the globe, raising concerns over unsustainable deforestation, thus creating a need to search for alternatives [3].

The components taken in to consideration in paper making are lignocellulosic material, cellulose, hemicellulose and lignin. Sugarcane bagasse is a World's largest crop grown in about 2.38 hectares followed by Corn. In sugarcane bagasse the percentage of following contents is as follows; 45-55% cellulose, 20-25% hemicellulose, 18-24% lignin [14]. Three main parts - Pith-5%, Fibers-73%, Rind-22%. Followed by corn husk this has the following percentage; Lignocellulosic material which is 80-87% cellulose and 18-24% lignin, Fibres- 10-30% [15]. Pineapple skin has Cellulose content-36-38%, Fibre per pineapple is 1.4g. These components contribute to tensile strength and folding endurance. Sugarcane bagasse, corn husk and pineapple skin are obtained all year round and since the vendors discard it after extracting the components of their choice of interest, thus freely available. Rather than disposing of crop residue in ways that have detrimental impacts on the environment, these waste by-products can instead be recycled and utilized in the production of paper, whilst also conveniently helping people like vendors discard their waste. Producing more paper from agricultural waste will yield further benefits, as the need to cut down trees in order to produce paper will be diminished, thus helping to alleviate the environmental concerns over unsustainability in regards to deforestation.

MATERIALS AND METHODS:

Step 1 - Treatment of Bagasse: 120g of corn husk, 80g of sugarcane bagasse & 30g of pineapple skin was collected from local vendors. The sample was cut into small pieces approx. 1inch. A solution of hot water and 40 g washing soda was kept for boiling. Once the water reached 100°C, the bagasse pieces were added to the boiling solution. After boiling, using a skimmer, the mixture was transferred to net bag for water removal.

Step 2 - Making the Pulp: In this step, the pith is separated from the rinds of the bagasse. The bagasse pieces are blended. For the separation of the pith, the mixture from net bag is transferred to the wet grinder or mixer. 600ml of water is added to it. All the three samples were blended separately. In this way, pulp was obtained. The pulp is mixed in order to get a homogeneous mixture. Obtained pulp was divided in two sections.

Section 1 – Steps 1-5 were followed with addition of 15-20 gm of Ararot powder for binding purpose. Section 2 – Steps 1-5 were repeated with addition of 15-20 gm of Cornflour for binding purposes.

Fig 1: Sample pieces soaked in water

Figure 2. Sample pieces cut in to 1 inch length

Fig 3

Fig 4

Fig 5: Pulp obtained after addition of binding

Step 3 - Paper Making: Pouring of the pulp by using a sieve to obtain homogeneity in texture. The pulp (section 1 and 2) obtained was placed on top of the mould and deckle. Using the palm, the pulp was spread as evenly as possible across the mould and deckle. The paper was kept for drying.

Step 4 – Paper Making And Testing: The two different paper sheet obtained from two different sections of pulps was cut in to selected size (approx. 2x15cm). The paper was further tested for its ability to soak ink and durability.

OBSERVATION AND RESULT:

1. **Consistency And Spreading Ability:** The pulp showed good consistency and spreading ability. Since the samples formed a homogenous pulp the paper developed had a good texture.
2. **Ink Sustaining Ability:** The developed paper showed ink sustaining ability thus proving that the developed paper can be beneficial for writing purpose.
3. **Binding Property:** As compared to cornflour, paper made by using Ararot as binding agent showed effective binding.
4. **Tensile Strength:** Developed paper showed minimum tensile strength.
5. **Durability:** Shelf life of the paper was around 4 days. After that paper started degrading as no chemicals or preservatives were used.

Fig 6: Developed paper using Ararot powder as binding agent and showing ink sustaining ability

Fig 7: Developed paper using cornflour

DISCUSSION:

Blending of long fibered pulp with short fibres. The extent of inter fibre bonding is considered the most important factor contributing to tensile properties. Sugarcane fibred pulp is one of the important aspects in this regard because this is expected to have attendant effect on the quality of paper produced from both materials. Papers made with long fibres generally have higher tensile strength properties than paper made of short fibres. The extent of inter fibre bonding is considered the most important factor contributing to tensile properties. Sugarcane bagasse paper is available in market for packaging purposes. Paper made only from corn husk is observed to have ink sustaining ability. So the two contributing properties along with Pineapple skin as novel ingredient can form a good quality paper.

CONCLUSION:

Sugarcane bagasse paper is available in market for packaging purposes. Corn husk, like several other raw materials suitable for paper making, is exclusively short fibered with an average fibre length <2mm [2]. It is well known that several properties of the paper produced from short fibered pulp are of inferior quality. It is the 'First Attempt' of making paper using pineapple skin. Each of the individual components contributes to different qualities in the paper. The paper made is environmentally friendly as no chemicals are used in making of the paper. Also, the paper is a cost-effective way towards sustainable development. The applications of this paper are – Packaging purposes, Gift articles, Substitutes for cardboards, Lampshades and Notepads.

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Chapter 5 – Plastic Degradation using Herbs, Spices and Essential Oils

Himakshi Vartak, Insiya Selia, Dr. Archana Thite*, Dr. Suvarna Sharma*

Department of Life Sciences, Kishinchand Chellaram College, Churchgate, Mumbai-400020.

Email: suvarna.sharma@kccollege.edu.in

ABSTRACT:

Plastics and plastic products have become an important part of our life. We can't imagine our life without them. Plastic being an inexpensive item, has made a huge impact in our day-to-day life, right since the Second World War. Today, it has become an inseparable commodity and has helped with our daily activities to a great extent. However, utilities also have ill effects such as pollution, hazardous health effects, disposing issues, improper waste management and many more. Plastic degradation is the natural and chemical change in properties of plastic. Plastic waste is one of the leading contributors to land, water and air pollution as well as known to have a varied effect on the guts of human and animal life. Over the years there has been tremendous research in the field of plastic degradation using incineration, UV rays and effects of microbial or enzymatic actions and many more.

India is rich in diversity in plants of spices and herbs. In this research we studied the action of herbs, spices and essential oils on Plastic degradation. We used herbs because they are known to be an important and rich source of secondary metabolites in plants. We examined the action on the different samples of plastics like High Density Polyethylene (HDPE), Low Density Polyethylene (LDPE) and Polypropylene (PP). So far, the measurable degrading effects on Plastics are not seen, but we are hopeful for positive results in the long run.

KEYWORDS: Plastic degradation, Secondary metabolites

INTRODUCTION:

Plastics are made up of wide range of semi synthetic compounds. They are polymers, these polymers are made of a basic structure given by macromolecule chains, which are from monomer reactions. They have a high molecular mass and they are malleable. Caused by the macromolecular structure and the temperature-dependent physical properties plastic materials are distinguished into different classes. Taking the chemical structure and the degree of crosslinking between the macromolecules, plastics can be classified as thermoplastics, elastomers and thermosets (Moore, C.J, et al. 2001). Polymer stabilizers are added to the plastics to prevent their degradation processes. Thermoplastics are in the application range of hard or tough elasticity and can be melted by energy input (mechanical, thermal or radiation energy). Elastomers are of soft elasticity and usually cannot be melted. Thermosets are in the application range of hard elasticity and also cannot be melted (Mato Y; Environ. Sci. Technol. 2001).

Due to plastic's resilience against degradation and its proliferative use in industry, the issue of plastic pollution has evolved to become a threat to global ecology. Plastic pollution arises from both terrestrial and marine sources. Plastic pollution in the marine environment is the cause of several hazardous and ecologically damaging effects. Plastic debris poses a direct threat to wildlife, with many and varied species documented as being negatively impacted by plastic items (Hayden K, et al.2013). As we know, widely used plastics do not naturally degrade to a large degree when released into the environment. Plastics contain quite high levels of organic pollutants and toxic chemicals, such as Polychlorinated Biphenyls (PCBs), Nonylphenol (NP). When scientists first discovered plastics, they thought they would be able to use plastics to preserve natural resources or animal killings. But soon, it turned to be a bane, then a bane, and what we fail to realize is the detrimental effects of plastic pollution, not only on environment, but also on humans. Plastic usage is a vicious cycle; we generate more plastic waste, that doesn't decompose, which is consumed by animals and fishes, ultimately consumed by humans.

Over years, scientists and scholars have been trying to degrade plastics and although very difficult, there have been no research on environment friendly degradation of plastics. Even degradation of plastic on large scale, can lead to environmental pollution. Since, India is one of the largest producers of spices and herbs, plastic degradation using spices and herbs can be a better and safer alternative. Spices, herbs and Essential oils, are rich in secondary metabolites like tannins, alkaloids, etc. and these metabolites can help break the polymeric bonds.

MATERIAL AND METHODS:

1. **Preparation of extract from spices:** To make concentrated extracts from the following spices that are red chili, turmeric, black pepper, clove, cinnamon, ginger garlic paste. Freshly packaged spices are taken and they are weighed 6 gm. 100 ml of distilled water is taken and these spices are added to it. The mixture is boiled until it becomes concentrated. The concentrated extracts are allowed to cool and then poured above the plastic sample into the petri plate in between the burners. In case of ginger and garlic the paste was directly used.
2. **Preparation of extract from herbs:** To make concentrated extracts from the selected herbs that are tea powder, Mesua ferrea, licorice powder, Triphala, Sitopaladi and tobacco. Freshly packaged herbs are taken and they are weighed 6gm. 100ml of distilled water is taken and these herbs are added to it and the mixture is boiled until they become concentrated extracts. The concentrated extracts are allowed to cool and they are then poured above the plastic sample in the petri plate in between the burners.
3. **Preparation of extracts from essential oils:** In case oils readymade oils available is used. By using 10ml graduated pipette 5ml oil is taken and then were heated and allowed to cool. Once the oil is cooled it is poured on the plastic sample into the petri plate in between the burners. The petri plates are kept in incubator at 40 degrees Celsius.
4. **Preparation of extracts combining with chemical:** To prepare extracts that are combined with concentrated HCL. Three spices are

taken that are red chili turmeric and cinnamon. Freshly packaged spices are taken and they are weighed 6 gm. 100 ml of distilled water is taken and these spices are added to it they are boiled until they become concentrated extracts. The concentrated extracts are allowed to cool and then they are poured above the plastic sample into the petri plate in between the burners. After pouring of the extracts into the petri plate 2ml concentrated HCL is added to it.

DEGRADATION BY SPICES, HERBS AND OILS

1. Spices (sterile & and non- sterile)

Spices	Duration	Result
Red chili	10 Days	Weight Increased
Turmeric	10 Days	Weight Reduced
Cinnamon	10 Days	Weight Reduced
Black pepper	60 Days	No changes
Cloves	60 Days	No changes
Ginger & Garlic Paste	60 Days	No changes

2. Herbs (sterile & non- sterile)

Herbs	Duration	Result
Tea Powder	90 days	No Changes
Nagkeshar	90 days	No Changes
Jeshthamad	90 days	No Changes
Sitopaladi	90 days	No Changes
Triphala	90 days	No Changes
Tobacco	90 days	No Changes

3. Essential Oil

Essential Oil	Duration	Result
Lemon Grass Oil	30 days	No Changes
Clove Oil	30 days	No Changes

4. Set Of Spices Combining with Chemical

Spices + Chemical	Duration	Result
Red Chili + HCl	30 days	No Changes
Turmeric + HCl	30 days	No Changes
Black Pepper + HCl	30 days	No Changes

Observations based on weight (spices)

As we see, according to the weight of the plastic observed, there is a slight increase of weight in case of red chili. The reason for the increase of weight is unknown and we are looking for the possible explanations, or it can be a human error. On the other hand the weight of the plastic, as observed in turmeric and cinnamon, shows a slight decrease. Some changes might have happened at molecular level, which cannot be observed with the naked eyes. Following table shows the change in the weight:

	Red Chili	Turmeric	Cinnamon
Before	0.0319g	0.0377g	0.0275 g
After 10 Days	0.0325g	0.036 g	0.025 g
Difference	0.0006g	0.0017g	0.0025 g

CONCLUSIONS:

Plastic degradation is any chemical and physical change in polymer as a result of environmental factors, such as light, heat, moisture, chemical conditions or biological activity and sometimes you cannot see the degradation by naked eyes. After the study and findings, this is what we concluded.

Degradation by Spices

We used six different spices to observe the degradation of plastic, in which there was a slight increase in weight in case of red chili. Although we do not know the actual reason behind the increase, there can be a possibility of human error. With respect to turmeric and cinnamon there was a slight decrease in weight. This decrease might be because of some changes happening at the molecular level, which cannot be observed by

naked eyes or even a microscope. We couldn't detect any quality loss/ thinning of the plastic sample or even changes in texture of the surface.

Degradation by Herbs

In this set of degradation using herbs, we used six different herbs to observe the degradation of plastic, but we could not observe any changes as we were focusing only on the visible changes in the plastic. But we do believe that some changes could have taken place which was not observable by the naked eyes. We also wish to further repeat this set, and check the change in weight of the plastic sample, if any.

Degradation by Essential Oils

We used two common essential oils, Lemongrass oil and Clove oil, to observe the degradation but again, we could not observe any visible changes. As mentioned earlier, we would like to repeat and check if the secondary metabolites present in the oil can break the polymeric bonds.

DISCUSSION:

As we know, the positives behind the discovery of plastic quickly turned into negatives. Researches on the reduction of environmental pollution emanating from the use of plastics as packaging materials have been on for many years. The difficulty of biodegradation of polyethylene material has been attributed to its hydrophobicity, high water repellency, high molecular weight and lack of functional groups that are prone to hydrolytic cleavage by the microbial enzymes (Chiellini et al., 2003). Hence, we decided to work with the degradation of plastic by using the spices, herbs and essential oils, as it gives us a safer alternative. India being one of the largest producers of spices and herbs, these extracts can be easily accessible as well. These spices, herbs and essential oils are rich source of secondary metabolites, as they contain strong aromatic compounds that could show activity against plastic polymers and help in breaking the polymeric bonds.

Till the date, no studies have been conducted on usage of herbs, spices and oils for plastic degradation. We made extracts and suspended the plastic samples to check the degradation in plastics, in any form. We also

combined spices with concentrated Hydrochloric acid to check its combined activity on the plastic. In case of, Red Chili, there was slight increase in the weight, while with Turmeric and Cinnamon; there was slight decrease in the weight of the plastic. We are looking for the possible explanations for the increase, we can say that there might be human error, while the decrease in the weight, explains that there were some changes at the molecular level which is not possible to observe with naked eyes or even a microscope. To addition of this study and out of curiosity, we also used bacteria and fungi to check degradation of plastic. We inoculated the bacteria and fungi, on the media, under which plastic was placed. We made 4 nutrient media, with reducing carbon source in each, and finally without any, to observe if the bacteria can penetrate the media and disintegrate the sample, to obtain food. Interestingly, there was a steady decrease in weight of the plates, every day, as noted. Not just change in the weights, but there was also a change in the weight of the plastic samples, in 8 days. Although we had kept the replicates, for observations, but due to sudden Pandemic, COVID 19, we could not continue further observations and conclusions. Plastics poison the environment while degrading, and this is the safest method, when it comes to plastic degradation. We further aim at repeating the procedures, and also at using more plant extracts rich in secondary metabolites, to know, if these metabolites have any potential to break the polymer bonds. Due to limitations in infrastructure, we couldn't find out the disintegration or any textural change on the surface of plastic polymer.

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Chapter 6 – Herbal Juices using Natural Preservatives

Himakshi Vartak, Insiya Selia, Dr. Suvarna Sharma*, Dr. Archana Thite*

Department of Life Sciences, Kishinchand Chellaram College, Churchgate, Mumbai-400020.

Email: suvarna.sharma@kccollege.edu.in

ABSTRACT:

With the changing lifestyle and our inclination towards the fancy foods and beverages from the West, we have somewhere forgotten the traditional food and ways to stay healthy. With the new form of diets and workouts, we have started consuming the packaged as well as instant foods, in turn harming our body indirectly. So here we made an attempt of making herbal juices, using Natural preservatives are safe and effective alternatives for Class I or synthetic preservatives. We studied and compared the brands and tested for shelf life as well as for nutritional value. The two drinks which we prepared are good for digestion, skin and detoxification. The first juice, which is the Digestive juice, is made from Gooseberry, Mint leaves, fennel seeds and Neem. While the ingredients for the second juice, Glow Juice, are pineapple and turmeric. Raw sugar and honey were used instead of white sugar. Natural preservatives are a way to preserve taste, retain color and restore flavor. Citric acid from Lemon and Honey prevents browning, while essential oils from neem extract preserve flavor. Although, the artificial preservatives have longer shelf life and are tastier to consume, natural preservatives, on the other hand, are harmless and have soothing effect on the body. As it is always said, "EAT LOCAL, THINK GLOBAL".

KEY WORDS: Natural Preservatives, Health juices, Herbal

INTRODUCTION:

With the new form of diets and workouts, we have started consuming the packaged juices, which are heavily packed with the class II preservatives and high amounts of white sugar, harming our body directly or indirectly. The consumption has increased in recent times due to their known

beneficial health effects and it is a new trend to nourish ourselves with extra supplements. (Arti Pandey et al, 2018). The juices which we normally buy are packed with class II preservatives.

One such traditionally used preservative for juices is Sodium Benzoate, which links to deficit-hyperactivity disorder in children. (Mohammad Javad Khoshnoud et al, 2017). The combination of natural antimicrobial compounds with other preservation techniques could be a new trend in preservation of juices with improved microbiological safety and acceptable organoleptic properties. Quality loss in fruit juices occurs as a consequence of microbiological, chemical and physical alternations.

Natural and processed foods are fragile and can become unpalatable and/or rotten. The processed food industry uses preservatives to enable distribution, even to different continents, and to extend the useful life of their products. Preservatives impede oxidation, a mandatory step in rotting, either by aerobic or anaerobic mechanisms. (Rafael Franco et all, 2019). Consumers are looking for healthier and artificial preservatives free products today, and many food companies are trying to adapt more specialized and sensitive techniques to meet the needs of the consumers. (Norma F Santos-Sánchez et al, 2017). The sources of natural antioxidants can be extremely varied, because practically all plants contain antioxidants that allow them to protect themselves from solar radiation and pests, as well as to regulate the production of chemical energy. (Santos-SÃ¡nchez et al, 2017). Natural antimicrobials ensure food safety without impairing organoleptic or nutritional properties. Natural preservatives could also constitute a viable alternative to address the critical problem of microbial resistance, and to hamper the negative side effects of some synthetic compounds, while meeting the requirements for food safety, and exerting no negative impact on nutritional and sensory attributes of foodstuffs. (Aurelia Magdalena Pisoschi et al, 2018).

Natural Preservatives like Lemon juice, Vinegar, Honey, rock salt, Turmeric and spices like cinnamon, cumin, clove etc. or even essential oils have the potential to be used as preservatives in many foods namely in processed meat to replace chemical preservatives. The uses of spices

have been known since long time, and the interest in the potential of spices is remarkable due to the chemical compounds contained in spices, such as phenylpropanoids, terpenes, flavonoids, and anthocyanins. (De La Torre Jessica Elizabeth et al, 2017). Ascorbic acid, citric acid, honey prevent browning, while essential oils from neem extract or even a squirt of lemon preserve flavour and prevent browning.

This study focuses on using Natural Preservatives to enhance the taste, to increase the shelf life and to preserve for at least a week using traditional preservation colours were added and only local ingredients were used in order to make the two sets of beverages.

MATERIALS AND METHODS

1. Composition

Sr. No.	Blush Juice	Digestive Juice
1.	Pineapple	Amla (Phyllanthus emblica)
2.	Turmeric	Saunf (Foeniculum vulgare)
3.	Lemon Juice	Mint Leaves
4.	Honey	Neem Extract
5.	Water	Lemon Juice
6.		Black Pepper
7.		Coriander Seeds
8.		Jaggery
9.		Rock Salt
10.		Water

2. Collection

All the raw ingredients were collected from the local market, washed thoroughly with the salt water, cleaned and used them for making the juices.

a. Blush Juice

- Pineapple was sliced and cut into cubes.
- Turmeric powder (available at home) was used.
- Lemon juice was extracted from fresh bought, clean Lemon.

- Potable water was used.

b. Digestive Juice

- Amla was bought, washed and cleaned and wiped it dry.
- Saunf, black pepper, Coriander Seeds, Rock salt and Jaggery was used from the shelves.
- Mint leaves were soaked in salt water and cleaned thrice with running water.
- Neem leaves were wahed and boiled for the extract.
- Lemon Juice was extracted from Lemon.
- Potable water was used for dilutions.

3. Functional Properties

a. Blush Juice

1. Made of pineapple, turmeric, lemon juice and honey.
2. Pineapple boosts immunity, prevents colon cancer, helps fight eyes and skin and helps in digestion.
3. Turmeric boosts Digestion, has anti- inflammatory properties, excellent antiseptic, good for skin, blood purifier and manages diabetes; helps prevent cancer and lower heart risks.
4. Honey is rich in antioxidants, better than sugar, help improve cholesterol and promote healing properties.
5. Lime juice rejuvenates skin, Improves digestion, Fights infections, helps with weight loss, lowers blood sugar and reduces inflammation.

b. Digestive Juice

1. Made of Gooseberry, Mint leaves, Aniseeds, neem, jaggery, coriander seeds and rock salt.
2. Mint helps improve brain function, relieve indigestion, decrease breast pain, improves cold symptoms.
3. Aniseeds (saunf) help in boosting immunity, anti-inflammatory, prevents constipation, lowers the risk of cancer, controls body weight and increases oestrogen levels.

4. Gooseberry (Amla) is rich in calcium, helps in hair growth, manages diabetes and reduces cholesterol levels.
5. Jaggery contains more nutrients than refined sugar because of its molasses content.
6. Table salt is heavily processed to eliminate minerals and hence rock salt was used.

Fig 1: Blush Juice

Fig 2: Digestive Juice

4. Natural Preservatives

a. Lemon or lime juice is the best natural ingredient to preserve any food or beverage with. The lime juice contains ascorbic acid and citric acid which are naturally antibacterial and antioxidants. Hence it helps in retaining the colour or flavour of any dish.

b. Rock salt effectively absorbs the moisture from a food dish and reduces the amount of water in a food dish for bacteria to grow on.

It acts as a natural preservative to keep food items fresh for several months.

c. The presence of highly concentrated sugar in honey does all the work as a preservative. It absorbs the moisture out of any food item and also keeps it fresh for a longer time. Honey is mostly effective for fruits and vegetables.

d. Jaggery is a natural preservative that helps food get rid of water and microorganisms. Sugar follows the same science as salt that is osmosis or dehydration. It soaks up the water that may lead to the growth of bacteria and further keep the food well preserved for a longer period.

e. Antimicrobial activity of turmeric (Curcuma longa) extract has a potential in food industry.

f. Plant derived extracts (PDEs) are a source of biologically active substances having antimicrobial properties. There are studies on, on Neem oils/extracts as preservatives.

METHODOLOGY:

1. The two juices were made, in the most sterile conditions. The platform was sanitized and the glassware sterilized. All the raw ingredients were washed thoroughly
2. and treated with the salt, in order to kill the microorganisms.
3. The burners were kept on to maintain the aseptic working conditions.
4. They were stored in different glass bottles inn cold conditions, and oxidation was prevented by overfilling the glass jars.
5. Each bottle was opened every day, and monitored on the basis of sensory and organoleptic testing.
6. Another set was sent to the certified laboratory for testing.
7. Another set was sent to Equinox laboratory, FSSAI certified laboratory for testing, situated at Jogeshwari, Mumbai.

OBSERVATIONS AND RESULTS:

1. Determination of Shelf Life

By organoleptic and sensory testing, we got to know the shelf life of the Herbal Juices, which was 7 days, when stored in cold conditions or refrigerator.

We used glass bottles to store the juices, opened and checked every day, by the taste, look and smell of the sample at the two different temperatures. At room temperature, the microbial growth was observed after 4 days, with sour smell.

There was no contamination or oxidation as we opened one bottle at a time and oxidation was prevented by overfilling the glass jars.

2. Nutritional Tests

The samples were made fresh, in the equal proportions, and delivered to the Equinox laboratory, in glass bottles, maintaining the low temperature, in the surrounding. The tests for the two samples costed us INR 4720. The test was performed with 100 ml of the sample.

Following are the results of the Nutritional values of the juices, which was tested by a FSSAI certified laboratory.

<u>Test Results for Blush Juice</u>

Sr. No.	Parameters	Units	Results
1.	Energy	Kcal/100	32.32
2.	Carbohydrate	g/100	7.79
3.	Protein	g/100	0.29
4.	Total Fats	g/100	0.0
5.	Sugar	g/100	7.57

From the above values we understand that the overall Energy content per 100 ml is pretty high, which is 32.32 kcal/100 ml. Apart from the energy levels, the sugar levels are comparatively less, that is, 7.57 g/100ml and completely fat free.

<u>Test Results for Digestive Juice</u>

Sr. No.	Parameters	Units	Results
1.	Energy	Kcal/100	57.54
2.	Carbohydrate	g/100	13.6
3.	Protein	g/100	0.48
4.	Total Fats	g/100	0.1
5.	Sugar	g/100	11.86

The results of the second juice also follows the same pattern with high energy content (57.54 Kcal/100ml), and lesser sugar content (11.86 g/100ml). The fat level is negligible which comes to only 0.1 g/100ml. By comparing the nutrition facts of the studied sets of juices with the nearly comparable marketed juices, we found out that the sugar levels are as low as half the amount found in the marketed juices. While sugar levels are high in the commercialised beverages, the energy content is quite low.

CONCLUSION:

After testing and monitoring the juices on daily basis, we found out that the juices have a shelf life of 7 days, when stored in cold conditions.

After 7 days, there was a fungal growth observed and deterioration of smell. From the nutritional tests, it is clear that the sugars in both the juices are quite low, with high energy content and 0 fat levels. Instead of white sugar used by other brands, honey and jaggery were used and salt was replaced by rock salt. Despite being Herbal juices, they tasted good.

Nutrition is how food affects the health of the body. Food is comprised of macronutrients including proteins, carbohydrates and fats that not only offer calories to fuel the body and give energy but play specific roles in maintaining health. So it is important to choose right kind of diet and consume foods and beverages that are high are energy, but low in sugar content.

The juices which we made have no added colours or additives and are synthetic preservatives free, which have no harmful effects on our body. Also, the ingredients used are local and indigenous with the help of the age old foods and preservatives. We tried to combine fruits with the spices. The sugars in our juices are natural sugars, without usage of white sugar. Instead, Honey and Jaggery was used, which are necessary for the body and have medicinal properties. Often, juicing is touted as a way to "cleanse" the body and rid it of toxins. We believe in the many benefits of juicing, and we believe that our bodies are intelligent - not inherently flawed. Each of us have effective and complex systems to rid the body of waste and the plants support our body's natural instincts to heal.

Of course, Grandmas pouch cannot replace a doctor, but at least help in taking precautions. Not only are these juices enriching and nutritious, but also a small step towards sustainable living. Eating local and seasonal food empowers you to face all environmental challenges. . A healthy and sustainable diet would minimise the consumption of energy-dense and highly processed and packaged foods, include less animal-derived foods and more plant-based foods and encourage people not to exceed the recommended daily energy intake. Sustainable diets contribute to food and nutrition security, have low environmental impacts and promote healthy life for present and future generations. There is an urgent need to develop and promote strategies for sustainable diets. As it is correctly said, "EAT LOCAL, THINK GLOBAL!!"

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SECTION 2 – MICROBIOLOGY

Chapter 7 - Study of Exopolysaccharide and Biosurfactant Production by Microorganisms

Shahida Gaziani, Tanisha Chouhan, Dr. Pratibha Shah*, Ms. Amina Dholkawala*

Department of Microbiology, Kishinchand Chellaram College, Churchgate, Mumbai-400020.

Email: Pratibha.shah@kccollege.edu.in

ABSTRACT:

Exopolysaccharides produced by microorganisms are biopolymers having diverse biological functions and industrial applications. Biosurfactants are a class of surfactants mainly produced by microorganisms enabling them to have many potential industrial and environmental applications related to emulsification, foaming, detergency, wetting, dispersion and solubilisation of hydrophobic compounds. The aim of this study is to isolate organisms that can produce both exopolysaccharides and biosurfactant thus having industrial and environmental significance. The isolation of exopolysaccharide and biosurfactant producer was carried out using a soil sample form dumping ground and marine water sample. 26 isolates were obtained after preliminary screening i.e. testing for production of mucoid colonies. Further confirmation was performed with blood haemolysis test and blue halo test on CTAB (cetyltrimethylammonium bromide) Agar. 23.07 % (06/26) isolates were confirmed to be exopolysaccharide producers. Further to check their ability to produce biosurfactants these isolates were tested for growth on Bushnell Hass media supplemented with diesel. All 6 isolates were able to grow on the media indicating their ability to utilize hydrocarbon as their nutrient source and thus their ability to produce biosurfactant. The isolates were identified using cultural, morphological and biochemical tests up to genus level and then were confirmed and identified up to species level using MALDI-TOF technique. The organisms identified were Bacillus

subtilis, Bacillus cereus, Acinetobacter baumannii, Stenotrophomonas maltophilia, Enterobacter hormaechei and Pseudomonas fluorescens.

Future prospects include extraction of the exopolysaccharide and biosurfactant, its stability studies at different temperatures, pH and salinity and to study its commercial applications and role in bioremediation.

KEYWORDS: Extremophiles, Exopolysaccharide, Biosurfactant, Hydrocarbon degradation, Bioremediation.

INTRODUCTION:

Exopolysaccharide: Microorganisms synthesize a wide spectrum of multifunctional polysaccharides including intracellular, structural and extracellular polysaccharides or exopolysaccharides (EPS). EPS generally consist of monosaccharides and some non- carbohydrate substituents (such as acetate, pyruvate, succinate, and phosphate) and secreted into its growth medium. They are mostly composed of polysaccharides and proteins but include other macromolecules such as DNA, lipids and humic substances [1].

Functions of Exopolysaccharides:

- They are the construction material of bacterial settlements and are important in biofilm formation and cells' attachment to surfaces. They constitute 50% to 90% of a biofilm's total organic matter [2].
- Capsular exopolysaccharides can protect pathogenic bacteria against desiccation and predation, and contribute to their pathogenicity [3].
- The physical and chemical characteristics of bacterial cells can be affected by EPS composition, influencing factors such as cellular recognition, aggregation, and adhesion in their natural environments [4].
- Owing to the wide diversity in composition, exopolysaccharides have found diverse applications in various food and pharmaceutical industries.

- Example: The exopolysaccharides of some strains of lactic acid bacteria, e.g., Lactococcus lactis subsp. cremoris, contribute a gelatinous texture to fermented milk products (e.g., Viili), and these polysaccharides are also digestible [5].

Biosurfactant:

Biosurfactants are amphiphilic compounds produced on living surfaces, mostly on microbial cell surfaces or excreted extracellular hydrophobic and hydrophilic moieties that confer the ability to accumulate between fluid phases, thus reducing surface and interfacial tension at the surface and interface respectively. They possess the characteristic property of reducing the surface and interfacial tension using the same mechanisms as chemicals surfactants.

Biosurfactants are mainly classified according to their chemical structure and their microbial origin. The main classes of biosurfactants are glycolipids, phospholipids, polymeric biosurfactants and lipopeptides (surfactin) [6].

Functions of Biosurfactants:

Biosurfactants can potentially replace virtually any synthetic surfactant and, moreover, introduce some unique physicochemical properties. Currently, the main application is for enhancement of oil recovery and hydrocarbon bioremediation due to their biodegradability and low critical micelle concentration (CMC). The use of biosurfactants has also been proposed for various industrial applications, such as in food additives, cosmetics, detergent formulations and in combinations with enzymes for wastewater treatment [7].

Significance of EPS and biosurfactants over their chemical counterparts:

Low toxicity, high biodegradability, good environmental compatibility, high foaming capacity, high selectivity and stability in extreme environments. The present study aims to isolate and identify the organisms that can produce both Exopolysaccharide and Biosurfactant so that there can be a reduction in the total dependence on the synthetic surfactant industry as Biosurfactant and exopolysaccharide

producing microbes can be the best tool for increasing the efficiency of remediation of heavy metal contaminated soil and have many applications in pharmaceutical, cosmetics, food industries and bioremediation. Some specific objectives of this study include:

1. Isolation and screening of organisms producing exopolysaccharide (EPS) and biosurfactant.
2. Identification of EPS and biosurfactant producers.
3. Extraction of the EPS and biosurfactant.
4. To Study the applications of EPS and biosurfactant.

MATERIALS AND METHODS:

1. Sample Collection:

a. Soil sample (from a dumping ground near Malad)

The soil sample was collected using a shovel from 15cm. beneath the ground. The sample was then transferred to the laboratory in sterile polyethene containers.

b. Marine water sample (from Marine Drive)

It was collected using a polyethene bottle with some stone in it and a long rope tied to it. The bottle was thrown to a distance and the water was drawn.

2. Enrichment of the Sample:

10gms of the soil sample was inoculated in each, 100ml of St. Nutrient broth and 100ml of St. Thioglycollate broth. Both the flasks were incubated at R.T. for 7days. 10 ml of marine water was inoculated in each, 100ml of St. Nutrient broth and 100ml of Halophilic broth. Both the flasks were incubated at R.T. for 7days.

3. Screening of EPS Producing Organisms:

Preliminary screening: The enriched sample from each flask were isolated on St. Nutrient agar plates and incubated at R.T. for 24-48 hours to check for the production of mucoid colonies [8].

Mucoid colonies are an initial visible indicator for the production of EPS due to their ropy and slimy nature.

Confirmatory screening:

a. Hemolytic activity (HA):

The isolates were inoculated on blood agar medium (5% of fresh human blood) and and incubated at 37°C for 24-48 hours. The hemolytic activity was assessed for conformation of EPS activity and results were recorded [9].

b. Blue agar plate method:

Bushnell- Hass agar medium (BHA) supplemented with glucose as a carbon source (2%) and cetyltrimethylammonium bromide (CTAB-0.5 mg/ml) and methylene blue (0.2 mg/ml) were used for determination of EPS production. The isolates positive for preliminary screening were isolated and incubated at 37°C for 48-72 hours. Blue colonies were observed and the results were recorded [9].

4. Detection of Biosurfactant Producer:

Degradation of hydrocarbons:

The isolates positive for both preliminary and confirmatory screening tests were checked for their ability to degrade diesel. Loopful of the culture was plated on Bushnell and Hass agar medium containing 2% (V/V) diesel oil as a carbon source and incubated at 30℃ for 7 days. For control, the isolates were plated on Bushnell and Hass agar medium without diesel [10].

5. Identification:

The isolates confirmed to produce EPS and biosurfactant were identified using morphological, cultural and biochemical tests up to genus level. Gram staining of the confirmed isolates was performed and based on the morphology obtained biochemical tests in accordance with the Bergey’s manual were performed. For further confirmation and identification up to species level, the isolates were outsourced and identified using MALDI-TOF technique.

6. Production and Extraction of the Crude Biosurfactant:

Method 1: [9]

a. Production of biosurfactant

The inoculum was prepared using Luria for overnight with 100 rpm agitation. The fresh overnight culture was used as an inoculum for the production of biosurfactant. To the 20 ml of production medium with 2% diesel oil, 1% inoculum was transferred aseptically and incubated at 37°C for 48 hrs at 120 rpm in shaking incubator.

b. Extraction of biosurfactant:

The culture supernatant of the bacterial isolates was obtained by centrifugation at 10000 rpm for 15 minutes and then filtered through a Millipore membrane system. The biosurfactant should be recovered from the cell-free culture supernatant by cold acetone precipitation in which three volumes of chilled acetone was added and allowed to stand for 10 h at 4°C. The precipitate should be collected by centrifugation and evaporated to dryness to remove residual acetone and should be re-dissolved in sterile water.

Method 2: [11]

a. Production of biosurfactant:

A minimal salt (MS) medium containing (g/l): KH_2PO_4, 1.4; Na_2HPO_4, 2.2; $(NH_4)_2SO_4$, 3; $MgSO_4.7H_2O$, 0.6; NaCl, 0.05; yeast extract, 1; $FeSO_4.7H_2O$, 0.01 and $CaCl_2.7H_2O$, 0.02; was used. The basal minimal medium was supplemented with 2 ml of trace element solution and glucose 2% (v/v) was used as the sole carbon source. The composition of trace element solution involved (g/l): $ZnSO_4$, 0.29; $CaCl_2$, 0.24; $CuSO_4$, 0.25 and $MnSO_4$, 0.17. The trace element solution was added after the production media were autoclaved, prior to inoculation by filtering it through membrane filters, sterilized by filtration. After incubating the cultures at 30°C for 7 days they were centrifuged and the culture filtrates were used for extraction.

b. Extraction of biosurfactant:

Extraction was carried out by adjusting the pH of the broth cell-free culture to 2.0 using 6 N HCl and keeping it at 4°C overnight. Pellet thus precipitated should be collected by centrifugation (8000 rpm for 15 min at 20°C) and dissolved in distilled water. Then the pH should be adjusted to 8.0 with 1 N NaOH, and the extract should be lyophilized.

RESULTS:

1. Screening of EPS producers:

26 isolates gave highly mucoid colonies which were considered positive for preliminary screening and subjected to confirmatory screening, out of which 17 isolates showed hemolytic activity and 13 isolates gave blue colonies indicative of positive blue agar plate test.

Screening	Isolates Obtained
Preliminary	26
Hemolytic	17
Blue Agar	13

Table 1: Results for the screening of EPS producers

Isolate	Gram Nature and Biochemical Test	Suspected Genus
1	Gram positive bacilli in chains and singles, citrate positive with central spores	*Bacillus sp.*
2	Gram positive rods in chains, citrate positive	*Bacillus sp.*
3	Gram negative coccobacilli in chains	*Acinetobacter or Moroxella*
4	Gram negative coccobacilli, catalase positive, oxidase negative	*Acinetobacter*
5	Gram negative short rods, catalase positive	*Enterobacter* or *Pseudomonas sp.*
6	Gram negative coccobacilli in chains, oxidase positive	*Pseudomonas sp.*

Table 2: Preliminary Identification Results

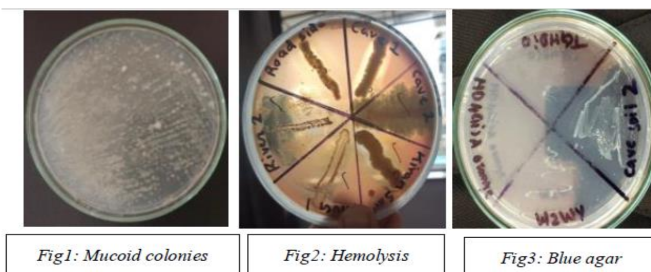

Fig1: Mucoid colonies *Fig2: Hemolysis* *Fig3: Blue agar*

2. Detection of biosurfactant producer:

<u>Degradation of hydrocarbons:</u>

Isolates positive for both hemolytic activity and blue agar plate method (6/26 isolates) i.e. 23.07% were checked for their ability to degrade hydrocarbons.

The control showed no growth and all the 6 isolates were able to grow on the media containing diesel indicating their ability to utilize diesel as their carbon source and thus to produce biosurfactant.

Fig 4: Hydrocarbon Degradation

3. Identification:

Isolates confirmed for production of EPS and biosurfactant were identified using morphological, cultural and biochemical tests up to genus level.

Their genus was confirmed and the organisms were identified up to species level using MALDI-TOF technique. The results were as follows:

1. *Bacillus subtilis/ amyloliquefaciens/ vallismortis*
2. *Bacillus cereus*
3. *Acinetobacter baumannii*
4. *Stenotrophomonas maltophilia*
5. *Enterobacter hormaechei*
6. *Pseudomonas fluorescens*

4. Production and extraction of biosurfactant:

Both the processes were unsuccessful as the biosurfactant could not be precipitated. It could either be due to an insufficient amount of biosurfactant production or human errors during the procedure. In order to get sufficient amount of biosurfactant the method should be repeated with larger quantities of production media.

DISCUSSION:

The study conducted by (Satpute S, et.al. 2010) states that marine biosphere offers wealthy flora and fauna which represents a vast natural resource of imperative functional commercial grade products among which are biosurfactants and EPS. Also (Liang T, et.al. 2013) stated that heavy metal and oil contaminated soil are good reservoirs of EPS and biosurfactant producers. For the present investigation soil from dumping ground and marine water sample was used [12][13].

Biosurfactant and exopolysaccharide producing microbe can be the best tool for increasing the efficiency of remediation of heavy metal contaminated soil and have many applications in pharmaceutical, cosmetics, food industries and bioremediation. In the current study Bacillus, Acinetobacter and Pseudomonas sps. were found to be producers of both which is similar to the studies performed by (Liang T, et.al. 2013) and thus the discovery of a potent exopolysaccharide and biosurfactant producing microorganism would enhance the use of environmental biodegradable surface active molecule and hopefully reduce total dependence or number of new application- oriented towards the chemical synthetic surfactant industry [13].

Also, the organisms were able to degrade 2% diesel which was similar to work done by (P. U. M, et.al. 2014) and to further find the best degrader the rate of degradation of all isolates needs to be compared [9].

CONCLUSION:

From the soil and water sample, 6 isolates were positive for preliminary and confirmatory screening and were able to degrade hydrocarbon and thus were confirmed to produce EPS and biosurfactant. They were identified up to species level and the results were as follows: *Bacillus subtilis*, *Bacillus cereus*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *Enterobacter hormaechei* and *Pseudomonas fluorescens*. Production and extraction of biosurfactant were carried out but was unsuccessful either due to use of less amount of production media or some other human errors. The study further aims to extract the exopolysaccharide and biosurfactant, carry out its stability studies at different temperatures, pH and salinity and to study its commercial applications and role in bioremediation.

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Chapter 8 - Extraction of Bacteriocin from Lactic Acid Bacteria and Checking Its Activity against Gastro-Intestinal Organisms

Netra Jain, Dr. Sejal Rathod*, Ms. Rajitha Satish*
Department of Microbiology, Kishinchand Chellaram College,
Churchgate, Mumbai-400020.
Email: sejal.rathod@kccollege.edu.in

ABSTRACT:

Bacteriocins are protein or protein complexes that are produced by bacteria and have antimicrobial activity against closely related species and are active against Gram positive and Gram-negative bacteria including food spoilage bacteria and pathogens. Bacteriocins from lactic acid bacteria (LAB), generally recognized as safe. Bacteriocins have a fast-acting mechanism, which forms pores in the target membrane of bacteria, even at extremely low concentrations. The aim of this research is to extract the bacteriocin from different lactic acid bacteria and checking its anti-bacterial activity on organisms like Escherichia coli, Shigella, Salmonella typhi, Staphylococcus aureus (food poisoning agent) and Pseudomonas aeruginosa. Lactic acid bacteria were isolated from various food products like paneer, idli batter, curd and dosa batter on MRS agar. Random colonies that were selected were inoculated in to the MRS broth and 5-6 days after incubation crude bacteriocin was obtained by centrifuging the broth. The activity of this crude bacteriocin was checked and later for confirmation pure bacteriocin was extracted using the ammonium sulfate method.

KEYWORDS: Bacteriocin, antibacterial, gastro-intestinal, lactic acid bacteria

INTRODUCTION:

Bacteriocins

Bacteriocins are synthesized by lactic acid bacteria (LAB) to inhibit the growth of similar or closely related bacterial strains. They are usually proteinaceous or peptidic toxins. Bacteriocins can be categorized in

several ways, including producing strains, common resistance mechanisms and mechanism of killing. Bacteriocins from LAB are generally safe [2]. Bacteriocins produced by LAB are important in preventing the growth of pathogenic and spoilage bacteria [4].

Lactic Acid Bacteria

Lactic acid bacteria are Gram positive, low GC, acid tolerant, generally non sporulating, anaerobic, either rod shaped or spherical bacteria. They are usually found in milk products, produce lactic acid as the metabolic end product and hence this trait has linked LAB with food fermentations.

Significance of Bacteriocin [1]

1. Bacteriocins have a fast-acting mechanism and works at very low concentration by making pores in the target membrane of the bacteria.
2. They can be degraded easily by proteolytic enzymes and hence do not stay in the body for long.
3. Bacteriocins are primary metabolites and are safe.

MATERIAL AND METHODS:

1. **Sample Collection:** Variety of milk samples and fermented food products were collected. They included paneer, milk, idli batter, dosa batter, curd and cheese.
2. **Isolation and Determination of Lactic Acid Bacteria from Food Samples:** The samples collected were isolated on MRS (De Man Rogosa and Sharpe agar) and incubated for 48-72 h under microaerophilic conditions to allow the colonies to develop. Random colonies were picked and tests were carried out to confirm the presence of lactic acid bacteria. The colony morphology, catalase test, gram staining and Homo-Hetero differential determination was carried out of all the selected colonies.
3. **Extraction of Crude Bacteriocin:** The selected colonies of lactic acid bacteria were inoculated in 100ml of MRS broth, and was kept

under micro-aerophilic conditions for 5-6 days. Since bacteriocin is produced extracellularly, the broth was then centrifuged at 5000 rpm for 20 minutes. The supernatant was collected and filtered through a 0.22 micron filter to assure removal of all living cells. This was then used as crude bacteriocin.

4. **Testing of Crude Bacteriocin on Test Organisms:** The activity of crude bacteriocin obtained was then tested against the gastro-intestinal organisms like Escherichia coli, Salmonella typhi, Shigella, food poisoning agent Staphylococcus aureus and Pseudomonas aeruginosa by two different methods.

   Method 1

   Agar Cup Method - The Mueller Hinton agar plates were swabbed with the test organisms and 50 micro litre of the crude bacteriocin was put in to the well made in the agar. The plated were incubated at 37°C for 24 h.

   Method 2

   Disc Diffusion Method - The test cultures were swabbed on the Mueller Hinton agar. A disc submerged in the crude bacteriocin was then placed on the agar and incubated for 37°C for 24h.

   A control was set up where sterile MRS broth was added to the wells.

5. **Extraction of Pure Bacteriocin:** After the testing of crude bacteriocin, further purification of the bacteriocin was carried out using the ammonium sulphate method [1]. In this method, the crude bacteriocin was taken and the pH was adjusted to 7 using 0.1N NaOH. After adjusting the pH precipitation with ammonium sulphate was carried out. The precipitate obtained was stored in 0.2M Sodium Phosphate buffer (pH 7).

RESULTS:

1. Gram Staining

After isolation of samples on MRS plate, the randomly selected colonies were subjected to catalase test. Colonies which were found to be catalase negative were further differentiated by Gram Staining method. Given below are the results that were obtained:

Sample	Colony	Gram Staining and Morphology
Paneer	1	Gram Positive cocci
	2	Gram Positive short rods
	3	Gram Positive cocci
Idli Batter	1	Gram Positive cocci
	2	Gram Positive short rods
Dosa Batter	1	Gram Positive short rods

Table 1: Gram staining results of isolated colonies

2. Homo- Hetero Differentiation

All the catalase negative colonies were further isolated on HHD agar. All the colonies showed white colonies on the HHD agar. This concludes that all the selected colonies of LAB were hetero fermentative.

3. Anti-Bacterial Activity of Crude Bacteriocin

Out of the 2 colonies whose activity was checked against the test organisms, bacteriocin produced from colony 1 from the paneer sample could inhibit all the test organisms. A zone of clearance was seen for all the test organisms. The crude extract from colony 2 that was tested showed no inhibition and hence we can say that isolate 2 from the paneer sample did not produce any bacteriocin. No inhibition was observed in the control that was carried out.

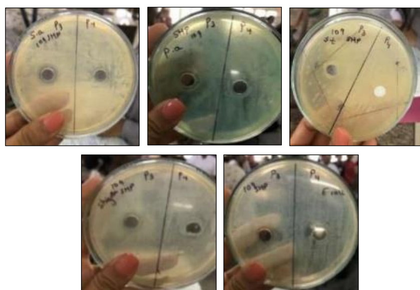

Fig 1: Results of Agar Cup method of crude bacteriocin

DISCUSSION:

In this study, lactic acid bacteria producing strain were isolated from various fermented food products. The bacteriocin produced from these strains was partially purified. The study showed that the incubation period of this LAB in the broth should not be more than 5-6 days since bacteriocins are primary metabolites and can be used or broken down for helping the bacteria to survive. Various factors affect the production of the bacteriocin such as the incubation time, culture medium and incubation conditions. The bacteriocin produced was able to inhibit all both Gram positive as well as Gram negative organisms. Study on the activity of bacteriocins were carried out in-vitro since certain factors in the food may reduce the activity of the bacteriocin. Hence laboratory tests give a better information on the bacteriocin activity.

CONCLUSION:

Out of all the selected lactic acid bacteria, the isolate 1 obtained from the paneer sample showed production of bacteriocin that could inhibit Gram positive as well as Gram negative organisms whereas the isolate 2 did not produce any compound that could inhibit the growth of the test organisms.

The extraction of the crude bacteriocin was carried out successfully. Further we can work with all the isolated colonies from all the food samples and identify the isolates upto genus and species level. Also the

aim is to check the activity of the bacteriocins at various factors like pH, temperature and at various concentrations.

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Chapter 9 - Isolation of Plastic Degrading Microorganism from Soil and to check its Activity

Jay Chaurasia, Mohit Kalani, Chaitanya Kadam,
Dr. Pratibha Shah*, Ms. Amina Dholkawala*
Department of Microbiology, Kishinchand Chellaram College,
Churchgate, Mumbai-400020.
Email: Shahpratij@gmail.com, amina.dholkawala@kccollege.edu.in

ABSTRACT:

Plastic waste is the major concern in today's world so the degradation and proper disposal is very essential. Microbial degradation is one of the ways to reduce the amount of plastic found in waste. In this study the soil samples were collected from three different location and enriched in mineral salt medium (MSM) containing polyethylene glycol (PEG) solution for the plastic degraders. After enrichment the isolation was carried out on MSM containing PEG solution of molecular weight 600 using different concentrations. On repeated subculturing well differentiated isolates were obtained on nutrient agar plate. The isolated organism was then gram stained and the activity is checked on 30 micron and 50-micron plastic strips in a liquid medium on shaker for 30 days. On gram staining three isolates were found to be gram positive and one was found to be gram negative. After 30 days of incubation the highest percentage degradation was found to be 33.33% for 30 micron plastic and 6.25% for 50 micron plastic by the microorganism isolated from dumping area and the lowest percentage degradation was found to be 6.66% for 30 micron and 2.5% for 50 micron by the organism isolated from playground area. According to statistical analysis of paired t test it was found that the difference in weight loss was significant since the $p < 0.05$ (0.0318 and 0.0123) for 30 micron and 50 micron plastic respectively. The results of anova for single factor states that there was no significant difference in the degradation rate between the four isolates since $p > 0.05$(0.252 for 30 micron and 0.250 for 50 micron).

KEYWORDS: Biodegradation, micron, plastic, micro-organism, soil.

INTRODUCTION:

Since last few decades the uncontrolled use of plastics for various purposes such as packaging, transportation, industry and agriculture in rural as well as urban areas has elevated serious issue of plastic waste, its disposal and pollution [1]. The efficient decomposition of plastic takes about 1000 years. Recalcitrant nature of plastic is due to its high molecular weight, complex three-dimensional structure, and hydrophobic nature, all of them hampers its availability to microorganisms [2]. Plastic is a synthetic polymeric material with a high molecular weight, made from a wide range of organic compounds such as ethylene, vinyl chloride, vinyl acetate, vinyl alcohol and so on. By origin, polymers are divided into: natural polymers (biopolymers), synthetic polymers (obtained by chemical synthesis), and modified polymers (natural or synthetic polymers whose structure was chemically or physically changed) [3]. Any physical or chemical change in polymer as a result of environmental factors such as light, heat, moisture, chemical conditions and biological activity is termed as degradation of plastic. [1][4]. The plastic that is being delivered into the oceans is toxic to marine life, and humans. The toxic components of plastic include diethylhexyl phthalate, bisphenol which is a carcinogen, as well as lead, cadmium, and mercury. Entanglement in plastic debris has been responsible for the deaths of many marine organisms. In human it causes serious illness of eyes, skin due to release of harmful chemical [5].

Microorganisms utilize polythene film as a sole source of carbon resulting in partial degradation of plastics. They colonize on the surface of the polyethylene films forming a biofilm. So the degradation depends upon the rate at which biofilm formation take place. Once the organisms get attached to the surface, starts growing by using the polymer as the carbon source [6]. In the primary degradation, the main chain cleaves leading to the formation of low- molecular weight fragments (oligomers), dimers or monomers. The degradation is due to the extra cellular enzyme secreted by the organism. These low molecular weight compounds are further utilized by the microbes as carbon and energy sources. The resultant breakdown fragments must be completely used by

the microorganisms, otherwise there is the potential for environmental and health consequences [3][7].

In 2019 a new report "Plastic and Climate" was published. According to the report, in 2019, production and incineration of plastic will contribute greenhouse gases in the equivalent of 850 million tons of carbon dioxide (CO_2) to the atmosphere [8]. The present study was undertaken because there was an issue regarding plastic ban due to increase in the plastic waste. The current study aims at (a) Isolation of plastic degrading bacteria from soil. (b) To calculate the percentage weight loss of 30 and 50 micron plastic in a liquid media after 30 days of incubation. (c) To compare the degradation efficiency of the isolates.

MATERIALS AND METHODS:

1. **Collection of soil samples**

10 grams of samples was collected from three different location such as dumping area (Malad), playground area (Churchgate) and college premises (K.C.College) from 15cm below the soil surface and stored in a container.

2. **Enrichment of soil samples**

10 grams of soil sample was added to 100ml Mineral salt medium (MSM) broth containing 2% of polyethylene glycol solution (MW 600) [9] as a sole source of carbon and kept for incubation period of two weeks at room temperature.

3. **Isolation of enriched soil samples**

A loop full of each enriched soil samples were then streaked on MSM having different concentrations of PEG (0.5%, 1%, 1.5%, 2%) [10] and kept for incubation at room temperature for 1 week. From the mixture of colonies appeared after 1 week of incubation four different colonies were selected and repeated subculturing was carried out and well differentiated isolates were obtained on nutrient agar plates.

4. Identification of the isolates by the gram staining

The isolates were then gram stained and the gram character, morphology and arrangements were studied. Rapid test such as catalase and oxidase were performed. For identification of the isolates till species level, various biochemical tests can be done according to Bergey's manual.

5. Checking plastic degrading efficiency

Plastic strips of 30 and 50 micron were cut into 3cm*3cm, washed, shade dried and the initial weight was measured. The plastic strips were then aseptically transferred into 50 ml of MSM broth and 1ml of saline suspension of each isolates is added to different flasks. A control was maintained without the culture in which only media and plastic strips were present. All the flask were kept on shaker for 30 days [11][12].

6. Calculating percentage degradation

After every 10 days both the strips were removed, washed, shade dried and the weight is measured. On 30th day the final weight was measured and the percentage weight loss of the plastic was calculated by the formula:

*Percentage weight loss = initial weight - final weight * 100 initial weight*

7. Statistical analysis

For statistical analysis, student paired t-test was performed to check the difference in the weight loss was significant or not. The Anova for single factor was also studied to check the degradation rates between the isolates was significant or not. Both the test were performed in MS excel 365.

RESULTS:

1. After repeated subculturing on MSM media and finally on nutrient agar plates, well isolated colonies were obtained on nutrient agar

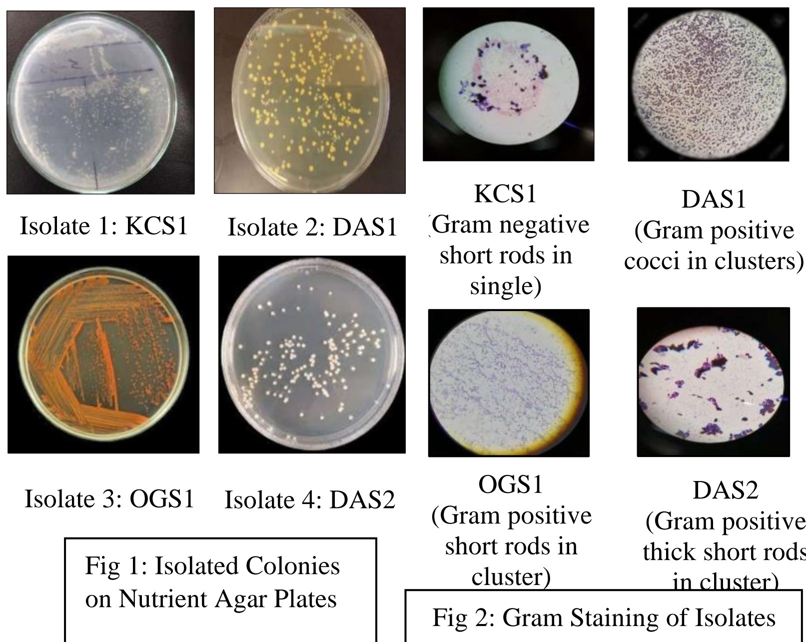


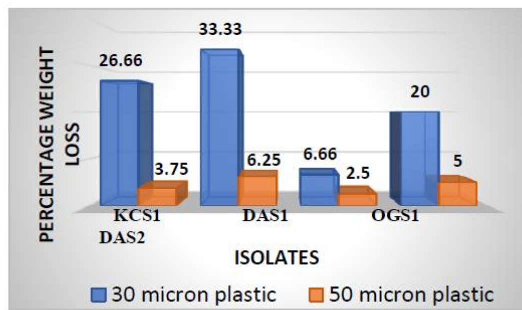
Fig 1: Isolated Colonies on Nutrient Agar Plates

Fig 2: Gram Staining of Isolates

plates (see Fig 1). The colony characteristics was studied for each isolate.

2. Gram staining of the isolates: Three isolates were found to be gram positive while one was found to be gram negative. The morphology and arrangements of each isolate were observed. (see Fig 2).

Comparative Study



	Initial Weight (g)	Final Weight (g)	Percentage Weight Loss
Control	0.015	0.015	-
KCS1	0.015	0.011	26.66%
DAS1	0.015	0.010	33.33%
OGS1	0.015	0.014	6.66%
DAS2	0.015	0.012	20.0%

Table 1: Percentage Weight Loss for 30 Micron

3. Calculating percentage degradation of plastic strips by each isolate. On 30th day the final weight was measured and the percentage weight loss was calculated (See Table 1 and 2). On comparing the degradation rate between the two types of plastic by the isolates, it was observed that the percentage weight loss is higher than 50-micron strips.

	Initial Weight (g)	Final Weight (g)	Percentage Weight Loss
Control	0.008	0.008	-
KCS1	0.008	0.0077	3.75%
DAS1	0.008	0.0075	6.25%
OGS1	0.008	0.0078	2.5%
DAS2	0.008	0.0076	5.0%

Table 1: Percentage Weight Loss for 50 Micron

4. Statistical analysis: The p value for the student paired t test was found to be less than 0.05 (0.0318 and 0.0123) for 30 micron and 50-micron plastic degradation, so it can be concluded statistically that the difference in the weight loss before and after inoculation was significant. For the Anova single factor, the p value was found to be more than 0.05 (0.252 and 0.250) for 30 micron and 50 micron plastic, so it can be stated that the degradation rate between the isolates was not significant.

DISCUSSION:

In the present study, plastic degrading bacteria were isolated from soil and the activity was checked on 30- and 50-micron plastic strips. In this study strips of plastics were inoculated in the liquid medium containing bacterial isolates and kept for 1 month on shaker at room temperature to observe the percentage weight loss of plastic strips by bacteria. The result shows the degradative ability of the microorganisms after one month of incubation. The percentage weight loss due to degradation was found more by DAS1. This shows it has the greater potential of degradation compared to other bacteria. In the current study the isolates were able to degrade at slower rate in comparison to the percentage degradation [11] which was 46.35% for 30-micron strips and 18.76% for 50-micron strips by Staphylococcus species. In the research [12] the highest percentage degradation by Bacillus species was found to be 23.33% and 30.0% for 10- and 40-micron plastic strips after 30 days of incubation in liquid media at laboratory condition. In a similar study by Asieh Nourollahi, it was found that the Pseudomonas species were able to degrade polyethylene strips up to 10.32% after 60 days of incubation in the liquid medium at room temperature. In the following study [18], the weight loss of 50-micron plastic strips by Aspergillus japonicus and Aspergillus niger was found to be 12% and 8% respectively after one month of incubation on shaker at room temperature.

CONCLUSION:

Four isolates were obtained. On gram staining three were found to be gram positive while one was found to be gram negative and their morphology and arrangement were observed. The DAS1 was much more efficient in degrading 30- and 50-micron strips. The percentage degradation was found to be 6.25 % and 33.33% for 50- and 30-micron strips. The DAS2 was efficiently able to degrade 50-micron strip and percentage found to be was 5.0%. The KCS1 was also efficiently able to degrade 30 micron strip and percentage found to be was 26.66%.The OGS1 was least effective in degrading both types of plastic strips.

These aspects need further analysis on different types of plastic and polyethylene glycol of different molecular weight, checking the degradation rate of the plastic by bacteria when used in consortium and also to identify the isolates till species level.

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Chapter 10 - Factors Affecting Biofilm Formation in Bacteria

Janice Elangikal, Dr. Pratibha Shah*, Mrs. Rajitha Satish*
Department of Microbiology, Kishinchand Chellaram College,
Churchgate, Mumbai-400020.
Email: janiceelangikal@gmail.com

ABSTRACT:

With a rise in antibiotic resistance since the past decade, the study of biofilm has emerged as a field of importance. Bacteria embedded in biofilm are more resistance to anti-bacterial agents in comparison to planktonic bacteria. In this project, various factors affecting biofilm formation in bacteria was studied. Research was carried out using four motile bacterial species: *Bacillus subtilis*, *Escherichia coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa*. The effect of temperature on biofilm formation in bacteria was studied using the Liquid interface coverslip assay method at temperatures of 4°C, 27°C and 37°C. It was observed that lower the temperature, greater was its anti-biofilm effect. The effect of Citrus sinensis peel extract (obtained by Soxhlet extraction) and juice extract (obtained by methanolic extraction) both showed greater anti-biofilm activity when subjected to higher concentrations of extract. Through this study we focus on gaining a better insight into the various factors influencing biofilm growth.

KEYWORDS: Biofilm, temperature, Citrus sinensis. inhibition, natural factors, resistance.

INTRODUCTION:

A biofilm comprises of a collection of microorganisms, containing cells of same or different species in which cells attach to each other and also to a surface. These cells become encased within a slimy extracellular matrix that is composed of Extracellular Polymeric Substances [EPS][1][2].

The cells of the biofilm generate EPS substances, which are a conglomeration of extracellular polysaccharides, proteins, lipids and DNA [1][2][3].

Microbes form a biofilm in response to a variety of cues, which may include cellular recognition of adhesion sites on a surface. Nutritional factors, or in some instances, by exposure of planktonic cells to antibiotics below concentrations capable of inhibiting their growth [5][6]. Biofilm formation help cells and colonies of cells to grow increasingly resistant to antibiotics, enabling them to grow on surfaces of medical devices, equipment in sterile operation theatres, skin grafts, body implants and cause extensive damage.

Studying the various factors will help to study whether they enhance or inhibit biofilm. Cells respond to several environmental cues, thus altering the way they express their genes. Biofilm formation is greatly shaped by the conditions they are exposed to. Factors like temperature, humidity, pH are found to have an effect on formation of EPS, thereby altering the adherence capacity of the bacterial cells [7]. Several studies have been undertaken to develop natural products as potential anti-biofilm agents [8]. The anti-biofilm properties of an extract depend on the following factors:

1. Inhibition of adhesion and attachment.
2. Reduction in production of polymer matrix.
3. Inhibition of quorum sensing and cell to cell communication [9].

In this study four motile bacterial species were selected: *Bacillus subtilis*, *Escherichia coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa*. The anti-biofilm effect of temperature and anti-biofilm activity of natural product (*Citrus sinensis*) was studied.

MATERIALS AND METHODS:

1. **Hanging drop (Robert Koch, 1989):** In this method, a loopful of culture suspension (0.1 OD at 540 nm) of test organisms was placed on a cavity slide and studied under the microscope.

2. **Stab inoculation in Nutrient agar butt containing Triphenyl Tetrazolium Chloride Dye (TTC):** A loopful of test organisms was taken (0.1 OD at 540 nm), and stab inoculated on Nutrient agar slants having 0.05g of 1% solution of Triphenyl Tetrazolium Chloride. The slants were then incubated at 37°C for 24 hours. Pattern of growth was observed.

3. **Detection of biofilm by tube adherence method (Freeman, et al., 1989):** A loopful of test organisms (0.1 OD at 540 nm) was inoculated in 10ml of Nutrient broth with 1% glucose in test tubes. The tubes were incubated at 37°C for 24 hours. Tubes were then washed with phosphate buffered saline pH 7.3, dried and stained with crystal violet (0.1%), excess stained was washed and slide was dried [9].

4. **Effect of temperature on biofilm formation by Liquid interface coverslip assay (Merritt, et al. 2005):** Wells were made on agar plates, sterile glass coverslips streaked with test culture (0.1 OD at 540 nm) was inserted into each well at a 90° angle relative to the bottom of the well. Plates were incubated at temperatures of 4°C, 27°C and 37°C for 24 hours.

5. **Preparation of peel extract by Soxhlet extraction (Negi, et al. 2003):** The orange peels were weighed, dried in a hot air oven for 12 hours. It was then ground to a fine powder and subjected to extraction in a Soxhlet apparatus using methanol as a solvent. It was run for 6 hours at a temperature of 50°C. The extract obtained was then subject to air drying (1-2 days), weighed, then stored in Dimethyl sulfoxide (DMSO). 5g of extract was dissolved in 20ml DMSO. From this stock solution, 4 dilutions of 100%, 75%, 50%, 25% were made. The anti-biofilm activity of the fruit peel extract was tested by taking 1 plate each for an organism and sectioning it into four quadrants. In each quadrant a coverslip is placed upon which 50ul of the orange peel extract (different concentrations) was added. After drying, 50 ul of culture (adjusted to 0.1 OD at 540nm) was added. Bacteria was stained by submerging coverslip in 0.1% Crystal violet for 10 minutes. Excess dye was rinsed and coverslips

were dried. Bacteria at the air- liquid interface on each coverslip was visualized under a microscope [10].

6. **Methanolic extraction of Citrus sinensis juice extract (Larson, et al. 2016):** Peeled oranges were washed with distilled water and then their weight was determined by using weight balance. After this fresh juice was filtered by using Whatman Filter Paper, then again, their weight was determined. Methanolic extract was prepared by mixing equal amount of orange juice and methanol 1:1. After vigorously shaking, three layers were separated. Collected three layers separately into three different clean beakers. All three layers were subjected to air drying for the collection of extract powder. After getting powder, a stock solution was made, by dissolving 1g of extract in 50% Dimethyl sulfoxide (DMSO). From this stock solution, 4 dilutions of 100%, 75%, 50%, 25% were made. One plate each for an organism and sectioning it into four quadrants. In each quadrant a coverslip is placed upon which 50ul of the orange juice extract (different concentrations) was added. After drying, 50 ul of culture (adjusted to 0.1 OD at 540nm) was added on to it. The plates were then incubated at 37°C for 24 hours and observed the next day [11].

7. **The effect of temperature on biofilm formation by Liquid Interface coverslip assay:** More biofilm was observed at higher temperatures of 37°C and 27°C for Escherichia coli, Bacillus subtilis, Proteus mirabilis and Pseudomonas aeruginosa as indicated by a denser field obtained upon crystal violet staining. Lower the temperature, greater was the inhibition of biofilm seen. To further confirm, quantitative assays must be done (Haney, et al. 2018). The plates were then incubated at 37°C for 24 hours. The pattern of growth was observed after the incubation period[12].

B.s at 4°C *B.s* at 27°C *B.s* at 37°C

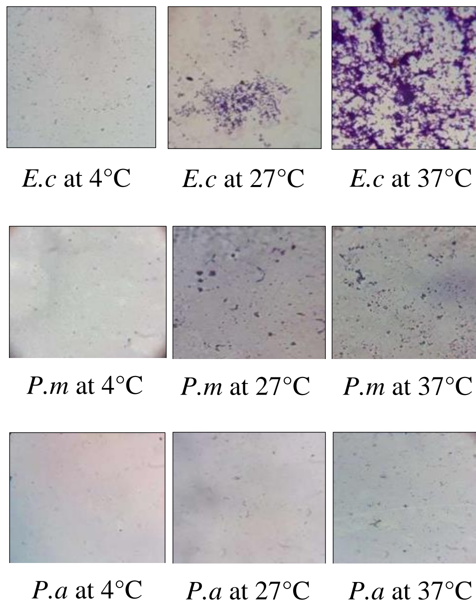


Fig 1: The effect of temperature on biofilm formation by Liquid Interface coverslip assay.

RESULTS:

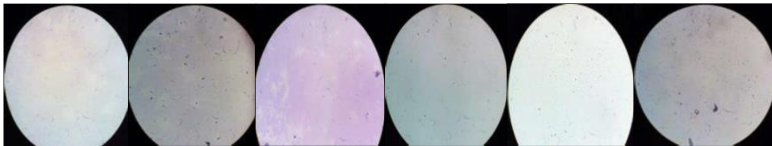
Motility tests:

Name of Organism	Motile	Type of Motility
Bacillus subtilis	Yes	Gliding motility
Escherichia coli	Yes	Swimming, Swarming and Sliding
Proteus mirabilis	Yes	Swarming
Pseudomonas aeruginosa	Yes	Swimming, swarming and Twitching

Table 1

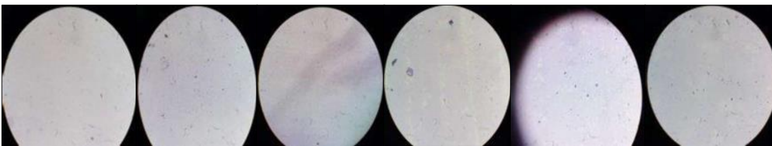
Anti-biofilm effect of concentrated Fruit juice and fruit peel extract:

Higher the concentration of (concentrated juice extract/ peel extract), more its anti-biofilm activity. To further confirm the inhibition of biofilm, microtiter biofilm inhibition assay or quantification of adhered biofilm cells in microtiter plates must be carried out [14].



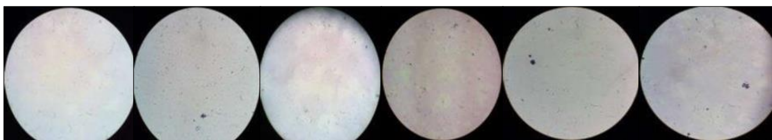
Negative Control	Positive Control	100%	75%	50%	25%

Fig 2: Antibiofilm activity of concentrated juice extract on *Bacillus subtilis*: (crystal violet staining)



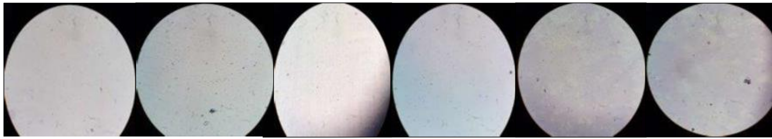
Negative Control	Positive Control	100%	75%	50%	25%

Fig 3: Antibiofilm activity of fruit peel extract on *Bacillus subtilis*: (crystal violet staining)



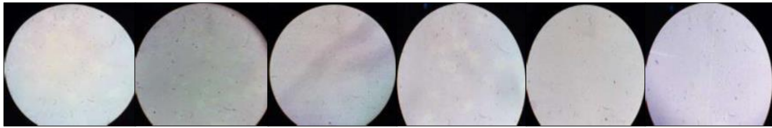
Negative Control	Positive Control	100%	75%	50%	25%

Fig 4: Antibiofilm activity of concentrated juice extract on *Escherichia coli*: (crystal violet staining)



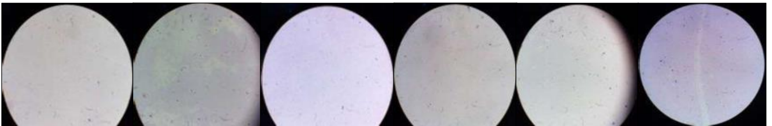
Negative Control | Positive Control | 100% | 75% | 50% | 25%

Fig 5: Antibiofilm activity of fruit peel extract on *Escherichia coli*: (crystal violet staining)



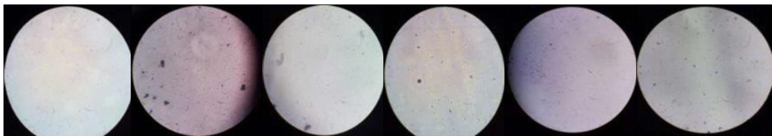
Negative Control | Positive Control | 100% | 75% | 50% | 25%

Fig 6: Antibiofilm activity of concentrated juice extract on *Proteus mirabilis:* (crystal violet staining)



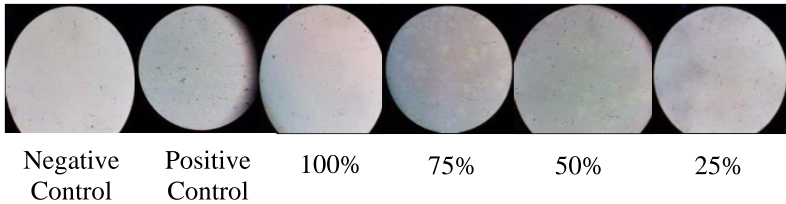
Negative Control | Positive Control | 100% | 75% | 50% | 25%

Fig 7: Antibiofilm activity of peel extract on *Proteus mirabilis*: (crystal violet staining)



Negative Control | Positive Control | 100% | 75% | 50% | 25%

Fig 8: Antibiofilm activity of concentrated juice extract on *Pseudomonas aeruginosa*: (crystal violet staining)



Negative Control	Positive Control	100%	75%	50%	25%

Fig 9: Antibiofilm activity of peel extract on *Pseudomonas aeruginosa*: (crystal violet staining)

DISCUSSION:

Rational for selecting motile species:

According to a previous study by (Houry, A., et al.) it has been stated that motility plays a major role in biofilm formation and presence of motility influences biofilm formation in several ways. It helps bacteria reach surfaces more easily thus giving rise to better adhesion. Motility helps give planktonic bacteria an edge over static bacteria with respect to invasion of the whole biofilm [15].

Therefore, in this project motile species were selected and the influence of factors on its biofilm growth formation was studied. In a study by (Al Kafaween et al. 2019) it has been stated that temperature is critical for formation of biofilm in bacteria. Therefore, in relation with the results obtained in this project, further study is needed to determine the ideal time duration for effective inhibition of biofilm at the specific temperature [13].

The orange peel extract shows a better activity than the concentrated orange juice extract by visual observation through crystal violet staining (further quantitative tests must be done to confirm) as it contains alkaloids, saponins, terpenes, resins, flavonoids, phenols, tannins, and sugars. It also comprises of cellulose, hemicellulose, lignin, pectin (galacturonic acid), chlorophyll pigments and other low- molecular weight compounds (e.g. limonene). (El- Desoukey, et al. 2018) [16]. The main organic acids found in orange juice are citric, malic, and ascorbic acid. The major sugars found in orange juice are sucrose, glucose, and fructose.

The two main compositional differences between peel and juice components are that the peel contains a higher concentration of ascorbic acid than the juice, and that the peel also contains higher concentrations of active components (d-limonene, hesperidin, naringin, and auraptene) than do the juice and pulp [14].

CONCLUSION:

Thus, the anti-biofilm effect of temperature and the activity of the natural product Citrus sinensis was studied on the test organisms. Lower the temperature, greater was the inhibition of biofilm seen. Thus, regulation of several factors including temperature has been found to have an important role in prevention of biofilm. This plays a major role in prevention of damage to medical equipment, skin grafts, implants in the medical sector. The natural concentrated juice extract and peel extract of Citrus sinensis showed us maximum antibiofilm activity at 100% concentration with less anti-biofilm activity at lower concentrations of extract. Further quantitative tests must be performed to confirm the same.

Citrus sinensis proves to have tremendous scope as an antibiofilm agent and reduces the problem of antibiotic resistance. It is sustainable and has energy efficient extraction methods. It does not give rise to any adverse effects on the environment and helps reduce the overall biofilm growth thus reducing the bioburden of microorganisms. Some of the organisms used in this study like Pseudomonas aeruginosa and Escherichia coli are pathogenic in nature and are major causes of infections. These extracts have proven to be successful in inhibiting their biofilm formation as well.

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Chapter 11 - Evaluation of Antimicrobial and Prebiotic Properties of *Averrhoa Bilimbi*

Gitika Kothari, Mrs. Rajitha Satish*, Dr. Sejal Rathod*
Department of Microbiology, Kishinchand Chellaram College,
Churchgate, Mumbai-400020.
Email: sejal.rathod@kccollege.edu.in

ABSTRACT:

Antimicrobial resistance is currently a major global issue. This has led to an increased interest towards discovery and development of antibacterial agents from plants having natural antimicrobial properties. *Averrhoa bilimbi* has been an important source of medicine since antiquity. Methanol extract of dried leaves of *Averrhoa bilimbi* was prepared using soxhlet apparatus. The antibacterial activity and the minimum inhibitory concentration of the methanolic extract were determined against various organisms at a range of 0.1% and 0.5% by agar dilution method. Further, silver nanoparticles were synthesized using fresh leaves of *Averrhoa bilimbi*, by green synthesis method. The antibacterial activity of the synthesized silver nanoparticle was determined using agar cup method. The prebiotic properties of aqueous extract of *Averrhoa bilimbi* was studied based on its probiotic growth stimulation, using strains of *Lactobacillus casei*.

KEYWORDS: *Averrhoa bilimbi*, antimicrobial activity, silver nanoparticles, prebiotic property

INTODUCTION:

Plants are the main source of drugs that being used from the ancient times as herbal remedies for the health care, prevention and cure of various diseases and ailments [7]. Natural products are the valuable sources of structurally diverse chemical compounds, as they possess therapeutic potential for treatment of human diseases. Among the natural resources, plants have been widely studied for the discovery of antimicrobial, antioxidants, anti-inflammatory, immunomodulatory and others therapeutic classes of chemical entities. The increasing prevalence of

multi-drug resistant organisms as well as strains with reduced susceptibility to the available antibiotics prompted us for the search of new effective therapeutic agents from plants [8]. *Averrhoa bilimbi* (commonly known as bilimbi, cucumber tree, or tree sorrel) fruit-bearing tree of the genus *Averrhoa*, family Oxalidaceae. It is a close relative of the carambola tree. *Averrhoa bilimbi* is essentially a tropical tree, attractive and is long lived that grows in Moluccas, Indonesia, and cultivated throughout Malaysia, Indonesia, Singapore, Philippines, Thailand, Bangladesh, Myanmar and India. It also extends to other countries like US, Argentina, Australia, Brazil, Colombia, Jamaica, Tanzania [1][8]. Traditionally the leaves are used as paste on itches, swelling, rheumatism, mumps or skin eruptions, after-birth tonic and also in cold and cough, bites of poisonous creatures, etc [8].

Nanotechnology is mainly concerned with the synthesis of nanoparticles and their application in various fields of medicine, chemistry, materials science, etc. Synthesis of nanoparticles are usually carried out by various physical and chemical methods which are not environmentally friendly. Among the various noble metal nanoparticles, gold and silver have several applications in sensors, detectors, and antibacterial agents. In ancient Indian medical system (Ayurveda), Silver has been described as therapeutic agent for many diseases and an efficacious antibacterial and antifungal agent. Recently, the studies are focused towards greener methods for the synthesis of nanoparticles. Biosynthesis of nanoparticles gained lots of interest during the past several years, production of metallic nanoparticles using low cost biological resources such as plants, algae, fungi, and bacteria are reported. As an alternative to synthetic chemicals, in the synthesis of nanoparticles plant extracts are used successfully [2][5].

Various types of microorganisms, known as gut microbiota, are inhabitants of the human gastrointestinal tract. The resident microbial groups in the stomach, small, and large intestine are crucial for human health. Human diet is the chief source of energy for their growth. Particularly, non-digestible carbohydrates can highly modify the composition and function of gut microbiota. Beneficial intestinal

microbes ferment these non-digestible dietary substances called prebiotics. Moreover, they can suppress pathogens in healthy individuals through induction of some immunomodulatory molecules with antagonistic effects against pathogens by lactic acid that is produced by *Bifidobacterium* and *Lactobacillus* genera [6]. These microorganisms function as probiotics because of their potential to inhibit pathogenic microorganisms in the gastrointestinal tract. In addition, prebiotics compounds are gastrointestinal tolerant in the presence of salivary amylase, gastric juice or bile extract, maintaining properties to activate the beneficial microbes for host health [3]. Prebiotic supplements are food for probiotic organisms. They are effective for treating diarrhoea, irritable bowel syndrome, allergic disorders & even the common cold. Prebiotic and Probiotics have been suggested as treatments for Obesity [6].

MATERIALS AND METHODS:

Preparation of Methanolic Extract

The fresh leaves of *A. bilimbi* was collected, washed and air dried in the Hot Air Oven at 60°C followed by making its coarse powder with a grinder. 10g of leaf powder was extracted with methanol using Soxhlet extraction method at 50°C. The pigmented extract is then dried in the petri plate and then scratched after the evaporation of the solvent and dissolved into DMSO solution and then the antibacterial activity was checked using microorganisms [1].

Preparation of Aqueous Extract

Fresh clean leaves of *A. bilimbi* leaves was cleaned. 10grams of fresh leaves were soaked into 40ml of Sterile distilled water(w/v), boiled at 100°C. for 15 minutes. It was then filtered using Whatman Filter Paper. The filtrate was the used as aqueous extract for further studies [1].

Synthesis of Silver Nanoparticles

For Green Synthesis of Silver Nanoparticles, 0.2ml of the prepared aqueous extract was dissolved into 50ml of 0.001molar solution of Silver Nitrate and then this mixture was incubated for 8 hours in the dark. Pale

yellow coloured solution was obtained [2][5]. The presence of silver nanoparticles was checked using UV Spectrophotometer [2] [Graph-1]. The antibacterial activity of the synthesised silver nanoparticles was confirmed using by Agar Cup Method on 2 Gram positive and Gram-negative organisms each [2].

Prebiotic Properties

Aqueous extract of Averrhoa bilimbi was supplemented in Man Rogosa Sharpe (MRS) broth for the cultivation of probiotic strains of *Lactobacillus casei*, then the prebiotic properties was determined based on probiotic growth stimulation [3]. *L. casei* Shirota was cultured at room temperature for 72 hours under anaerobic conditions on MRS agar and then the culture suspension was adjusted to 0.1 OD at 540nm. [Graph 2]

Five different flasks were taken for the test:

1. Mix flask contained 50ml of MRS broth + 1.25ml of Inulin + 1.25ml of aq. bilimbi extract + Culture of *L. casei* Shirota.
2. *A. bilimbi* flask contained 50ml of MRS broth + 2.5ml aq. bilimbi extract + Culture of *L. casei* Shirota
3. Inulin flask contained 50ml of MRS broth + 2.5ml of Inulin + Culture of *L. casei* Shirota
4. Positive flask contained 50ml of MRS broth + culture of *L. casei* Shirota
5. Negative flask contained 50ml of MRS broth (no culture added)

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of methanolic extract of A. bilimbi was determined by Agar Dilution Method, using 0.1% and 0.5% concentration, against Gram-positive and Gram-negative organisms. Organisms used were *S. para typhi B, S. aureus, C. diphtheriae, K. pneumoniae, S. typhi, E. coli, S. pyogenes* [1]. [Table 1]

Antimicrobial activity

The antimicrobial activity of methanolic extract and silver nanoparticle was evaluated by Agar Cup Method (Agar Well Method) against various

Gram positive and Gram negative organisms such as *S. para typhi B, S. aureus, C. diphtheriae, K. pneumoniae, S.typhi, E. coli, S. pyogenes, P. aeruginosa* using St. Mueller Hinton's Agar Medium. The antimicrobial activity was determined by measuring the diameter of zone of inhibition in mm [1][2]. [Table 2,3]

RESULTS AND DISCUSSION:

During the MIC Determination, the methanolic extract of A. bilimbi at 0.1% concentration showed no inhibition while at 0.5% concentration, organism C. diphtheriae showed very less inhibition. The antimicrobial activity was done by Agar Cup Method on several organisms such as *S. para typhi B, S. aureus, C. diphtheriae, K. pneumoniae, S. typhi, E. coli, S. pyogenes, P.aeruginosa* and it was found that maximum inhibition was observed in *C. diphtheriae* and *E. coli.* Synthesis of Silver Nanoparticles was done by Green Synthesis Method and hence to confirm the synthesis of nanoparticle the absorbance was determined at various wavelength (425-435nm), and maximum peak was observed at 430nm and 431nm.

Similarly, the UV-Visible spectrophotometer showed the maximum absorbance at 433 nm using the *A. bilimbi* leaf extract [2]. Antimicrobial activity for silver nanoparticle extract was checked by Agar Cup Method on 2 Gram positive such as *C. diphtheriae* & *S. aureus* and 2 Gram negative organisms such as *E. coli* & *S. typhi* in which *C. diphtheriae* showed maximum zone of inhibition. The biosynthesized silver nanoparticles showed excellent antimicrobial activity against Gram Positive organisms i.e., *C. diphtheriae* & *S. aureus*. It has been demonstrated that the extract of *Averrhoa bilimbi* leaves are capable of producing silver nanoparticles extracellularly and the Ag nanoparticles are quite stable in solution [2].

Lastly to check the prebiotic activity of the aqueous extract on the probiotic gut microbe i.e. *L. casei* was done by Probiotic Growth Stimulation Method. The aqueous extract of *Averrhoa bilimbi* showed maximum growth as compared to the commercial prebiotic compound i.e. Inulin. The Growth curve reading was plotted on the graph to see the

desirable difference in the commercial product and extract and its activity towards probiotic organism i.e. *L. casei* Shirota.

Name of Organism	**Concentration in %**	
	0.1%	**0.5%**
S. para typhi B	-	-
S. aureus	-	-
C. diphtheriae	-	+
K. pneumoniae	-	-
S. typhi	-	-
E. coli	-	-
S. pyogenes	-	-

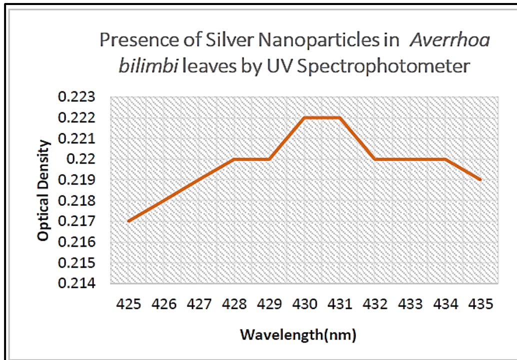
Key - (+) : Inhibition (-) : No inhibition

Table 1: The MIC results of the methanolic extract

Name of Organism	**Zone of Inhibition in mm**	
	Control	**Test**
S. para typhi B	-	16.5
S. aureus	-	12.0
C. diphtheriae	-	20.5
K. pneumoniae	-	12.5
S. typhi	-	13.0
E. coli	-	20.0
S. pyogenes	-	12.0
P. aeruginosa	-	15.0

Key: (-) is No Zone

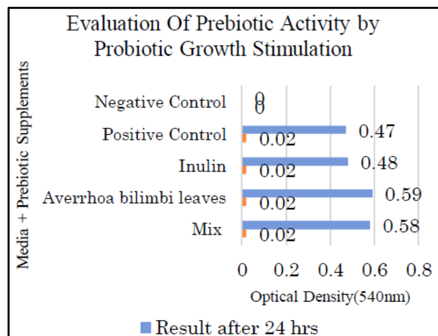
Table 2: Results of Agar cup method of methanolic extract in 24 hours



Graph 1: Results obtained from UV Spectrophotometer

Name of Organism	Zone of Inhibition in mm		
	Control	**Test**	**Test - Control**
S. aureus	23	33	10
C. diphtheriae	33	50	17
S. typhi	32	32	-
E. coli	23	25	2

Table 3: Results of Agar cup method of silver nanoparticles extract in 24 hours



Graph 2: Evaluation of Prebiotic Activity by probiotic growth stimulation

CONCLUSION:

It is evident from the present studies that *Averrhoa bilimbi* plant have various significant antimicrobial activity. No such research has been done till date on prebiotic properties and silver nanoparticles on leaves of *Averrhoa bilimbi*. By present studies it has been found that the fruits of this plant are very useful and many such research papers are available for it and some of them are listed below as well. By the above study it shows that *Averrhoa bilimbi* plant inhibits harmful bacteria by its antimicrobial activity and at the same time it allows the growth for beneficial organism. Further to strengthened the upcoming studies on *Averrhoa bilimbi* leaf, the size and characterization of obtained silver nanoparticles extract can be checked, the antimicrobial activity of obtained silver nanoparticles can be checked more organisms, the Pathogenic Growth inhibition and Gastrointestinal Tolerance of gut flora with reference to prebiotic activity can be checked and identified.

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SECTION 3 – BIOTECHNOLOGY

Chapter 12 - A Sequence Comparison and Structure Prediction Based In Silico Study on Protein Function Annotation of Smim23

Arunika Sherry, Ms. Hajra A. S. Gupta*, Dr. Pratibha Shah*
Department of Biotechnology, Kishinchand Chellaram College,
Churchgate, Mumbai-400020.
Email: Pratibha.shah@kccollege.edu.in

ABSTRACT:

In the post-genomic era, proteomics studies are picking up pace. Even though genomics has been studied thoroughly in detail, certain proteins still have no functional annotation and predicted structural design. Research shows that 520 molecular structures remain as proteins of unknown functions and 1097 proteins annotated as uncharacterised proteins by UniProtKB. Small Integral Membrane Protein 23 is one such protein. It is expressed in 49 organs in human beings and shows highest expression in the testes. Lack of information regarding proteins, poses as a hindrance towards metabolic reconstruction in silico and further research in genetic engineering. Comparative genomics and in silico structure prediction are the two most efficient methods for further research and studies of unknown proteins. On performing BLAST, around 100 orthologs were obtained for SMIM23 and a genetic comparison helps in deducing the function of the protein. Amino acid sequence comparison or the FASTA sequence comparison of an unknown protein and a well-studied protein is done to ascertain the function of the unknown protein. This is possible as it is highly likely that the proteins that have similar amino acid and genetic sequences have similar functions as well. Molecular graphic visualisation is performed in order to study the structure of the protein of interest further. The research conducted showed that the protein is a transmembrane structure with helices and its function can be deduced from a similar protein found in testes of Mus musculus (mouse or rodent). Parallels drawn from functions of known orthologs ascertains the function of protein of

interest. The extracellular domain of the protein was found to be similar to 1,4-alpha glucan branching enzyme in *Dorea* species and *Lachnospiracea* family. Similarity to this enzyme suggests that the protein is involved in the catabolism of carbohydrates by sperms or Sertoli cells and may play a role in motility of sperms.

KEYWORDS: SMIM23, PDB, UniProt, BLAST, Functional annotation

INTRODUCTION:

Certain proteins have unknown functions and structure. Such proteins are called Orphan Proteins. These proteins impede and cause a hindrance in research work pertaining to functions revolving around such proteins Considering that each gene encodes for a single protein an estimate of about 20,000 proteins can be made for number of proteins present in the human body. However, research shows that out of these proteins, 520 proteins are of unknown functions and 1097 proteins are annotated as uncharacterised proteins as suggested by UniProtKB (Valencia, 2005). Protein molecules are essential to study as even though it is the DNA that contains the genetic information, it is the protein that implements functions, sustains life and maintains the cells and the human body.

Functions largely depend on the structure of the protein. To understand the importance of protein functions, one can cite the example of proteins that actively take part in DNA replication, which is the most important life process as it affects cell division. Protein functions give an idea about how proteins and their specific functions are essential for the body to continue working efficiently and constantly. Any error or change in the function performed may adversely affect the body. Even though huge databases are available to proteins whose function and structure is in order to deduce the structure and subsequently the function and compare it to already existing information about Small Integral Membrane Protein 23 (Loewenstein, 2006).

It is a part of a class of proteins called “Integral Membrane Proteins”. As the name suggests, they are embedded in the cell membrane and usually function as linkers, channels, receptors, enzymes, structural

membrane anchoring domains, accumulation of energy etc. Such proteins usually have one extracellular end, one cytoplasmic end and the middle part of the body embedded in the membrane.

MATERIALS AND METHODS:

1. **NCBI BLAST:** National Centre for Biotechnology Information is a part of National Library of Medicine. Both NCBI and NLM are based out of the United States and are databases and have Bioinformatics applications that are available worldwide. NLM is a branch of the National Institute of Health. NCBI BLAST refers to Basic Local Alignment Search Tool. This tool is available on the NCBI website. NCBI BLAST was chosen for this research project as a heuristic Basic Local Alignment Tool is ideal for retrieving preliminary information about a protein. BLAST works by detecting local alignments between sequences that are similar to each other. The BLAST computers begin with a small set of three letters known as "query word". These letters represent three amino acids or nucleotides. The BLAST search then looks for the number of times (and places along the sequence) in which this three-letter "word" appears. It will also look for closely related "words" in which one letter letter is different, i.e. those codons or words that have only one different amino acid residue (Schmutz, 2004). Each query is scored to determine which proteins are similar in sequence with the query sample provided.

2. **PDB:** Databank repository is a protein database that gives information of those proteins whose origin is known and they have been translated from known and sequenced genes. The Protein Data Bank (PDB) is a database for the three-dimensional structure of large biological molecules such as proteins. The data obtained is generally through experimental means and may include the following techniques - X-ray, crystallography, NMR spectroscopy and so on. Information derived from these experiments gives the actual structure of the protein or biomolecule. This structure can then be visualised in silico using Bioinformatics applications and computer software. The structure includes particular motifs, alpha

and beta chains, hydrophobic and hydrophilic chains, metallic central atoms and other characteristics making the protein unique from other proteins. The structures are usually movable and can be seen in 3D which helps in better understanding of the protein. The format in which a PDB file is saves is called a PDB file format. There is a PDB identifier that is specific for certain proteins. Each protein, however, may have the same identifier. This is because many proteins may have similar conformations in different environments.

The saved PDB file format can be transported to 3D biomolecule imaging applications such as Pymol or Rasmol. These applications allow the researcher to study the biomolecules in 3D and helps one to understand more about the structure.

3. **UniProt:** Uniprot is a protein database that contains all the information about a particular protein. It is commonly used for structural research projects based on proteomics. It provides protein sequences and their functional information (Aloy, 2001). However, not much has been referred to about Small Integral Membrane Protein 23. Since, not many experiments have been conducted to ascertain function of SMIM23, there is limited information as to how the protein works. UniProt enables the researcher to answer the following questions about a protein, provided it contains the information.

- What is the location of the protein?

This information helps the researcher understand where the protein is expressed most and thus correlate this expression to function and structure.

- What is the function of the protein?

This answer may or may not be provided depending on the extent of experimental research carried out for the particular protein. UniProt provides freely accessible high-quality information about protein functions and sequences. Thus, Uniport makes for an excellent

Bioinformatics tool whose application is best suited for research projects aiming towards protein functional annotation and structure prediction (Polacco, 2006). UniProt provides with details of a protein and most of these details are similar or same as the ones seen on PDB.

4. **Raptor X:** For proteins such as Small Integral Membrane Protein 23, whose structure, location and function are unknown or have not been thoroughly researched on, predicted molecular imaging is necessary to get an idea about the structure, hydrophobic chains, hydrophilic chains, alpha sheets, beta pleated structures, cytoplasmic, membrane or extracellular regions etc.

   The function can further be deduced from the predicted structure to give an idea about what the protein may function as (Roy, 2012). As stated before, proteins may function as linkers, channels, receptors, enzymes, structural membrane anchoring domains, accumulation of energy etc. This function is directly linked to the function of the protein. Thus without the understanding of the structure, function may be difficult to assess in silico. A very integral part of research based on protein functional annotation and structure prediction is the usage of 3D molecular imaging, thus RaptorX is an integral part of structure prediction. RaptorX predicts structure of a protein and is a server developed by Xu group at University of Chicago. They usually predict structures of those proteins whose information is not known, and they have less or no homologs in PDB. Structure prediction is carried out by sending the PDB FASTA sequence on the website. The prediction server takes up to 2 days for response. The detailed image of the predicted structure is emailed to the recipient.

RESULTS:

NCBI BLAST results were obtained by entering the protein FASTA sequence of SMIM23 as the query sequence. The query sequence was then compared with all database FASTA sequences by the BLAST computers. BLAST scores each sequence the sequences are related to

each other. When the FASTA sequence was submitted, 100s of orthologous protein sequences were returned, suggesting that these sequences are very similar to the query sequence that were returned in the results suggested the presence of a hypothetical protein the proteins were named SMIM23 in different species, with unknown functions. Only two proteins were found to be named differently, SMIM23 in *Mus musculus* and Testes specific protein 4 in *Mus musculus*.

Protein Databank is a repository source for the 3D structural data of specific proteins, whose FASTA sequence has been submitted. It has approximately 8000 entries of biological molecules. The FASTA sequence of the protein of interest was retrieved and submitted to PDB. The results returned, showed that the protein has 4 main domains namely - Cytoplasmic region, Helical region, Extracellular region and Super Coiled region. The UniProt knowledgebase is a resource to retrieve protein sequences and their detailed annotation. The database contains over 60 million sequences. The experimental and predicted data enables scientists to navigate the vast amount of sequence and functional information of proteins. On submitting the retrieved FASTA sequence, UniProt states that there are a total of three regions in the protein of interest, namely Cytoplasmic region, Helical region and Extracellular region.

Another interesting result on UniProt states that the protein is expressed in 49 organs in the human body and is highly expressed in the human male right testes. A similar result was observed when further research was done on the Testes specific expressed protein 4. The results stated that this protein is highly expressed in the testes, suggesting a similarity with the function of SMIM23. Futher analysis using UniProt BLAST suggests that the extracellular region of SMIM23 is highly similar to 1,4-alpha glucan branching enzyme of *Dorea* species and *Lachnospiraceae family.*

RaptorX is an online software developed by the Xu Group. It is generally used for those proteins that have no structural and functional annotation. Due to the unavailability of data regarding SMIM23, RaptorX was used to deduce the structure of SMIM23. The software requires the researcher

to send the FASTA sequence of the protein of interest. The structure prediction showed that there are about 4 helical regions on either end of the protein and there are loops and turns throughout the protein.

CONCLUSION:

Conclusions regarding the structure and function of the protein of interest, SMIM23, can deduced from the results obtained from online databases and softwares. NCBI BLAST was carried out for the FASTA sequence of the protein and it was found that the protein has orthologs arising from various species. It suggests that SMIM23 is a protein in Human Testes. This conclusion is also supported by UniProt results which state that the protein is highly expressed in the right Testes of Human Male.

Raptor X suggests that the two terminal has loops and turns throughout. The super coiled structures act as Signal anchors for relay of activity taking place due to the glycogen present in the testes.

similar to the 1,4-alpha glucan branching enzyme present in *Dorea* species and *Lachnospiraceae* family, it can lead to the following conclusions or possibilities, regarding the function of SMIM23. The presence of Glycogen or any other carbohydrate may be relaying a specific unknown function in the cells related to the development of sperm cells. Apart from this, it may also be possible that the protein SMIM23 may be activated during the catabolism of Glycogen or any other similar carbohydrate.

Further, activity of SMIM23 during spermatogenesis can also be studied to understand the function in detail. The function of the said enzyme and protein can also be studied further in the pathway of development and hereditary characteristics.

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Chapter 13 - Isolation of *Pseudomonas Cichorii* from Garden Soil and Evaluation of its Potential as a Bacterial Inoculum for Production of 6-Aminopenicillanic Acid, Precursor to Penicillin

Harsh Gala, Ms. Hajra A. S. Gupta*, Dr. Sejal Rathod*
Department of Biotechnology, Kishinchand Chellaram College,
Churchgate, Mumbai-400020.
Email: sejal.rathod@kccollege.edu.in

ABSTRACT:

Aminopenicillanic acid (6-APA), the precursor for the penicillin antibiotics that are used against the infection or disease caused by the gram-negative pathogens. Due to over usage the pathogens have gained resistance against the antibiotics which led to the need for the new medicines which would be effective and after research semi-synthetic antibiotics were prepared. Various acyl groups were added to the 6-APA to prepare semi-synthetic antibiotics. This led to increase in the demand for 6-APA for mass production of the antibiotics. Traditionally, 6-APA was obtained by fermentation of penicillium mold but now recombinant E. coli cells are used for the production of the acid. This study is based on the soil organism that infects plants, to confirm that it naturally produces 6-APA. The organism, i.e., *Pseudomonas cichorii*, was isolated from the leave sample tests, also using phenol broth mannose broth and a *Pseudomonas cichorii* specific media. Separation and confirming the presence of 6-APA by thin layer chromatography (TLC) was carried out. Results for the tests were obtained, colonies were observed on specific media and brown spot was observed on TLC plate. Comparative studies will be carried out between the quality of 6-APA extracted from *Penicillium chrysogenum* and *Pseudomonas cichorii* and it can be used as a replacement for the 6- APA obtained from the penicillium mold and recombinant E. coli. This could provide an easy way to supply the 6-APA and meet the huge industrial demand, it would also make the process short and cut down the production cost, which would in-turn lead to the cheaper supply of antibiotics.

KEYWORDS: Penicillin, 6-APA, *Pseudomonas*, *Pseudomonas cichorii*, Semi-synthetic antibiotics, TLC

INTRODUCTION:

Today world's biggest industries are known to produce some of most important entities, of which Penicillin doesn't need any introduction. It has been and still is one of the most widely produced antibiotics in the world. It almost has an annual production of around 3x107 kg/year and sales of about $15 billion which makes pharma-industries huge (Nandi et al., 2014). Antibiotics are a type of antimicrobial substances that are used against the infection caused by the bacteria. They can also work against protozoa and sometimes also against certain viruses. The first antibiotic that was discovered was penicillin. Later, due to over use of the antibiotics the organism stated getting resistant to the antibiotics and therefore, new derivatives were prepared using chemical reactions which could be used against resistant pathogens. The mechanism of action of these antibiotics is by inhibiting the biosynthesis of the cell wall in the bacterial culture. These property makes them most effective against the gram-negative bacteria, since they have thin cell wall.

The cross linking of the peptidoglycans, that is, NAM and NAG, is affected, which is the final step of the bacterial cell wall formation. Due to this the cell wall weakens and the internal contents move out of the cell uncontrollably or water moves into the cell due to osmosis and it cannot maintain the concentration gradient. This can either lead to the lysis or bursting of the cell. This prevents them from causing infection in the host body.

Penicillin are the most widely used b-lactam antibiotics, broad spectrum of antibacterial activity, low toxicity, and outstanding efficacy against various bacterial strains. Currently, the only method of overcoming the resistance problem is the use of newer semisynthetic antibiotics. (Parmar et al., 2000) Most of the new semisynthetic penicillin are produced from 6-aminopenicillanic acid (6-APA), which in turn is produced mainly by enzymatic or chemical deacylation (Chisti and Moo-Young, 1991) of the natural benzyl penicillin (Parmar et al., 2000).

The penicillin molecule consists of the two important parts that are, the 6-aminopenicillanic acid (6-APA) and the N-acyl group. It's the nucleus of the penicillin molecule. It consists of the beta lactam ring to which other molecules or groups can be added to produce a variety of antibiotics. This compound is used in the production of semi-synthetic and synthetic antibiotics such as amoxicillin, ampicillin and many more.

The enzyme that is used is the penicillin G acylase (PGA). The enzyme catalyses the reaction of hydrolysis of the amid bond present in the molecule for the production of the 6-APA, the recombinant *E. coli* was made using genetic engineering which produced the enzyme PGA in the medium. There are a lot of steps involved in the production process and it leads to chemical load on the environment due to the use of hazardous chemicals such as pyridine, phosphorous pentachloride, and nitrosyl chloride which in turn harms the environment. The enzymatic process has a lot of factors has to be considered like the preparing of recombinant organism, growth of organism for the production of the enzyme PGA, the substrate production that is penicillin G or penicillin V, maintaining the organism in the lag phase of the growth curve, the physio-chemical factors that affect the production of 6-APA and also the growth of the organism. Even the recombinant organisms are not reliable as they can lose their ability after few generations. The *penicillium* fungi takes long to grow and require specific conditions such as temperature and pH which are favourable and support growth. The maintenance becomes really expensive. The production of penicillin also a long process and tedious. The waste water from the process has detrimental effect on the environment.

Pseudomonas cichorii is a gram-negative bacterium that is found in the soil and is a plant pathogen *P. cichorii* was first isolated from the plant *Cichorium endivia*. According to the 16s rRNA analysis this organism was placed in the group of *Pseudomonas syringae*. The organism produces 6-APA. This organism can be used for the production of the 6-APA. Based on phylogenetic analysis using gyrB and rpoD, *P. cichorii* is grouped within the *P. syringae* pathogen complex (Yamamoto et al. 2000) and more recently was placed in Phylogroup 11 based on

multilocus sequence analysis (MLSA) of housekeeping genes (Berge et al. 2014). Standard LOPAT assay that tests for levan production, oxidase and proteolytic activity, arginine dihydrolase utilization and tobacco hypersensitivity is used to differentiate *P. cichorii* strain from another fluorescent pseudomonas (Timilsina et al., 2017). This organism can destroy the crop fields and can lead to the extensive economic damage.

It causes irregular leaf blight and spots on the leaf surface. Such lesions appear to be water soaked. They can develop either on the centre of the leave that is mid-vein or at the edges. They turn brown or black and may also have white or yellow halo concentric rings around them. As the leaf dries, the lesions get brittle and they easily crack.

MATERIALS & METHODS:

1. Sampling location and collection of Samples:

The samples for the experiment were the infected leaves. The leaf samples were collected from the 2 different locations such as the Garden area (Dheeraj Presidency, Kandivali-W) and plant pot from College premises (Kishinchand Chellaram College). The locations for the samples were randomly selected. The leaves which showed leaf blights and spots or lesions were selected. These spots or lesions had turned brownish-black surrounded with yellow and white halo rings. All the leaves were collected in clean polythene bags and taken to the laboratory the next day.

2. Isolation of the culture:

The isolation was carried by the spread plate technique of the sample. The 0.5g weighed and separate batches of leaf were added to the sterile saline of about 5mL in a sterile medium-sized test tube. The last three dilutions were plated on the supporting media. The media that was used for isolation was King’s B media. The fluorescent isolated colonies were observed on the plates under UV trans-illuminator. The colonies were picked and streaked on the cetrimide agar.

3. Identification and characterization of the isolate

The gram staining was carried out and looked under 100x oil immersion microscope. The biochemical tests were performed such as Indole test; Methyl red test; Voges-Proskauer test and Catalase test (IMViC) and other tests like KOH test and Oxidase test. Tryptone water broth is used for the indole test. The Kovac's reagent is added after 24-hour incubation. If a red colour ring is observed on top of liquid than the test is positive. The methyl red and Voges-Proskauer test is carried using the same broth, that is, Glucose Phosphate (GP) broth. The broth is also called MR-VP Broth, the Omera's reagent is added which contains KOH solution and in the presence of KOH, the acetoin gets oxidized and the colour change is observed. If the pinkish-red colour is developed then the test is positive. The catalase test is performed on Simon's citrate agar slant to check the ability of the organism to utilize citrate present in the media and it also contains bromothymol blue, which is a pH indicator. The colour of citrate agar is green but if the colour changes to royal blue after inoculation and incubation for 37°C then the test is positive.

To differentiate between *Pseudomonas* fluorescence and *Pseudomonas cichorii*, the colonies were inoculated in the phenol red maltose broth. The maltose acts as the carbon source, the phenol red acts as the pH indicator. It turns orange in acidic medium and remains red in basic medium. *P. fluorescence* can utilize the mannose and colour change is observed, while, on the other hand *P. cichorii* cannot utilize, so no colour change is observed. Three tubes were maintained, one was control with no inoculation, second was inoculated with *P. fluorescence* an third with isolate.

The isolate was streaked on the *Pseudomonas cichorii* specific media which is a specific media as it supports the growth of *Pseudomonas cichorii*. The plate is placed at room temperature for 24 hours at 37°C and placed under UV-trans illuminator. If the colonies are present, it shows fluorescence. The composition of the media was 0.5g KH_2PO_4, 3g $Na_2HPO_4.12H_2O$, 8g sodium tartrate, 5g $(NH_4)_2SO_4$, 25mg $MgSO_4.7H_2O$, 24mg $Na_2MoO_4.2H_2O$, 10mg EDTA-Fe, 50microg L-cysteine, 1mg methyl violet, 50mg pheneticillin potassium, 10mg

ampicillin sodium, 10mg cetrimide, 25mg cycloheximide, 20mg phenol red, 25mg potassium tellurite, 15g agar in 1 litre of distilled water.

4. Detection of 6-APA by Thin Layer Chromatography (TLC)

The TLC is a solid-liquid technique for the separation of biomolecules such as amino acids, proteins, fatty acids and other metabolites produced by the organism. The separation is accomplished by the distribution of the mixture between two phases: one that is stationary and one that is moving. The TLC was used to identify the component- 6-APA produced by the organism that was isolated from the leaf sample. The 0.1 OD bacterial cultures were enriched in 100ml of the KB broth for about a week. 1mg Cetrimide was added in the broth to prevent contamination by growth any other organism except pseudomonas. After one week, 10ml of the broth was transferred into the sterile centrifuge tubes. They were centrifuged at 5000 RPM for 20 minutes. The supernatant was collected and the spot was made on the silica-g TLC plate. The mixture of glacial acetic acid, n-butanol and water was added in the ratio of 1:3:1. The solution of the entire chemical was poured in a glass chamber and left for saturation for about 30-45 minutes. After spotting, the plate was placed in the saturated chamber. The iodine vapours were used for the detection of the compound. The brown spots would be visible if the expected compound was present. The Rf value is calculated, the standard Rf value of the 6-APA is 0.53. The Rf is calculated using a formula, that is,

$$Rf = \frac{\text{Distance Travelled by Solute}}{\text{Distance Travelled by Solvent}}$$

RESULTS:

6-Aminopenicillanic acid (6-APA) is the part of the penicillin antibiotics. Other beta-lactam antibiotics can be produced by attaching a chemical compound to the 6-APA. These antibiotics can be used for the treatment of many infections caused by the non-lactamase producing streptomyces or staphylococcus by retarding the growth or killing the organism. Thus, preventing the spread of the infection.

1. Isolation of the organism

The fluorescent colonies were observed under UV trans-illuminator on KB media plates. The colonies were observed in the plate with 10^{-3} and 10^{-5} dilution. The fluorescent colonies were picked from both the plates and T-streaked on the cetrimide media. Fluorescent colonies were observed in both the cetrimide plates showing that the isolated organism maybe of Pseudomonas species.

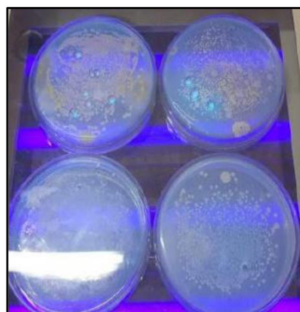

Fig 1: Fluorescent Colonies on KB Media

2. Identification and characterization of the isolate

The Gram staining was carried out and the slide was observed under 100x oil immersion lens and the isolate was gram negative. The pink, coco-bacilli were observed. The biochemical tests were carried out to performed to confirm whether the isolate is *Pseudomonas*. Indole Test Negative, Methyl Red Test Negative, Voges-Proskauer Test Negative. Citrate test Positive, Catalase Test Positive, Oxidase Test Positive and KOH Test Positive.

The Phenol Mannitol Broth was used to differentiate the isolated organism from *Pseudomonas fluorescence*. The second tube of three was inoculated with *P. fluorescence* and the other tube was inoculated with the isolated organism. There was no colour change in the control tube which means there was no contamination during the preparation of the broth.

The colour change was observed from red to yellow in the second tube, while no colour change was observed in the third tube which shows that the isolated organism is from *Pseudomonas* species but not *Pseudomonas fluorescence*. The isolated organism was also T-streaked on *Pseudomonas cichorii* specific media and after incubation at room temperature for 24 hours. The fluorescent colonies were observed under the UV light which shows that the isolated organism maybe *Pseudomonas cichorii.*

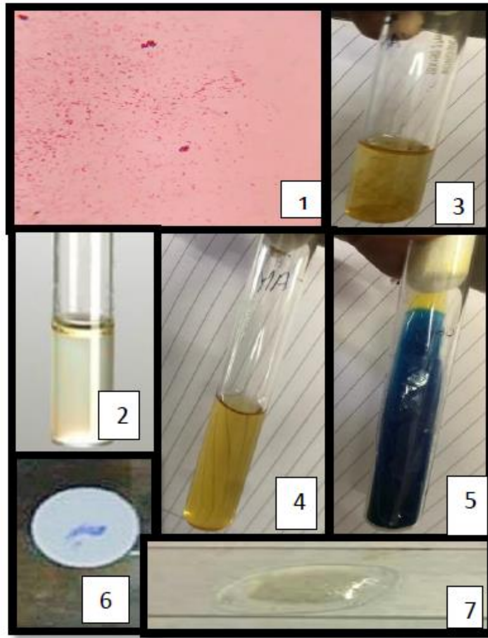

Fig 2: (1) Gram staining, (2) Inodle-Negative, (3) Methyl Red-Negative, (4) Voges Prauskauer-Negative, (5) Citrate-Positive, (6)Oxidase-Positive, (7) Catalse-Positive

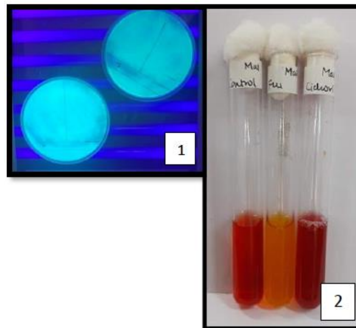

Fig 3: (1) Fluorescent colonies on PCSM (2) Phenol Broth Mannose Broth Test

3. Detection of 6-APA by Thin Layer Chromatography (TLC)

The TLC was carried out on a glass slide and silica G was used as the stationary phase, while the moving phase was a solution prepared by the mixture of glacial acetic acid, n-butanol and water in proper ratio. The brown spot was observed on the TLC plate after treating with iodine. The rf value was calculated and found to be 0.533. The standard Rf value of the 6-APA is 0.53. This shows that the separated compound can be 6-APA.

Fig 4: Brown spot of 6-APA on TLC

CONCLUSION:

The antibiotic consists of two parts, that is, an acyl group and 6-APA. *P. cichorii* was isolated from the leaf sample using and identified by biochemical tests and using specific growth media. The isolate was cultured in the KB broth. Separation of spot of broth was carried on TLC; it showed a brown spot at the Rf of 0.533. This shows that the organism can produce 6-APA. The culture could be confirmed by carrying out 16srRNA sequencing. We can conduct a compare study between 6-APA obtained from *P. cichorii*, *Penicillium chrysogenum* and one by action of PGA on Penicillin G. This would cut down the production cost and price of antibiotics.

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Chapter 14 - The Gut Feeling: Isolation of Dopamine producing microorganisms, from the Gut Microbiome

Kavya Zaveri, Dr. Pratibha Shah*, Ms. Prachi Bhatia*
Department of Biotechnology, Kishinchand Chellaram College,
Churchgate, Mumbai-400020.
Email: pratibha.shah@kccollege.edu.in

ABSTRACT:

There exists a bidirectional communication system between the gastrointestinal tract and the Brain. Increasing evidence shows that the gut microbiota can play a critical role in this communication, forming the brain-gut microbiome axis. Gut microbes make around 95% of the body's stock of dopamine, which impacts both state of mind and gastrointestinal tract movement.

This study centres on dopamine synthesizing microbes, which form the normal flora of the gut microbiome. Probiotic bacteria such as *lactobacillus sp.* and fungi such as *saccharomyces sp.* were each isolated from food sources on selective media like DeMan, Rogosa & Sharpe (MRS) agar and Sabouraud's agar respectively. Following isolation and identification, screening for production of dopamine was carried out by allowing the organisms to grow in appropriate broths in bulk for about a week. A crude dopamine extract was obtained after the broths were subjected to ultra-sonication and centrifugation. A standard solution of dopamine hydrochloride was used to make comparisons for both qualitative and quantitative tests. Qualitative tests for dopamine detection included the enzyme polyphenol oxidase and Thin-Layer Chromatography (TLC). TLC analysis used Glacial Acetic Acid: Butanol: Water (1:4:1 v/v) as a mobile phase and yellow bands were observed when bromophenol blue was sprayed onto the wet TLC plate. Quantification was carried out using UV spectrometry at a λ max of 300nm, the results demonstrated that *lactobacillus sp.* yields the highest quantity of dopamine.

KEYWORDS: Dopamine, Gut-microbiome, *Lactobacillus sp.*, *Saccharomyces sp.*, Probiotics

INTRODUCTION:

Mental health is a problem worldwide. When the gut microbiomes of individuals struggling with psychiatric issues were analysed, every one of them showed to have great differences concerning the composition of the microbiota. Recent studies within the subject of neuropsychology and those studying mental health issues includes a hypothesis that bipolar disorder, schizophrenia, and other psychological or neurological conditions may additionally be associated with shifts/alterations in the microbiome. There is a hypothesis that any disruption to the normal, healthy balance of microorganisms within the microbiome can be the reason for the immune system overreact and cause inflammation of the GI tract and in turn, the effects of which are seen not only physiologically on your body but also neurologically. As research continues to gain an insight into the intricate details of how the gut-brain microbial network functions, it could be a breakthrough in treating psychiatric disorders [1][2].

The human gut contains 10^{13}-10^{14} microorganisms, and 100 times more genes than our genome, weighing about 1kg in the adult. The communication between the brain and the microbiota is bidirectional, through multiple pathways: neural through the Vagus nerve [3][4] and/or spinal cord, endocrine (through the hypothalamic-pituitary-adrenal, HPA, axis), immune (cytokines), and metabolic [4]. The possibility that microscopic organisms teeming the gut can influence the body, yet in addition the psyche, offer the tantalising possibility of using beneficial, or probiotic, microscopic organisms to treat disorders and mood, for example, ADHD, Alzheimer's and Schizophrenia [6] either by regulating helpful microorganisms themselves or by creating drugs that emulate their metabolic capacities.

Gut microscopic organisms additionally produce several neurotransmitters that the cerebrum uses to control essential physiological functions such as cognition, sleep, motor control and reward and reinforcement behaviour [7]. It is the main organ to flaunt its autonomous sensory system, a system of 100 million neurones implanted in the gut lining.

Bacteria such as Lactobacillus species are found to yield acetylcholine and gamma-amino butyrate (GABA), *Bifidobacterium* is a known producer of GABA, *Escherichia* secretes norepinephrine, serotonin and dopamine, *Streptococcus & Enterococcus* are producers of serotonin and *Bacillus* yields norepinephrine and dopamine [8]. Dopamine (DA), a monoamine catecholamine neurotransmitter, is naturally produced in the substantia nigra, ventral tegmental area, and hypothalamus of the brain. Dopamine acts on dopamine receptors to regulate motor and non-motor function in a specific manner [9].

DA has numerous functions in the brain affecting: Cognition, voluntary movement, motivation, punishment and reward, sleep, dream, memory and learning. As well as other functions that affect physiology: improving blood flow to the kidney and increasing the pumping strength of the heart [10].

The chief source of DA in the brain are the neurons of the midbrain. Faulty dopamine neurotransmission leads to several pathological conditions, such as Hyperprolactinaemia [11], depression, autism spectrum disorder, schizophrenia, ADHD (Attention Deficit Hyperactivity Disorder) and Parkinson's disease [1][12]. Conditions stated above have no permanent cures but rather heavy doses of antidepressants and antipsychotics prescribed that in the long run do more harm than good. An alternative could be a daily intake of probiotic cultures of gut microbes that synthesise DA in moderation along with other essential compounds that are a requirement of the body. Known DA producers are *Lactobacillus sp. & Saccharomyces sp.*

Nutritional research has been standing at the core of medical, economic, cultural and social research. "Let food be thy medicine" was coined by Hippocrates over 2,000 years ago [13] and based on accumulating scientific evidence, emerging dietary supplement/treatment can to some extent be used for, stopping depression, bipolar disorder, schizophrenia, and anxiety disorders, attention deficit disorder/attention deficit hyperactivity disease (ADD/ADHD), autism, and addiction [14]. Frequently prescribed medication (mostly anti-depressants) cause uncomfortable side effects this causes the patients to pass on taking their

medications. Such non-compliance is a frequent prevalence encountered via psychiatrists [15]. Supplementing the diet with probiotic bacterial strains aspires to replenish the gut with bacteria that are greatly expedient, which produce metabolites advantageous to the host. These affiliations provide strong evidence for a whole new outlook on the importance and the functioning to the gut microbiota [16].

MATERIALS AND METHODS

1. **Isolation of Gut microorganisms:** To study dopamine production, *Lactobacillus sp* and *Saccharomyces sp* were isolated from Yakult and homemade curd. A loopful of the sample was collected and inoculated on a Sterile MRS (DeMan, Rogosa and Sharpe) [17] agar plate and Sabouraud's agar (Sab) [18] plate (with chloramphenicol) respectively. The curd used was 24 hours old. After incubation under anaerobic conditions at 37°C, appropriate colonies that were selected, purified, sub-cultured, stored for further studies and subjected to identification tests.

2. **Identification of the isolates:** The isolates of the selected strains were identified based on the cultural, morphological and biochemical tests such as Gram staining, sugar fermentation, catalase test and monochrome staining, as outlined in Bergey's Manual of Systematic Bacteriology [19].

3. **Culture medium and Conditions:** Following purification, a 0.1 ml culture (adjusted to 0.1 O.D) was inoculated in 100ml of Sterile MRS broth which consisted trace amounts of amino acid, tyrosine and was incubated at 37°C under anaerobic conditions for 4-5 days [20]. Uninoculated Sterile MRS broth was used as a control.

4. **Extraction of Dopamine:** A crude extract was obtained by sonication and centrifugation. This served as the sample for further qualitative and quantitative tests.

5. **Ultra-Sonication for cell lysis:** 10ml was aliquoted from the broths into a Petri plate. An ultrasonic bath sonicator with the temperature set to 35°C and a timer for 3 minutes and was used (repeat for 7

cycles, with intermittent cooling). After sonication, the broth was transferred to a medium-sized falcon tube [20].

6. **Centrifugation:** The tubes were centrifuged for 40 minutes at 5000rpm or 23 minutes at 8000rpm. The supernatant was subsequently collected in saline and stored at 4°C [20].
7. **Thin Layer Chromatography:** Crude sample extracts and standard 1mg/ml dopamine hydrochloride solution were spotted onto a hardened TLC plate. The mobile phase used was Glacial Acetic Acid : Butanol : Water in the ratio of 1:4:1 (v/v). Bromophenol Blue in 5% NaOH was used as the visualising agent, [20] was prepared before use and stored in dark. The plate was removed and allowed to dry for 15 minutes. The dye prepared was then sprayed onto the plate. The samples were compared to the standard.
8. **UV Spectroscopy:** A standard dopamine hydrochloride solution of 1mg/ml (1mg standard to 1ml MRS broth) was used to find the λ max using a UV-Vis Spectrophotometer [21]. A dopamine standard chart was drawn for different working solutions ranging from concentrations of 1-30mg/ml. The diluent was used as the blank. Absorbance at λ max was measured against a reagent blank for the samples, concentration estimated and. compared.

RESULTS:

1. **Identification *Lactobacillus sp.*:** Gram staining was carried out along with Catalase test, Sugar Fermentation test, Indole test, Methyl Red test, Citrate test and Voges Proskauer test and, on the basis of the Bergey's Manual of Systemic Bacteriology we can indicate that the isolated culture is *Lactobacillus sp.*

Colony Characteristics	
Shape	Round
Margin	Wavy
Elevation	Flat
Surface	Mucoid
Texture	Dry
Opacity	Translucent
Size	1-2mm
Gram Nature	Gram-Positive
Cell Shape	Slender Rods

Table 1: Colony Characteristics for *Lactobacillus sp.*

Biochemical Tests	Standard	Observed
Indole	Negative	Negative
Methyl Red	Negative	Negative
Voges Proskauer	Negative	Negative
Citrate utilization	Negative	Negative
Catalase	Negative	Negative
Oxidase	Negative	Negative
Sugar Fermentation		
Glucose	Positive	Positive
Maltose	Negative	Negative
Mannitol	Negative	Negative
Sucrose	Negative	Negative
Xylose	Negative	Negative
Lactose	Negative	Negative

Table 2: Identification tests for *Lactobacillus sp.*

2. **Identification for *Saccharomyces sp.*:** Was characterized by its growth as round, white pearly colonies on Sterile Sabouraud's agar with chloramphenicol and then performing monochrome staining and observing the eukaryotic cells under the microscope.

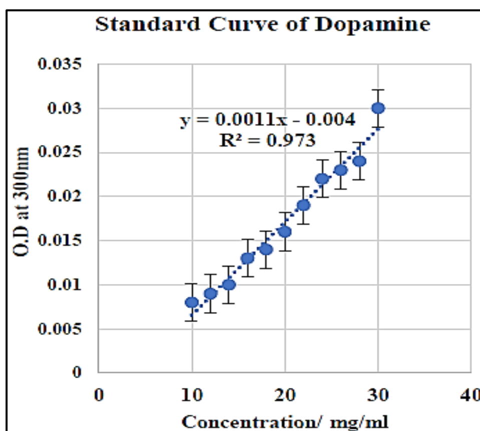
Fig 1: Saccharomyces sp. colonies on Sab agar

3. **Qualitative analysis by TLC:** The presence of dopamine was analyzed using bromophenol blue prepared in 5% NaOH. Rf values were calculated for both.

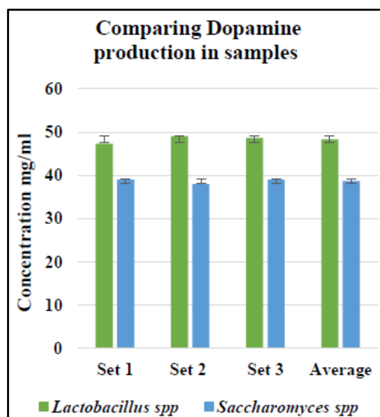
Sample	Rf Value
Standard Dopamine	0.70
Lactobacillus sp.	0.71
Saccharomyces sp.	0.71

Table 3: Rf values for TLC

4. **Quantification by UV Spectroscopy:** λ max was found to be 300nm and the standard graph (refer Graph 2) was used to calculate the concentration of dopamine in samples.



Graph 1: Standard Curve of Dopamine at 300 nm



Graph 2: Comparative Dopamine production in samples

The graph obtained from standard dopamine concentrations aided in yielding the equation of the line and in turn the R^2 value. The equation was then used to calculate the concentration of dopamine in samples.

Table 4 shows the statistical analysis of the data. A single mean/sample t-test was carried out which is a form of an inferential statistical tool. The t statistical value for the samples was calculated from the standard deviations. A high value of the sample is indicative that the results obtained were significant. Thus, the null hypothesis can be rejected i.e., the organisms do not produce any dopamine.

Organism	**Dopamine concentration (mg/ml)**			**Average Dopamine Produced**	**Sample Standard Deviation**	**Single Mean**
	Set 1	Set 2	Set 3			
Lactobacillus Sp.	47.3	49	45.6	48.3	0.888819	94
Saccharomyces Sp.	39	38	39	38.67	0.577350	116

Table 4: Dopamine concentration in samples (T-Test Value (T crit= 2.92) at α= 0.05)

DISCUSSION:

Scientists have been looking forward to substituting synthetic drugs with natural products for a long time now, essentially for diseases that have no apparent cure and have a lifelong dependence on heavy doses of medicine. The current study highlights the importance of probiotic organisms, which are a part of the normal flora of the gut, in the regulation of several neuroactive compounds.

Food items consumed daily by the majority of the population such as curd and probiotic drink, Yakult, were used for isolation of microbes which have proven to be advantageous to humans. These organisms are capable of secreting Dopamine as seen, which is an essential neurotransmitter required by the body for various cognitive, neuroendocrine and physiological purposes.

Lactobacillus sp. isolated from Yakult was characterised by Gram staining where Gram-positive, short purple rods were seen. Biochemical tests were in accordance with the Bergey's Manual of Systemic Bacteriology [19], hence, on the basis of these results and those seen in [17] we can safely say that the isolated organism belongs to *Lactobacillus sp.*

Saccharomyces sp., isolated from the sample was identified up to the genus level using colony characteristics and morphological studies. Identification was based on microscopic studies. Existing literature [18] which have isolated different strains of yeast from edible sources such as fermented goat milk have yielded comparable results.

Dopamine presence was qualitatively assessed by using thin-layer chromatography. When bromophenol blue was sprayed, all 3 samples showed yellow coloration. To confirm that the analyte eluted was dopamine, Rf values were calculated and the samples had an identical value to that of the standard. Since TLC works on the principle of separation by polarity, the mobile phase and visualising agent used are highly specific to the analyte in question and it unlikely that any other substance present in the crude extract will give a similar result. These results were obtained when the experiments were repeated as well. Previous studies [20] have also yielded similar results.

UV spectroscopy was used to assess the quantity of dopamine produced by the isolates. Studies have previously quantified dopamine presence by using dyes such as Prussian blue [20] or potassium ferrocyanide [21]. This particular study focused on obtaining the λ max and obtaining a standard chart for dopamine without the use of a dye. Amount of dopamine proved to be significantly higher than in studies previously conducted, such as 0.34mg/ml [20], as compared to this study which obtained 48.3mg/ml.

Although studies have highlighted the importance of the balance of gut microbiota in healthy adults, it is even more essential to maintain them in diseased individuals. This study has further implications on nutrition, such that's foods rich in probiotic organisms and those in essential amino acids can be the alternative to synthetic medicine everyone is waiting for. The effects of modulation of these microbes can be studied further on several diseases and behaviour.

CONCLUSION:

Lactobacillus sp. isolated from the probiotic drink, Yakult. Along with this *Saccharomyces sp.* was isolated from curd. The isolates were found

to be capable of producing the neurotransmitter, Dopamine. The crude extract served as a sample to run TLC analysis and UV Spectroscopy. Dopamine standards were prepared for both the techniques to use for comparison. UV spectroscopy revealed that *Lactobacillus sp.* produced 48mg/ml. Dopamine *Saccharomyces sp.* yielded 38mg/ml.

Dopamine producing organisms belonging to the gut microflora can be a promising alternative to conventional treatment methods for autism, depression, anxiety, Parkinson's and ADHD. Future studies will provide insight into the development of novel treatment strategies (probiotics or pharmacological), for gastrointestinal disorders that are associated with altered signalling from the bowel to the brain.

The study outlined above indicates that there is an increasing need to understand the molecular, cellular and physiological basis of enteric microbiome-gut-brain communication. Certain probiotic strains can modulate various aspects of the microbiome-gut-brain axis. However, these effects are bacterial strain-dependent, the accumulating data suggest a clear ability of probiotic and potential probiotic strains to modulate brain and behaviour.

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Chapter 15 - A Study on Comparison of Efficiency of Low Density Polythene (LDPE) Degradation by Microorganisms Isolated from soil under Aerobic and Anaerobic Conditions

Ranjika Bhattacharya, Dr. Sejal Rathod*, Ms. Hajra AS. Gupta*
Department of Biotechnology, Kishinchand Chellaram College,
Churchgate, Mumbai-400020.
Email: sejal.rathod@kccollege.edu.in

ABSTRACT:

Plastic has many desirable properties and thus, possesses several varied applications. This has led to a rise in its production and over the years, it has caused plastic pollution. Biodegradation is an affordable and more environment friendly method compared to other techniques currently available to get rid of plastic. The aim of this study is to isolate LDPE degrading microbes from soil samples collected from 4 different sources in Mumbai and compare their respective LDPE % degradation rates. Isolation of potential plastic degraders was carried out on Bushnell Haas agar containing LDPE strips. The LDPE biodegradation rate was calculated by weight loss measurements after an incubation period of one month, under aerobic or anaerobic conditions. For identification of the bacteria, gram staining and MALDI-TOF were performed and colony characteristics were studied. For identification of fungi, Potato Dextrose agar containing chloramphenicol was used as the selective media, followed by LPCB (lacto phenol blue) staining and referring to keys by Raper and Fennell. Two facultatively anaerobic bacterial isolates, Bacillus sp. and Staphylococcus cohnii ssp. urealyticus with LDPE degradation rates of 9.8% and 5.57% and a highly aerobic fungus, Aspergillus niger, with a degradation rate of 12.13% were found. Fungi showed maximum rate of biodegradation. Bacillus species exhibited a degradation capacity of almost double to that of Staphylococcus species. To improve the biodegradation capacity, the optimum conditions for microbial growth and enzyme production can be assessed and these findings can be applied commercially on a larger scale.

KEYWORDS: Low density polythene, biodegradation, plastic, eco-friendly, aerobic, anaerobic, *Bacillus*, *Staphylococcus*, *Aspergillus niger*

INTRODUCTION:

Solid waste consists of unwanted solid materials produced as a result of human activities in housing, industrial or commercial regions. It has been found that solid waste comprises 10% of the total waste generated. Out of this, up to 60-80% is plastic waste which collects in seas and on land. In 1950, 0.5 million tonnes per year of plastic waste was generated. Since then, figures have skyrocketed and have reached an alarming rate of 230 million tonnes per year [1][2]. Central Pollution Control Board (CPCB) reports state that currently plastic usage in India stands at about 8 million tonnes per year out of which 5.7 million tonnes of waste is produced [3].

Plastic waste has escalated over the years due to a surge in demand, sales and consumption of plastic. This is mainly because plastic has many desirable properties such as it is quite strong, durable, light weight, elastic, cheap to produce, resistant to corrosion and has very high thermal and electrical resistance, making it ideal for varied applications [4].

A major problem caused due to plastic pollution is ingestion of microplastics (fragments smaller than 5 mm in size) [5] by aquatic animals such as turtles, whales, seals that accidentally mistake them for food. This can cause blockage of their digestive systems and may lead to death by suffocation or starvation through reduced feeding stimuli, gastrointestinal blockage, decreased secretion of gastric enzymes and low levels of steroid hormones, causing reproduction problems in the future [6]. Marine creatures may also get caught in discarded plastic items like fishing nets [7]. Suspended plastic particles in the ocean may consist of highly toxic chemicals like polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), bisphenol A (BPA), etc. [8]. Many of these compounds can undergo severe biomagnification and may be a direct risk to human health and in some severe cases may even lead to developmental impairment, cancer, endocrine disruption, neurobehavioral changes, arthritis, breast cancer, diabetes and DNA hypomethylation [9].

Since plastic is non-biodegradable or may potentially take several years to degrade [10], it continues to accumulate in landfills or dumping grounds which may have an overall negative impact on the economy since it imposes an additional cost of cleaning and disposal to the community and affects tourism adversely [11]. Plastic pieces were also found to interact with water and form hazardous chemicals which then seep underground and degrade the groundwater quality [12].

Polyethylene (PE) is the most common type of solid waste and makes up to 64% of total synthetic plastic. It is produced by addition or radical polymerization of of ethylene (C_2H_4) molecules, in the presence of Ziegler Natta and Phillips catalysts [13].

The current majorly used methods used to get rid of plastic include incineration and dumping in oceans or landfills. During incineration, toxic substances like dioxins, furans, heavy metals (e.g.: chromium, copper, cobalt, selenium, lead cadmium, mercury) and greenhouse gases are released into the environment that contribute to severe air pollution. It was also found that this led to an increase in cardiovascular diseases, respiratory ailments, rashes, and damage to nervous system [14]. Improper dumping of plastic wastes contaminates the environment and may have an adverse effect on the quality of the surroundings which impacts the biodiversity [15]. These methods have major limitations making them ineffective, thus, alternatives for polymer degradation are necessary. Oxidative degradation occurs in presence of oxygen free radicals.

It is of 2 types: Photo degradation (occurs in presence of UV light) and thermos degradation (occurs at elevated temperatures) [16]. Comparatively, biodegradation is more affordable and environment friendly method making it widely accepted, even though, its efficiency is moderate. This is a natural process wherein different types of microorganisms such as bacteria, fungi and algae degrade complex organic materials. Organic material can be degraded either aerobically or anaerobically. Aerobic biodegradation mostly occurs in landfills and it leads to the production of water and CO2 whereas anaerobic biodegradation occurs mainly in composts and results in the formation of water, CO2 and methane as end products [17]. Different microbes play

different roles throughout the process such as breakdown of the polymer into smaller constituents or utilization of monomers or excretion of simple waste compounds as by products or usage of the by-products. Some examples of such microbes are: bacteria (*Psuedomonas, Streptococcus, Micrococcus and Moraxella*), fungi (*Aspergillus*) and actinomycetes [18].

The main objective of this study is to isolate, analyze the LDPE degradation rates and identify the potential isolates exhibiting plastic decomposition activity from various soil samples from Mumbai city.

MATERIALS AND METHODS:

1. **Sample collection:** Approximately, 20-30 grams of soil samples (4-5 cm from the soil surface) were collected from 4 different locations in Mumbai and stored in sterile, airtight containers.

Soil Sample	Location
Garden Soil	Lokhandwala
Forest	Versova
Dumping Ground	DN Nagar
Mangrove Soil	Lokhandwala

Table 1: Soil sample type and location

2. **Enrichment and isolation of potential LDPE degrading microbes:** 20 g of each soil sample type was added separately to a flask containing sterile 200 cm^3 Bushnell Haas broth [19] and 0.5 g of sterile 1cm^2 LDPE strips, in order to prepare 4 sets. After incubation for 30 days at room temperature, a loopful of inoculum from all 4 sets was streaked separately on sterile Bushnell Haas agar plates coated with sterile 0.5 g 1cm^2 LDPE strips [20]. These four plates were further incubated at 37 °C for 5 days.

3. **Obtaining pure culture and checking growth under aerobic and anaerobic conditions:** appropriate colonies with fast growth on the LDPE strips were selected and purified on sterile Bushnell Haas agar plates under aseptic conditions and incubated at 37°C for 5 days. They were sub-cultured on sterile nutrient agar slants and

stored for further studies. The growth of these isolates was further checked under anaerobic conditions. Each isolate was streaked on a sterile Bushnell Haas agar plate which was then placed in an airtight, anaerobic glass jar saturated with CO_2 for 5 days [21].

4. **Bulk growth of pure isolates:** a loopful of each pure isolate was inoculated in sterile 100 cm3 nutrient broth [22].

5. **Determination of LDPE degradation rates of isolates:** The bulk growth of each pure isolate (approximately 0.25g) was added to a conical flask containing sterile 100 cm^3 Bushnell Haas broth and sterile 0.5 g 1cm^2 LDPE strips. The flasks containing aerobic cultures were incubated at 37°C for 30 days and the flasks containing facultative anaerobic/ anaerobic cultures were placed in an airtight anaerobic glass jar for 30 days. A control flask was simultaneously maintained. After 30 days, the LDPE strips were extracted from the broth, washed with tap water first and then distilled water, cleaned thoroughly using 70% alcohol and finally dried on filter papers. The final weight of the strips was recorded and the LDPE degradation rates, i.e., % weight (wt) loss for the respective isolates was calculated by the given formula [19]:

$$LDPE\ deg\ rate = \frac{Initial\ wt - final\ wt\ x\ 100}{Initial\ wt}$$

6. **Identification of the isolates:** the bacterial isolates were identified based on gram staining, colony characteristics and MALDI-TOF MS (matrix-assisted laser desorption/ionization time of flight mass spectrometry) results [23]. Fungal isolates were identified by using potato dextrose agar (with chloramphenicol) as a selective media [24], LPCB (Lacto Phenol Blue) staining and referring to keys by Raper and Fennell [25].

RESULTS:

1. **Isolation of potential LDPE degrading microorganisms:** After enrichment for 30 days, only sets 2, 3, 4 showed the presence of microbial colonies growing on LDPE strips overlaid on solid

Bushnell Haas agar media. These colonies were selected and studied further.

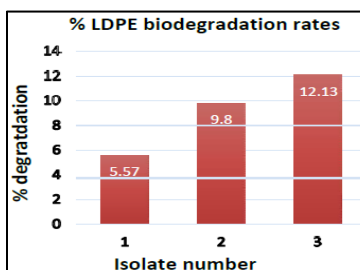
2. **Checking growth of pure isolates under aerobic and anaerobic conditions:** 3 pure colonies that were rapidly seen to be growing on LDPE were selected and their oxygen requirements were determined. The bacterial isolates were found to be facultatively anaerobic while the only fungal isolate was highly aerobic.

3. **Determination of LDPE degradation rates of isolates:** Sets 1 and 2 (Fig 1) contained bacterial isolates 1 and 2, respectively and set 3 (Fig 2) had isolate 3 which was a fungus. The final weights of LDPE strips were recorded for all 3 isolates and the LDPE biodegradation rate was calculated and compared (Graph 1).

Fig 1: Control, Set 1, Set 2 (Left To Right); Fig 2: Set 3

Table 2: LDPE Biodegradation Rates

Isolate	Initial wt (g)	Final wt (g)	LDPE% Biodegradation Rate
1	0.503	0.475	5.57
2	0.510	0.460	9.8
3	0.445	0.391	12.13
Control	0.520	0.520	0



Graph 1: Comparison of biodegradation rates of isolates

4. **Identification of bacterial isolates:** Gram staining performed, colony characteristics studied (Table 3) and MALDI-TOF MS done to identify the isolates. Isolate 1 was *Staphylococcus cohnii sp. urealyticus* (Fig 3) and isolate 2 was *Bacillus subtillis/ amyloliquefaciens/ vallismortis* (Fig 4).

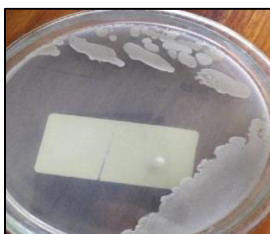


Fig 3 and 4: *Staphylococcus sp*. on NA and *Bacillus sp*. on NA

	Isolate 1	**Isolate 2**
Size	1mm	3mm
Shape	Circular	Circular
Colour	White	White
Margin	Entire	Entire
Elevation	Raised	Raised
Surface	Smooth and Shiny	Smooth
Consistency	Butyrous	Butyrous
Opacity	Translucent	Opaque
Pigmentation	Absent	Absent
Gram Nature	+	+

Morphology	cocci	baicilli
Arrangement	Singles or in pairs	Singles or in pairs
Motility	Non-motile	Motile

Table 3: Colony characteristics of bacterial isolates

5. **Identification of fungal isolates:** LPCB staining was done, colony and morphological characteristics (Table 4) studied and keys by Raper and Fennell to identify the fungal isolate as *Aspergillus niger* (Fig 5) were followed [25].

Fig 5: *A. niger* colonies on PDA

Details	**Observations**
Differential media used	Potato Dextrose Agar (PDA) + chloramphenicol
Colony colour	Initially, cottony white. Brownish-black after 2-3 days
Morphology	Globular vesicles covered by metulae and phialides, Long conidiophores, Spores produced from conidial heads, Septate hyphae
Margins	Entire

Table 4: Colony and morphological characteristics of *A. niger*

DISCUSSION:

Plastic pollution is one of the major problems that the world faces today. Scientists are looking for methods that can help eliminate this ecological risk. Biodegradation has gained popularity over the years since it is an affordable and eco- friendly process [18]. The current study highlights the importance of microbes which are naturally present in soil, in the degradation of LDPE.

Soil is abundant in various types of microorganisms; thus, it was the ideal sample for isolation of desired isolates exhibiting LDPE degradation activity. Soil was collected from 4 locations: garden, forest, dumping ground and mangroves. Bushnell Haas broth was used as an enrichment medium since it contained all the nutrients necessary for microbial growth except a carbon source. Hence, in this case, the LDPE strips added were the sole hydrocarbon source available to microbes. Enrichment was done for 30 days which gave the microbes in the soil adequate time to get acclimatized to utilizing LDPE as carbon source.

3 colonies that were potential plastic degraders were isolated from forest, dumping ground and mangrove soil. 2 bacterial isolates (facultatively anaerobic) and 1 fungal isolate (highly aerobic) were found. Bacterial isolate 1, *Staphylococcus cohnii sp. Urealyticus*, was characterized by gram staining where gram-positive cocci were seen. Bacterial isolate 2, *Bacillus subtillis/ amyloliquefaciens/ vallismortis*, was characterized by gram staining where gram-positive rods were seen. 16s rrna sequencing can be performed in the future to accurately determine the species. For both isolates, colony characteristics were studied and MALDI-TOF MS was carried out to confirm the identity of the isolates. On observing the fungal isolate under 40X, conidial heads, long conidiophores and septate hyphae could clearly be seen. Metulae and phialides were seen to be covering the globular vesicle. Spores produced from conidial heads were seen as well which is a distinguishing feature of *Aspergillus niger*. On purification on potato dextrose agar, it was seen that the colonies were initially colourless and after 2-3 days, they became brownish-black. To confirm that the fungus was *A. Niger*, keys by Raper and Fennell were referred to as well [25].

A.niger had the greatest LDPE biodegradation rate of 12.13%. This result is in accordance with existing literature that states fungi have a much higher biodegradation capacity compared to bacteria [26][27]. Bacillus sp. Had a rate of 9.13% which was almost double that of staphylococcus sp. That had a rate of 5.57%. Previous studies have yielded similar results [26][28][29].

CONCLUSION:

Various polythene degradation/disposal techniques are available such as incineration and recycling but they may not always be effective. In this case, biodegradation is a suitable alternative since it is the cheapest, eco-friendly and widely accepted. Microbes release extracellular enzymes such as lignin peroxidase, manganese peroxidase to degrade the polythene. There is a huge demand in exploring these microbes which can grow in different conditions and, under specific stress conditions, may be directed to grow and use the plastic carbon polymers as energy source.

Bacillus sp., *Staphylococcus sp*. and *Aspergillus niger* were isolated and grown under laboratory conditions from soil samples and checked for their LDPE degradation capacity. Bushnell Haas media was used primarily for this study for screening of these microbes since it contains all the necessary nutrients required for growth but does contain a carbon source. LDPE was the sole hydrocarbon source so only microbes that were capable of utilizing this plastic could grow. After an incubation period 30 days, the data obtained from the microbial population measurements showed significant differences between the inoculated and un-inoculated treatment (control) with the selected microorganism. The highly aerobic fungus *Aspergillus niger* was found to be the most efficient LDPE degrader with a total degradation rate of 12.13%, followed by *Bacillus subtillis/ amyloliquefaciens/ vallismortis* with an overall degradation rate of 9.8% and finally, *Staphylococcus cohnii sp. urealyticus* with a mere degradation rate of 5.57%.

The above bacterial isolates were identified by gram staining, studying their various colony characteristics and conducting MALDI-TOF MS.

The fungal isolate was identified by LPCB staining, studying its various morphological characteristics and following the keys of Raper and Fennell. The results of this study showed that the isolates obtained exhibit high efficacy in degrading LDPE.

In further studies, these microbial strains can be used to reduce the LDPE content in the natural environment where plastic waste is rapidly accumulating. Biodegradation can be enhanced by processes such as abiotic hydrolysis, photo-oxidation and physical disintegration which will enhance the surface area of the polymer and reduce its molecular weight; facilitating microbial degradation (Singh et al. 2007). It is also important to consider the role of microbial interactions, as well as physical and chemical parameters in the biological degradation of LDPE in real ecosystems. Since biological degradation in natural environments can be a cooperative process, the role of each factor must be determined, including the negative impact on the biodegradation rate.

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SECTION 4 - ECONOMICS

Chapter 16 - Economic Thought of Kautilya's Arthashastra and its Contemporary Relevance on Taxation

Akanksha Milind Vaidya, Dr. Hiral Sheth*
Department of Economics, Kishinchand Chellaram College,
Churchgate, Mumbai-400020.
Email: hiral.sheth@kccollege.edu.in

ABSTRACT

Kautilya, also known as Chanakya is the most illustrious political economist of all times. Kautilya wrote Arthashastra around 300 B.C. In Arthashastra he has not only focused on political and economic aspects but also on socio-culture and life lessons too. Even though Kautilya wrote Arthashastra two thousand and three hundred years ago, it has contemporary relevance in political and economic aspects. This paper demonstrates that the current structured tax system is derived from Arthashastra itself. The interpretation of this paper is to leave a mark on the minds that all economic system and activities (Taxation) trace back to Kautilya's Arthashastra and it has contemporary relevance in the 21st century. This research is based on Secondary data. Most of the findings have been taken from journals, books and articles. This research paper is an attempt to prove that Kautilya's Arthashastra's economic thoughts hold contemporary relevance in the area of Taxation.

"The nation should have a tax system that looks like someone designed it on purpose" - William Simon

KEY WORDS: Kautilya, Economics, Arthashastra, Taxation, Revenue.

INTRODUCTION

Arthashastra is India's ancient treatise. The Arthashastra of Kautilya, written by Chanakya, in the year 321 A.D, the original name of Chanakya was Vishnugupta, but it was also called Chatur; which was known as "Chanakya" in the state. It is known as "Kautilya" because of administration of the state. The Arthashastra is a famous Indian ancient treatise encompassing many aspects of human life. It was written around 300 B.C, Artha means "Wealth" and Shastra means "Science". Thus, Arthashastra literally implies the science of acquiring and managing wealth. The Arthashastra deals with Economics, Administration,

Political Ideas, Ecology and various other topics. Kautilya's Arthashastra is fundamentally a book of state management. Arthashastra comprises of fifteen books of which the first five deal with internal administration, the next deals with relations with bordering states and rest two are miscellaneous in character. Apart from Kautilya's Arthashastra he has written several other books such as Chanakya-Sutra (rules of science) and Chanakya-Rajanitishastra (science of government policies). Kautilya is India's most illustrious political economist of all times. He was a true statesman who bridged the gap between experience and vision. For him good governance was paramount, the discussion in Arthashastra is as relevant today as it was in Kautilya's times. He emphasized the importance of the accounting method in economic enterprise to properly measure economic performance. Kautilya presents a view of purpose of economics and functions of the king before examining his views about the treasury and taxation.

Kautilya's works can also be used to illustrate several aspects of economics, such as the principle and agent problems, information asymmetry, urban bias, principle of taxation, the importance of national economic, accounting and census data. Such aspects are outlined and before concluding, further attention is given to Kautilya's views on Principle of Taxation and its contemporary relevance.

Objectives of the Research Paper

- To explore Kautilya's economic view on taxation.
- To investigate the relevance of Kautilya's economic ideas on taxation in modern times.
- To acknowledge that, the Principle of Taxation & Revenue Sources given by Kautilya are still pertinent in Tax structure of India.

Rationale of the Research Study

Arthashastra was written by Chanakya over 2300 years ago. Though it has contemporary relevance in today's time, Kautilya's writings were not discovered until early twentieth century. Later on, economists and philosophers introduced Kautilya's economic ideas which Arthashastra embodies. Hence this research is an approach to demonstrate that Kautilya's economic thoughts on taxation are still pertinent in modern times. The principle of taxation given by Kautilya and ways of collecting revenue are still relevant today.

Research Methodology

This is an analytical study. It is based on secondary data collected from books, journals and research articles.

FISCAL MANAGEMENT

Chanakya paid supreme importance to the maintenance of a rich treasury, which favourably affected entire activities of the administration. He paid a lot of attention to good fiscal management and the ways to development all the sectors of the economy. To him public revenue did not exist for the pleasure of the king but as a fund to be utilised and to augment the wealth of nations. Kautilya admitted, taxation is the main source of revenue. He argued that both the provisions of infrastructure and having a larger army were dependent on tax revenue. Kautilya understood the importance of tax revenue as he wrote "All state activities depend first on treasury." Therefore, a king shall devote his best attention to it. A king with a depleted treasury eats into the very vitality of the citizens and the country. Kautilya suggested that a king starts his day by receiving 'reports on defence, revenue and expenditure.' So that it will bring prosperity to the people and they will never face financial crisis.

Revenue Sources

"*Kosha Moola Danda*." Chanakya wrote in the first chapter of Arthashastra; which means "Revenue is the backbone of the administration." This verse in the Devanagari script is also a part of the official logo of the Income Tax Department of India. In Arthashastra, Kautilya has mentioned various types of taxes and duties such as those imposed on agricultural produce, trade, tolls and custom duties. According to Kautilya, the collector general shall attend the collection of revenue. Kautilya imposed tax on every commodity. The revenue was supposed to be collected from Durga (forts), Rashtra (country parts), Khrai (mines), Setu (buildings and gardens), Vanam (forests), Vraja (herds of cattle), and Vanikpattha (roads of traffic).

Kautilya identified an exhaustive list for the sources of revenue and put the actual administration of revenue collection on the department under which that particular activity was covered, the collector general was to consolidate the revenue from all departments into the central treasury. Thus, an institutional mechanism was set up. The system is very similar to the modern system of revenue collection and management, whereby

the actual collection was decentralized while the consolidation and audit was centralized.

Sources of revenue mentioned in Arthashastra under their corresponding modern categories can be highlighted as below:

1. **Corporate Taxes:** The corporations or guild of artisans and handicraftsmen (Karusilpiganah).
2. **Indirect Tax:** Liquor, slaughter of animals, threads, ghee, sugar, oils (Kshara), the state goldsmith (Sauvarnika), the warehouse of merchandise, rivers, ferries, boats and ships, road cess (Vartani), ropes (Rajju) and ropes to bind thieves (Chorarajju) and minerals.
3. **Land and Property Tax:** Town houses and building sites (Vastuka), pasture ground, agricultural produce, flower gardens, vegetables gardens, fruit garden, wet fields (Setu) and a large part of tax on agriculture was received in kinds.
4. **Custom Duty or Sulkas**: It was collected on all imported duty at the city gates and the work of collecting customs duty was supervised by Sulkadhakshya or the superintendent of customs.
5. **Fees and Government Service Charges:** Tolls, fines, weight and measures, the town clerk (Nagaraka), the superintendent of coinage (Lakshanadhyakshah), the superintendent of seal and passport.
6. **Income Tax:** Produce from crown land (Sita) and the portion payable to government charges (bhaga).
7. **Entertainment Tax:** Sex workers' taxes and gambling taxes.

Most of these taxes are still there even under the present tax structure of Indian public finance. Kautilya believed "Just as one plucks fruits from a garden as they are ripen, so shall a king have revenue collected as it becomes due." Just as one does not collect unripe fruit, he shall avoid taking tax that is not due because that will make the people angry and spoil the sources of revenue. He also understood that taxation is the most important sources of income for the state, which is why revenue collection officials must be honest, dedicated and professional. According to him, negligence of duty, ignorance, corruption, arrogance, greed and non-professional attitude leads to the main reasons behind loss of revenue collection.

Principle of Taxation

Kautilya suggested ways to increase the tax base and not the tax rate to increase revenue. He was against putting any excessive tax burden on the people. He used to say that, “The king must collect taxes like honey bee, enough to sustain but not too much to destroy.” He emphasized fairness, stability of tax structure, fiscal federalism, avoidance of heavy taxation and ensuring of tax compliance and subsidies to encourage capital formation. For example, he suggested for the king, “He shall protect agriculture from being harassed by fines, taxes and demands of labour.” Similarly, he did not want the tax collection to be overzealous and collect only what was due.

Kautilya's insights into compliance issues are remarkable. According to him, ignorance of the work, neglect of duty, timidity, corruption, arrogance and greed on the part of tax officials were the main features for causing the loss of revenue. Clearly Kautilya emphasised on both revenue and efficiency. He noted that it was not easy to detect corruption. He stated “Just as it is impossible to know when a fish moving in water is drinking it, it is impossible to find out when government servants in charge of undertaking misappropriated money.” He added, “It is impossible to know even the path of birds flying in the sky but not the ways of government servants who hide their [dishonest] income.”

Therefore, Kautilya suggested heavy penalties on those officials, who misappropriated revenue. He suggested, “Those who have amassed money [wrongfully] shall be made to pay it back; they shall be transferred to other jobs where they will not be able to misappropriate and be made to discourage again what they had eaten.” On the other hand, according to Kautilya, “An officer who accomplished a task as ordered or better shall be honoured with promotion and rewards.”

It appears that Kautilya did not recommend any punishment for bribing. Since he considered the people more like victims. In fact, he suggested compensating them for their losses. He wrote, “A proclamation then be issued calling on those who had suffered at the hands of the dishonest official to inform the investigation officer. All those who respond to proclamation shall be compensated according to their losses.” That could be effective way to combat corruption since the person, who is forced to bribe might be more than willing to provide some solid evidence against the corrupt officials. The current law by treating both the giver and receiver of bribes as criminals unnecessarily protects corrupts officials.

He recommended that some enterprises such as liquor sales, betting and gambling be run by the government to generate some surplus to complement the tax revenue. He wrote "Income due to profit on sales: increase in price on commodity at times of sale, profit from the use of differential weights and increased income due to competitions from buyers." Kautilya would not under any circumstances, have approved the continued operation of public undertakings draining tax revenue by generating huge losses.

Kautilya on Growth Oriented Government Expenditure

Two points are noteworthy. First, according to Kautilya, most of the tax revenue should be used to the provisions of infrastructure. He wrote, "The [total] salary [bill] of the state shall be determined in accordance with the capacity [to pay] of the city and the countryside and shall be [about] one quarter of revenue of state."

> It implies that according to Kautilya India will get more mileage out of the tax revenue by constructing a few additional miles of highway than squandering resources on overstaffed government offices and some outdated and unproductive institutions/organisation, such as planning commission.

1. Second, Kautilya emphasised the need for tax incentives to encourage investments. However, those were very few and only for a very short duration.
2. He suggested the following:

    - Tax holidays: "Anyone who brings new land under cultivation shall be granted exemption from payment of agricultural taxes for a period of two years. Similarly, for building or improving agriculture facilities, exemptions from waters rates shall be granted.
    - Subsidised loan: "[on new settlement], the cultivators shall be granted, grains cattle and money which they can repay at their convenience."
    - Exemptions from import duty: "Any items that, at his discretion, the chief controller of customs, may consider being highly beneficial to the country. (Such as rare seeds)" are to be exempt from import duties.

Kautilya envisioned a ‘Dharmic social contract’ between the king and the citizens. His discussion of taxation includes several underlying principles: the taxation power of the state should be limited; taxes should not be heavy or excessive; tax hikes should be introduced gradually; tax should be levied in the proper place, time, and forms; and tax levels should be equitable and reasonable. Ideally, the government should collect taxes like a honeybee that sucks just the right amount of honey from the flower so that both can survive. Kautilya's scheme of taxation involved the element of sacrifice by the taxpayers, direct benefit to the taxpayers, and redistribution of income, as well as tax incentives for desired investments.

Kautilya realized the critical role of the tax system in ensuring the economic wellbeing of society. The hallmark of his tax system was the “certainty” of time, rate, and mode of payment. Stability in the tax regime was an important factor in ensuring active trade and commerce in the Mauryan empire. This, in turn, strengthened the revenue base of the state and enabled it to maintain a huge standing army and welfare apparatus. Kautilya advocated for the taxing power of the state to be limited, and taxes should be equitable and just. According to him, taxes should not be heavy and excessive. He suggested that the tax rate should not exceed sixteen percent to twenty percent [1/6th or 1/5th] of economic activities. He was aware that taxes beyond a certain limit would hamper economic activities and encourage tax evasion. However, he advised a tax rate of up to fifty percent or more for goods or services that were harmful to society in nature, such as liquor and gambling. Moreover, it is worth noting that most of the taxes were levied on land and various commodities.

The state was overzealous in the collection of taxes and trapped virtually every source. Citizens paid a toll tax, and farmers had to pay 1/6th of the produce as land tax. There was a land census at periodic intervals, and records were maintained. Traders had to pay 1/10th of the value of merchandise as tax. There was also an entry tax to enter the fort, a tax on the use of roads and waterways, and for getting a passport. Even hermits living in the forest had to pay 1/6th for grain gleaned by them as they, too, needed the protection of the king. The service industry was also taxed - actors, dancers, soothsayers, prostitutes, and auctioneers were all subjected to taxation. Pilgrims had to pay a yatra vetna (pilgrimage tax). Kautilya also emphasized the role of the intelligence department, stating

that a kingdom cannot be successful only with administrative officers. In the Arthashastra, he mentioned that the intelligence department was responsible for preventing economic offenses. He was also concerned about the expenditure side of the budget and suggested differential wage rates depending on skills, quality of work, and the nature of jobs.

Kautilya identified and discussed the many sources of state revenue and concluded that the state was zealous in the collection of revenue from all possible sources so that the accumulated wealth could be spent on the production of the state from external and internal changes, social services, and productive enterprises such as building forts, roads, planting colonies, villages, asylums, orphanages, and educational institutions.

Tax Administration: Interdependence of Ethical Conduct

1. According to Kautilya, "When people are impoverished, they become greedy; when they are greedy, they become disaffected; when disaffected they either go to the enemy or kill their ruler themselves."
2. According to Kautilya, a king should not only be honest and efficient but his administration should have those qualities too.
3. Kautilya also suggested, "Thus, the king shall first reform the administration by punishing appropriately those officers who deal in wealth; they, duly corrected, shall use the right punishment to ensure the good conduct of the people of the towns and the countryside."
4. The following table may be used to express Kautilya's ideas:

<table>
<tr><th colspan="4">Interdependence of Ethical Conduct</th></tr>
<tr><th colspan="2"></th><th colspan="2">Administration</th></tr>
<tr><th rowspan="3">Public</th><th></th><th>Honest</th><th>Dishonest</th></tr>
<tr><th>Honest</th><td>Ideal case I</td><td>Unstable case II</td></tr>
<tr><th>Dishonest</th><td>Unstable case III</td><td>Worst case IV</td></tr>
</table>

Thus, according to Kautilya either both the public and the administration were honest or both were dishonest. If the administration squandered the tax revenue, why would the taxpayer be honest in paying their taxes? Similarly, why would the administration be honest if taxpayer cheated on taxes? So Kautilya tried hard to avoid case IV.

He understood the disastrous consequence since that was harmful both to economic growth and national security by creating political instability and tempting an enemy to attack the kingdom. Kautilya did not discuss case II and case VI, perhaps realising that those were transitory. In case of aggression by an outside agency, the janapads (districts) could ask for tax remission as the king had failed in his duty to protect citizen.

CONTEMPORARY RELEVANCE OF KAUTILYA'S THOUGHTS AND TAXATION STRUCTURE IN INDIA

Kautilya, also known as Chanakya or Vishnugupta, is one of the most prominent Indian political thinkers. Although he lived a long time ago, certain principles from his theories are still relevant in today's tax system, such as Kautilya's tax system. Kautilya laid down specific terms for taxation without any scope of arbitrariness, fixed a timetable for payment of taxes, and determined what share of produce or produce value should be paid as tax. Furthermore, Kautilya's stance on progressive taxation and ability to pay principles are followed in modern days.

Kautilya's scheme of taxation involved the element of sacrifice by taxpayers, direct benefit to taxpayers, redistribution of income, and tax incentives for desired investments. Similar elements can be seen in today's tax structure. For example, a new tax reform introduced by the Modi government in August 2020, "Transparent Taxation Honouring the Honest," encourages direct taxpayers. This reform includes the idea that those who pay taxes will be honored sincerely. Two elements of Kautilya can be seen relevant here: sacrifice by taxpayers and benefit to taxpayers. Another example of the same element can be seen in the "Vivad se Vishwas Scheme," a direct tax scheme announced in the 2020 budget for settling tax disputes between individuals and the income tax department.

Kautilya advocated that the rich should pay higher taxes according to their paying capacity, and taxes should be levied once a year. Kautilya believed that taxes are a major source of revenue, so he suggested more tax on liquor and gambling during war or emergency periods to generate revenue. This principle is still relevant today. For example, due to the outbreak of Covid-19 in 2020, the government announced a lockdown for three months, which resulted in a slowdown of the economy. To boost the economy, liquor shops were opened after three months, resulting in a collection of 45 crore on the first day in Karnataka, 51 crore in Uttar Pradesh, 41 crore in Rajasthan, and 50 plus crore in Maharashtra, respectively. By taxing high on liquor, the government earns 2000 crore

tax monthly. Hence, Kautilya's principle of taxation is still relevant in the tax system.

Apart from these ideas, there are several other things in Arthashastra that are relevant, such as conservation of natural resources. Arthashastra provides basic knowledge about economics, and several of Kautilya's ideas are still applicable today.

CONCLUSION

Kautilya's Arthashastra provides valuable basis for economy. It contains useful insights about economies. It can be used as it holds relevance to our time and can be useful to illustrate several modern economic ideas. He offered a set of different economic policy measures to promote economic development in the economy. Though Kautilya's system of taxation is very comprehensive but some of the ideas expressed about taxation in Arthashastra predate it. Many of the principle of taxation that he outlined are still relevant today.

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LIST OF CONTRIBUTERS

1. Dr. Sagarika Damle
2. Dr. (Mrs.) Aashu Vajpai
3. Dr. Shalini Rai
4. Dr. Tejashree Shanbhag
5. Dr. Amruta Kothare
6. Dr. Archana Thite
7. Dr. Suvarna Sharma
8. Dr. Pratibha Shah
9. Ms. Amina Dholkawala
10. Dr. Sejal Rathod
11. Ms. Rajitha Satish
12. Ms. Hajra Gupta
13. Ms. Prachi Bhatia
14. Dr. Hiral Sheth