

IN VITRO DEGRADATION OF POLYTHENE USING MICROBIAL ISOLATES FROM PLASTIC CONTAMINATED WATER SAMPLES AND STUDYING THE EFFICACY OF MICROBIAL ENZYMATIC ACTIVITY

Project report

*Submitted in partial fulfillment of the requirements for the award of
Bachelor of Science degree in Biotechnology.*

by

Kosuru Bhavana Rao (Reg. No. 41770044)



SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

CATEGORY-1 UNIVERSITY BY UGC

**Accredited with Grade "A++" by NAAC | 12B Status by UGC | Approved by AICTE
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of Kosuru Bhavana Rao (4177044) who carried out the project entitled "*In vitro* degradation of polythene using microbial isolates from plastic contaminated water samples and studying the efficacy of microbial enzymatic activity" under my supervision from September 2023 to May 2024.

Internal Guide

Dr. J. Theboral

Head of the Department

Dr. V. Ramesh Kumar M. Phil, Ph.D

Submitted for Viva voce Examination held on 27-04-2024

27/4/24
Internal Examiner

(Dr. J. JAYASHREE)

27/4/2024
External Examiner

Dr. Rakesh S

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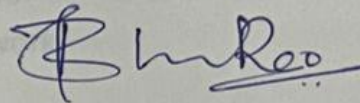
DECLARATION

I am pleased to acknowledge my sincere thanks to The Board of Management of Sathyabama Institute of Science and Technology for their encouragement in doing this project and for providing me with all the facilities. I am grateful to them.

I, Kosuru Bhavana Rao, here by declare that the Project Report entitled "***In vitro* degradation of polythene using microbial isolates from plastic contaminated water samples and studying the efficacy of microbial enzymatic activity**" done by me under the guidance of Internal guide Dr. J. Theboral at Sathyabama Institute of Science and Technology, Chennai is submitted in partial fulfillment of the requirements for the award of Bachelor of Science degree in Biotechnology.

I would like to express my sincere and deep sense of gratitude to my Project Guide Dr. J. THEBORAL for his valuable guidance, suggestion and constant encouragement paved the way for the completion of my project.

DATE: 25-04-2024



PLACE: Chennai.

SIGNATURE OF THE CANDIDATE

I wish to express my thanks to all Teaching and Non-teaching staff members of the Department of Biotechnology who were helpful in many ways for the completion of the project.

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I am pleased to acknowledge my sincere thanks to The Board of Management of Sathyabama Institute of Science and Technology for their encouragement in doing this project and for completing it successfully. I am grateful to them.

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I would like to express my sincere and deep sense of gratitude to my Project Guide **Dr. J. THEBORAL** for her valuable guidance, suggestions and constant encouragement paved way for the successful completion of my project work.

I wish to express my thanks to all Teaching and Non-teaching staff members of the Department of Biotechnology who were helpful in many ways for the completion of the project.

ABSTRACT:

Polyethylene glycol (PEG) is a versatile polymer widely used in various industrial and biomedical applications due to its water solubility, biocompatibility, and non-toxicity. However, its persistence in the environment poses significant challenges to ecosystem health. Microbial degradation of plastics, including PEG, offers a promising solution to mitigate environmental pollution. Microbes, particularly bacteria, possess the enzymatic machinery capable of breaking down complex polymer structures, rendering them more amenable to biodegradation. This study investigates the utilization of PEG by bacterial species from the genus *Paenibacillus*, focusing on its biodegradation potential. Water samples collected from various locations at Sathyabama University underwent serial dilution and were cultured on nutrient agar supplemented with different concentrations of PEG. Remarkably, bacterial growth was observed, particularly in PEG-containing media, indicating the adaptability of *Paenibacillus* species to utilize PEG as a nutrient source. Subsequent sub-culturing and RNA sequencing identified *Paenibacillus thiaminolyticus* as the predominant species. Moreover, the study determined a minimum inhibitory concentration (MIC) of 3g of PEG for bacterial growth.

These findings underscore the ecological significance of PEG-utilizing bacteria and their potential role in biodegradation processes, offering promising avenues for further research in environmental remediation and biotechnological applications.

Key words: Polyeththylene Glycol (PEG), Plastic, Biodegradation, Bacterial enzymes, *Paenibacillus thiaminolyticus*.

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REFERENCE

List of Abbreviations

BOD	- Biological Oxygen Demand
PAMCS	- Plastic associated microbial communities
PBS	- Polybutylene succinate
PDES	- Plastic degrading enzymes
PDMS	- Plastic degrading microbes
PEG	- Polyethylene Glycol
PHA	- Polyhydroxy Alkanoate
PHB	- Poly D-3-hydroxybutyrate
PET	- Polyethylene terephthalate
PLA	- Polylactic acid

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CHAPTER 1

1.INTRODUCTION

1.1 What Is PEG?

Polyethylene Glycol, a widely used synthetic polymer, poses a significant environmental challenge due to its persistence in the environment and resistance to natural degradation processes. This project focuses on the in vitro degradation of polyethylene using microbial isolates sourced from water samples contaminated with plastic debris. The primary objective of this study is to investigate the efficacy of microbial enzymatic activity in degrading polyethylene under controlled laboratory conditions. First the polymer is oxidized to carboxylated PEG by alcohol and aldehyde dehydrogenases and then the terminal ether bond is cleaved to yield the depolymerized PEG by one glycol unit. Most probably PEG is anaerobically metabolized through one step which is catalyzed by PEG acetaldehyde lyase, analogous to diol dehydratase. Whether aerobically or anaerobically, the free OH group is necessary for metabolization of PEG. Plastics in the environment represent new substrates for microbial colonization, and recent methodological advances allow for in-depth characterization of plastic associated microbial communities (PAMCS). Plastic degrading enzymes (PDES) and plastic degrading microbes (PDMS) are studied to understand the microbial mediated plastic degradation and discover biocatalysts for plastic processing (Peng *et al.*, 2017).

1.1.1 DEGRADATION PROCESS:

To use PEG for storing thermal energy, it needs to stay stable. When PEG breaks down due to heat, it creates smaller molecules, which makes the material melt at a lower temperature and reduces its ability to store heat (Bigger *et al.*, 1919). Heating PEG at 80°C in the presence of air leads to a notable alteration in its form. Following 100 hours of thermal exposure, PEG transitions from a powder with free movement to a pliable, waxy solid (Hans *et al.*, 1997). Tests conducted on the degradation of PEG in aqueous solutions using the photo-Fenton and H₂O₂/UV

processes show that both methods are viable options for treating wastewater containing PEG (Giroto *et al.*, 2010).

In another study done by (Glastrup *et al.*, 1996) has concluded that during PEG degradation, formic acid is typically formed, primarily through oxidation at the terminal hydroxyl group. Under dry conditions, formic acid and its esters are produced, along with an unstable hemiacetal of formaldehyde and triethylene glycol. Triethylene glycol, mono- and diformates are also observed. However, under wet conditions, formates are absent, resulting in the full oxidation of tetraethylene glycol.

1.2 BIO-DEGRADATION OF PLASTIC:

Plastics, consisting of carbon, hydrogen, nitrogen, oxygen, chlorine, and bromine, are synthetic or semi-synthetic organic compounds with high molecular mass, comprising long-chain polymeric molecules. They are inert, resistant to decomposition, and durable, owing to their lightweight, affordability, and malleability. Plastic's versatility allows it to be molded into various sizes and shapes, leading to its widespread application across diverse fields of life and technology. Commonly used plastics include polyethylene, polystyrene, polyvinyl chloride, polypropylene, and polyethylene terephthalate (Shah *et al.*, 2005)¹. Biodegradation is the breakdown of materials by microorganisms. Plastics degrade based on factors like mobility, crystallinity, molecular weight, and additives. Microorganisms colonize the plastic surface, reducing its molecular weight. This process starts with polymers breaking down into monomers, which then further degrade into carbon dioxide, water, and methane in a process called mineralization (Elahi *et al.*, 2021).

Some polymers are too large to pass through cellular membranes, so they first undergo depolymerization into smaller monomers before being absorbed by microorganisms. Enzymatic degradation involves hydrolysis, where enzymes break down the polymer substrate into smaller molecules like oligomers, dimers, and monomers. Microorganisms secrete enzymes like lipase, proteinase K, and dehydrogenase to aid in plastic biodegradation, with bacteria and fungi playing key

roles. Another degradation method involves the formation of clear zones when polymers penetrate agar medium, with enzymes diffusing through the agar to degrade the plastic into water-soluble compounds. Various synthetic plastics, such as PHB, PCL, and PBS, are degraded by specific bacterial strains, while natural plastics are broken down by corresponding microorganisms.

1.2.1 Biodegradable plastics:

Biodegradable plastics can include both bio-based and fossil-based polymers, depending on their degree of biodegradability and microbial assimilation. The degradation process involves enzymatic and non-enzymatic hydrolysis, with factors such as the type of organism, pretreatment, and polymer characteristics influencing efficiency. Important characteristics for degradation include mobility, crystallinity, functional groups, molecular weight, and additives. Microorganisms secrete enzymes that break down polymer complexes into smaller molecules like dimers and monomers, which are then utilized as energy and carbon sources. Biodegradation reactions occur through both aerobic and anaerobic mechanisms (Ahmed *et al.*, 2018).

1.2.2 Biodegradation mechanisms:

Various microorganisms, such as *Variovorax paradoxus*, *Comamonas sp.*, *Aspergillus fumigatus*, *Acidovorax faecilis*, and *P. lemoignei*, found in soil, have been identified as capable of degrading bio-based polymers under both aerobic and anaerobic conditions. Bacteria and fungi, predominant among these microbes, produce extracellular enzymes that facilitate the breakdown of both bio-based and fossil-based plastics. Through metabolic and enzymatic mechanisms, these microbes convert polymers into CO₂ and H₂O. Enzyme specificity varies among microbial species and strains, influencing the degradation of different types of polymers. For instance, *Bacillus spp.* and *Brevibacillus spp.* produce proteases involved in polymer degradation, while fungi containing laccases are adept at breaking down lignin through oxidation (Shah *et al.*, 2008).ⁱⁱ The activity of

microbial enzymes plays a crucial role in efficiently and sustainably degrading polymers in the environment. In aerobic conditions, bacteria utilize oxygen as an electron acceptor, leading to the synthesis of smaller organic compounds. Consequently, carbon dioxide and water are generated as final products. In anaerobic conditions, microorganisms break down polymers in the absence of oxygen. Anaerobic bacteria utilize sulfate, nitrate, iron, carbon dioxide, and manganese as electron acceptors during this process (Priyanka *et al.*, 2011).

Enzymatic degradation is the preferred method for treating plastic waste. This process involves two steps: enzyme attachment to the polyethylene substrate followed by hydrolytic cleavage. Fungi and bacteria produce intracellular and extracellular depolymerases to break down polyethylene. This results in the disintegration of complex polymers into smaller chains, which serve as a carbon and energy source for bacteria. Ultimately, mineralization occurs, producing carbon dioxide, water, or methane. Physical parameters such as temperature, pressure, and moisture can mechanically damage polymers, enhancing the effectiveness of biological degradation processes facilitated by microbial enzymes and metabolites (Tokiwa *et al.* 2004).

1.3 MICROORGANISMS INVOLVED IN BIODEGRADATION:

1.3.1 Microbes:

Bacteria: Studies mention *Pseudomonas chlororaphis* and *Comomonas acidovorans*. These bacteria produce enzymes (putative polyurethanases) that specifically target polyurethane plastics by breaking down the ester bonds (Chowdury *et al*).

Fungi: *Candida rugosa* is a fungus that produces a different type of enzyme (serine hydrolase) to degrade polyurethane. Interestingly, *Pestalotiopsis microspora*, another fungus, can utilize polyurethane as a food source, breaking it down entirely within days.

1.3.2 Enzymes:

Esterases: Break down ester bonds, commonly found in polyurethanes.

Lipases: Break down fats and oils, potentially aiding in degrading some plastics.

Proteases: Break down proteins, not directly involved in plastic degradation here.

Ureases: Break down urea bonds, not directly relevant to plastic degradation in this context.

Lignin-degrading fungi and manganese peroxidase: While not directly mentioned for plastic breakdown, these enzymes from *Phanerochaete chrysosporium* can partially degrade high-molecular-weight polyethylene under specific conditions.

TABLE: 1.1 SOURCE AND MICROBES FOR PLASTIC DEGRADATION

<i>Source</i>	<i>Microorganisms</i>	<i>Reference</i>
<i>Fungal</i>	<i>Aspergillus flavus</i>	(Bhardwaj et al., 2013)
	<i>Penicillium funiculosum</i>	
	<i>Aspergillus niger</i>	
	<i>Aspergillus oryzae</i>	
	<i>Streptomyces</i>	
	<i>Trichoderma sp.</i>	
<i>Bacteria</i>	<i>Paenibacillus thiaminolyticus</i>	
	<i>Rhizopus delemar</i>	
	<i>Firmicutes</i>	
	<i>Pseudomonas stutzeri</i>	

CHAPTER 2

2. LITERATURE SURVEY

2.1 Biodegradation of plastics:

Microbes convert complicated materials into simpler ones through a process known as biochemical transformation. Plastic polymers' physical properties are changing, and these changes most notably the loss of mechanical strength, the reduction of molecular weight, and the modification of plastic surface qualities indicate biodegradation (Ho *et al.*, 2018). The majority of studies on PEG degradation have focused on BOD and chemical oxygen demand testing of PEG and PEG-based surfactants; little attention has been paid to the enzymes involved in PEG degradation or the characteristics of PEG that influence PEG degradation. The size of these molecules is a key characteristic, and research has demonstrated that the larger PEGs with higher molecular weights are unable to penetrate the bacterial cell. As the current study has shown, it is most likely that an extracellular enzyme is the cause of the degradation due to the polymer's inability to enter the cell. However, a bacterium has been revealed to possess an internal flavin-linked enzyme that acts on tetraethylene glycol, and bacteria have been observed to utilize PEG oligomers with molecular weights of fewer than 600 (Haines *et al.*, 1975).

When PEG molecular weight increased, the overall rate of degradation generally decreased. Degradation percentages for the PEG-1000 and Methane buildup served as a major foundation for PEG-20,000 consortiums, as the other goods were primarily oxidized. *Methanosarcina sp.* was present in both consortia, and it's possible that this bacteria is what removed the acetate. During their growth phase, the pH of these consortia dropped below 6.0, but the pH of the PEG-1000 and PEG-20,000 consortia stayed between 7.0 and 7.2. This might have chosen to be antagonistic to *Methanosarcina sp* (Dwyer *et al.*, 1983). One of the biggest classes of thermoplastic polyesters, microbial polyhydroxyalkanoates (PHAs), is gaining a lot of interest as a biodegradable alternative to non-biodegradable plastics. PHB

stands for poly(D-3-hydroxybutyrate), and it is the most widely used and extensively researched PHA. These polyesters are broken down by a wide range of microorganisms in diverse conditions.

Many groups of microorganisms and enzymes with different features are currently awaiting different applications, despite the fact that different PHB-degrading microorganisms and PHB depolymerases have been investigated and characterized. This review covers the distribution of microorganisms that break down PHB, variables influencing PHB's biodegradability, and the microbial and enzymatic breakdown of PHB. Additionally, we suggest using *Streptomyces* strain MG, a recently identified thermophilic PHB-degrading microbe, to enzymatically break down D-3-hydroxycarboxylic acids like D-3-hydroxybutyric acid to produce pure monomers of PHA and other valuable compounds (Tokiwa *et al.*, 2004). The increasing use of plastics has intensified the need for biodegradable options and efficient plastic waste disposal. Advances include adding additives like pro-oxidants and starch to enhance biodegradability. Recent studies show that even traditionally resistant plastics, like polyolefins, can biodegrade after photo and chemical degradation. Microorganisms can also break down thermoset plastics, like polyester polyurethane, by cleaving ester or urethane bonds. Aliphatic-aromatic copolyesters are favored for their mechanical properties and biodegradability (Zheng *et al.*, 2005). The study done by Priyanka *et al.*, 2011; compared the biodegradation of polythene and plastic using soil samples from different sources (Medicinal Garden, Sewage Water, Energy Park, Sludge Area, Agricultural Soil). Bacteria and fungi were cultured from these samples and incubated with the materials. Weight loss was observed in both polythene and plastic, with polythene degrading faster. Various species of bacteria and fungi, including *Bacillus subtilis*, *Aspergillus niger*, and others, were found to efficiently degrade both materials by producing active enzymes. Biodegradable plastics, like aliphatic-aromatic co-polyesters, are attracting attention for their microbial degradability. New microorganisms and enzymes capable of breaking down various polyesters, including PBS, PLA, and PET, offer potential for waste treatment and bioremediation in polyester-contaminated environments (Shah *et al.*,

2014). Propylene glycol demonstrated efficacy against three organisms: *S. mutans*, *E. faecalis*, and *E. coli*, with bactericidal activity observed at concentrations of 50%, 25%, and 50%, respectively. PEG 1000, on the other hand, was effective against *S. mutans* and *E. coli* at a concentration of 25%. Therefore, propylene glycol exhibited effectiveness against a greater number of organisms, including *E. faecalis*, which is known for its resistance. Although PEG 1000 showed bactericidal effects at a lower concentration, it only targeted two organisms (Nalawade *et al.*, 2015). Microorganisms utilize the carbonyl group produced during polyethylene oxidation for its degradation (Cornell *et al.*, 1984; Awasthi *et al.*, 2017). Reducing the solubility of polymers diminishes the degradation capability of microorganisms. Plastics become less susceptible to microbial breakdown as their solubility decreases. They adapt to microbes through interactions with their cell membranes. Polymer degradation methods include chemical, thermal, photo, and biological processes. Proper waste management, along with controlled biodegradation facilities, makes biodegradable plastics appealing for packaging and healthcare applications, offering economic, environmental, and health benefits. Polymer properties, such as size and shape, influence degradation rates, with larger surface areas degrading more rapidly. A newly discovered bacterial strain, *Ideonella sakaiensis* 201-F6, efficiently degrades PET by utilizing it as an energy and carbon source. This strain produces two enzymes that break down PET into environmentally safe monomers: ethylene glycol and terephthalic acid (Ahmed *et al.*, 2018).

CHAPTER 3

3. AIM AND SCOPE OF THE PRESENT INVESTIGATION

3.1 AIM:

- To assess the activity of microbial enzymes on degradation of plastic from polluted water samples.

3.2 SCOPE:

- To isolate microbes from plastic-polluted water samples.
- To characterize and identify the isolated microbe by using biochemical tests and DNA sequencing
- To study the polythene degrading activity of the microbial isolate under invitro condition
- To elucidate the efficiency and kinetics of the degradation of polythene by microbial enzymes and assess the by-products of the mechanism.
- To analyze the changes in the chemical structure of plastic, the surface morphology of the samples before and after degradation, the gaseous by-products and changes in the microbial genes respectively through FTIR, UV-SPEC, RNA seq and MIC.

CHAPTER 4

4. MATERIALS AND METHODS

4.1 MATERIALS:

Nutrient Agar, Agar agar, Yeast extract and PEG-6000 have been procured from HiMedia Laboratories. Staining kits containing crystal violet, iodine, saffranin, Malachite green from Thermo Scientific were used. Distilled Water Conical flask Petri plates Parafilm and other facilities including Incubator, Autoclave, Gloves, Pipette, Water Bath, Microscope, 20 ml centrifuge tubes and 2 ml Eppendorf tubes. UV and FTIR Spectrometer were provided by IRC (International Research Park), Sathyabama University, Chennai.

4.2 METHODOLOGY:

This study was conducted at Sathyabama Institute of Science and Technology and was approved by the Institutional Review Board of the university. The methodology involved assessing the growth of bacterial colonies in water samples collected from different locations on campus, as well as their susceptibility to different concentrations of PEG.

4.2.1 PEG degrading bacterial isolate P1:

4.2.1.1 Culture media:

Nutrient media; Yeast extract plus PEG (Polythethylene Glycol) media.

Sample type: Sewage water samples were gathered from various locations across the campus of Sathyabama Institute of Science and Technology, including the boys' hostel, girls' hostel, and a designated dumping site.

4.2.1.2 Dilution method:

In order to reduce the high concentration of colonies from the collected water samples, serial dilution was obtained. The samples were serially diluted at dilution factors of 10^{-3} and 10^{-6} , later they were labeled according to their dilution factors and left at room temperature overnight.

4.2.3 PREPARATION OF MEDIA:

The process of preparing the media entailed combining 2.8 grams of nutrient agar and 2.4 grams of agar-agar in 100 milliliters of distilled water. Following this, six sterilized petri dishes were meticulously arranged: three were designated to harbor varying concentrations of PEG (0.5 grams, 1.0 gram, and 1.5 grams) (Fig., 5.1), while the remaining trio were marked as control specimens.

4.2.4 INOCULATION PREPARATION:

The growth method, also known as the log phase method, was executed in the following manner. The samples with highest dilution factor were chosen for inoculation due to smaller number of colonies. By using spread plate method, 2 mL of sample was taken from each test tube using a pipette and inoculated onto the culture plates containing nutrient media. The inoculated petri dishes were incubated at 37°C for 15 days in the laboratory. After the period of incubation, growth of bacterial colonies was observed in culture plates containing 1g and 1.5g. (Fig. 5.1)

4.2.5 Observation of Bacterial Growth:

After the incubation period, bacterial growth was observed, characterized by thick creamy white colonies on the media.

4.2.6 Minimum Inhibitory Concentration (MIC):

MIC determination involves exposing a standardized microbial inoculum to a range of concentrations of the antimicrobial agent of interest. The lowest concentration at which no visible growth occurs after a specified incubation period is recorded as the MIC. Bacteria from 1g culture plate was sub cultured

into three culture plates with media containing yeast extract (3g) and PEG (2g, 2.5g and 3g respectively) by streaking method. These culture plates were incubated at 37° C for 30 days. It has taken twice the amount of time without nutrient broth because bacterial degradation of PEG, a polymer, takes significant amount of time. Triplicates for each of the standard plate with three different concentrations were prepared.

Based on our studies, the concentration of microbial concentration decreased as there is an increase in the concentration PEG without any other nutrient source. In case of an additional nutrient supplement, there is a gradual growth in the concentration of bacteria. It was also observed that under given conditions, bacteria was able to grow until 2.5g and at 3g there was comparatively lesser growth with colony count under 100.

4.3 ANALYTICAL TESTS:

4.3.1 MOLECULAR LEVEL IDENTIFICATION OF BACTERIAL ISOLATE:

4.3.1.1 Bacterial DNA extraction:

50-100 mg bacterial cells that have been resuspended in 200 ul of water or isotonic buffer (e.g.PBS) or up to 200 mg of tissue were added to a ZR bashingbead lysis tube containing 750 ul Lysis Solution. It was secured in a bead fitted with 2ml tube holder assembly and processed at maximum speed for > 5 minutes. The ZR bashingbead lysis tube was centrifuged in a microcentrifuge at > 10,000 x g for 1 minute. 400 ul supernatant was transferred to a centrifuge tube and centrifuged at 7,000 x g for 1 minute. 1,200 ul of Bacterial DNA Binding Buffer was added to the filtrate in the centrifuge tube. 800 ul of the mixture was transferred to a Zymo-Spin™ IIC column in a collection tube and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IIC column has a maximum capacity of 800 ul. The flow was discarded through the collection tube. 200 ul of DNA pre-wash buffer was added to the Zymo-Spin™ IIC column in new collection tube and centrifuged at 10,000 x g for 1 minute. 500 ul bacterial DNA wash buffer was added to the Zymo-Spin

TM IIC column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin TM IIC column was transferred to a clean 1.5ml microcentrifuge tube and 100 ul (35ul) DNA elution buffer added directly to the column matrix. It was then centrifuged at 10,000 x g for 30 seconds to elute the DNA.

4.3.1.2 QUANTITY AND QUALITY CHECK:

The product was checked on a nanodrop of model 2000 from Thermo Scientific to quantify the concentration of the extracted DNA and also determine purity by measuring at 260/280nm amount of proteins left in the DNA extract.

4.3.1.3 POLYMERASE CHAIN REACTION (PCR) ANALYSIS:

The DNA was subjected to a cocktail mix and condition for the PCR. PCR reaction cocktail consisted of 10 ul of 5 x Go Taq colourless reactions, 3 ul of MgCl₂, 1 µl of 10 mM of dNTPs mix, 1 ul of 10 pmol each. The Internal Transcribed Spacer (ITS) gene for characterization of bacteria, ITS universal primer set which flank the 16S was used.

- 16SF: GTGCCAGCAGCCGCGCTAA
- 16SR: AGACCCGGGAACGTATTCAC

Polymerase Chain Reaction (PCR) conditions include a cycle of initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of each cycle comprised of 30 seconds denaturation at 94 °C, 30 seconds annealing of primer at 55 °C, 1.5 minutes extension at 72 °C and a final extension for 7 minutes at 72 °C.

4.3.1.4 PURIFICATION OF AMPLIFIED PCR PRODUCT:

The amplified fragments were purified with ethanol in order to remove the PCR reagents. Briefly, 7.6 ul of sodium acetate 3M and 240 ul of 95% ethanol were added to each about 40 ul PCR amplified product in a new sterile 1.5 ul eppendorf tube, mixed thoroughly by vortexing and kept at -20°C for atleast 30 minutes. Centrifugation was done for 10 minutes at 13000 Revolution per minute and 4°C followed by removal of supernatant (tube was inverted on tissue once) after which the pellet were washed by adding 150 ul of 70 % ethanol and mixed, then

centrifuged for 15 minutes at 7500g and 4°C. Again all supernatant was removed and tube inverted on paper tissue and allowed to dry in the fume hood at room temperature for 10-15 minutes. It was re-suspended with 20 µl of sterile distilled water and kept in -20°C prior to sequencing. The purified fragment was checked on a 2% Agarose gel run on a voltage of 120 V for about 1 hour, to confirm the presence of the purified PCR product. The ladder used for bacteria is hyper ladder 1 from BioLone while 1 kb plus ladder from Invitrogen was used for bacteria. The expected base pair of the amplicon is around 850 bp. The amplified samples were then sent for sequencing.

4.3.1.5 GENOME SEQUENCING AND ANNOTATION:

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturer's manual while the sequencing kit used was that of Big Dye Terminator v3.1 cycle sequencing kit. BioEdit software and MEGA 6 were used for all genetic analysis. The classification/identification of organisms was performed by a local nucleotide BLAST search against the non-redundant version of the NCBI ref database.

4.3.3 GROWTH CURVE ANALYSIS BY UV SPECTROSCOPY AND FTIR:

The sample preparation for UV spectroscopy and FTIR, 2ml Eppendorf tubes were taken in which bacterial isolates grown in three varying concentrations of PEG i.e., 2g, 2.5g and 3g were observed for three days with a gap period of 12 hours in between each observation by keeping them at 4°C. The solvent for UV spectroscopy was distilled water and for FTIR, Ethanol was used.

CHAPTER 5

5. RESULTS AND DISCUSSION:

5.1 ISOLATION OF PEG DEGRADING BACTERIA:

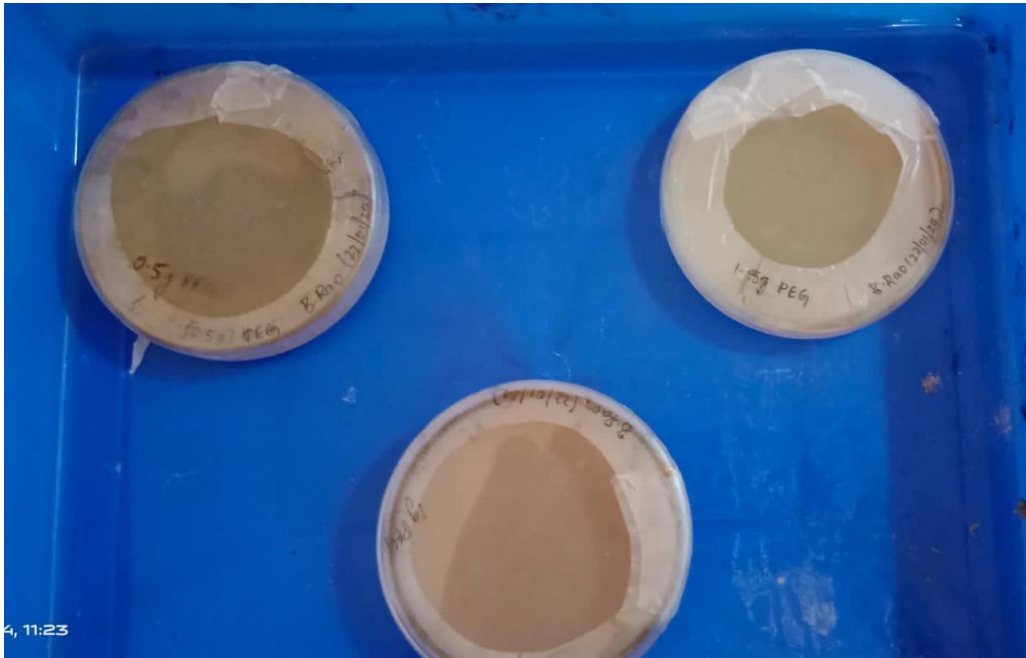


Fig 5.1: Bacterial growth on nutrient agar with 0.5g, 1.0g, 1.5g PEG that were kept under incubation for 15 days at 37 °C.

After 15 days of incubation, growth of bacterial colonies has been observed by thick white colonies on the media. Bacterial growth has been noticed above 0.5g PEG. There was growth in 1g and 1.5g PEG.. (Figure 5.1)

5.1.1 Sub-culture of the bacterial colonies:

Bacterial colonies have been sub-cultured into petri dishes with various concentrations and media. After this, three petri dishes were taken with 2g, 2.5g and 3g PEG and 3g yeast and sample was inoculated by streaking and kept under incubation at 37 degree Celsius for 30 days. We have observed that bacterial colonies grew in three of the culture plates at varying concentrations. (MIC). This proved that bacteria had grown by feeding onto PEG due to the absence of nutrient agar or any other nutrient supply. One petri dish contained Nutrient agar with bacterial colonies sub-cultured onto it, the other petri dish contained Nutrient agar, PEG and bacterial culture. We have observed growth of bacterial colonies in both cases.



Fig 5.2: Subculturing of the selected bacterial isolate on nutrient agar supplemented with PEG

Table 5.1: Growth analysis of bacterial growth on different concentration of PEG

Concentration of PEG	Growth Of Bacterial Colonies
0.5g	NO
1g	YES
1.5g	YES

5.2 MINIMUM INHIBITORY CONCENTRATION (MIC):

Minimum Inhibitory Concentration (MIC) is a pivotal parameter in antimicrobial susceptibility testing, providing crucial information about the potency of antimicrobial agents and the susceptibility of microbial pathogens. MIC determination involves exposing a standardized microbial inoculum to a range of concentrations of the antimicrobial agent of interest. The lowest concentration at which no visible growth occurs after a specified incubation period is recorded as the MIC.

- The culture was grown with PEG (2g, 2.5g and 3g concentrations each) as a nutrient source along with yeast extract for 30 days under incubation at 37 c.
- 2g culture plate has recorded the most bacterial growth while 3g culture plate had the lowest concentration of colonies, this concludes that for this bacteria under the conditions given, MIC is 3g.

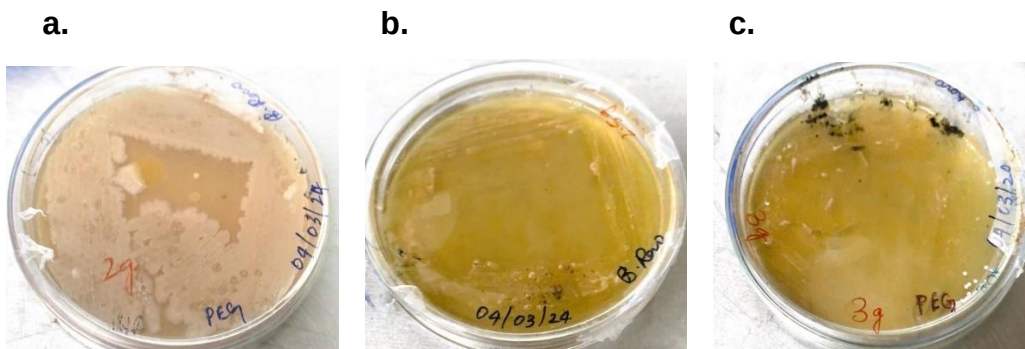
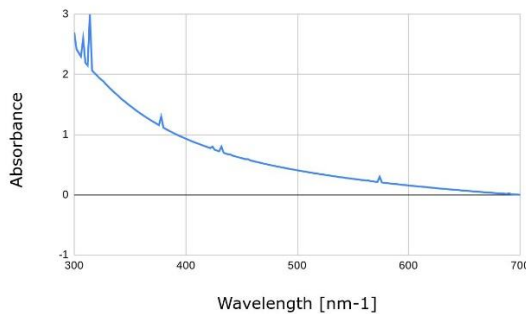


Fig 5.3 Growth pattern of bacterial isolate P1 on different concentrations (a. 2.0g, b. 2.5g and c. 3g) of PEG

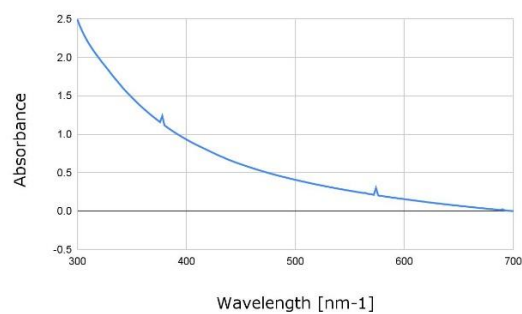
5.3 UV spectroscopy analysis of biodegradation of PEG by bacterial isolate P1:

UV- Spectroscopy results have depicted a graph where we can observe a peak between 300nm^{-1} and 600nm^{-1} with the absorbance to be the highest from 300nm^{-1} to 400nm^{-1} . The peaks in graph (A) with respect to absorbance denotes that there is a high concentration of bacterial isolates P1 or cell matter which indicates the presence of bacterial growth. By this we can say that bacteria has grown by feeding on PEG *in vitro*. Whereas, graph (B) shows only two peaks between 300nm^{-1} to 400nm^{-1} and 500nm^{-1} to 600nm^{-1} , this explains that bacteria wasn't able to degrade efficiently and grow at a higher concentration of PEG under given conditions. In graph (C) we see no peaks which indicates absence of isolate P1.

A)



B)



C)

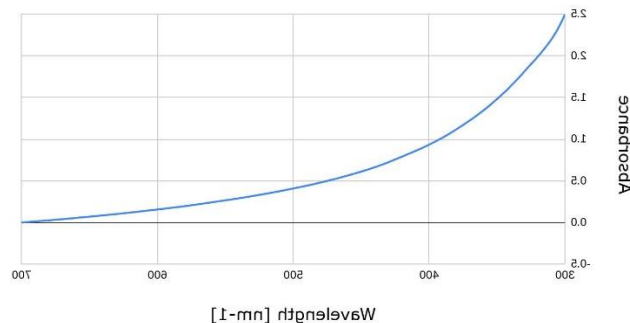


Fig 5.4 UV - Spectroscopy analysis of biomass of bacterial isolate P1 on different concentrations of PEG. A) 2g B) 2.5g C) 3g

5.4 FTIR SPECTROPHOTOMETRY:

FTIR spectroscopy has given us the graph (Fig: 5.4.1) in which we could deduce all the peaks and intensities (Fig: 5.4.2). These readings have given us all the functional groups and bond types (Table 5.2) that could show the degradation of polymer into monomers.

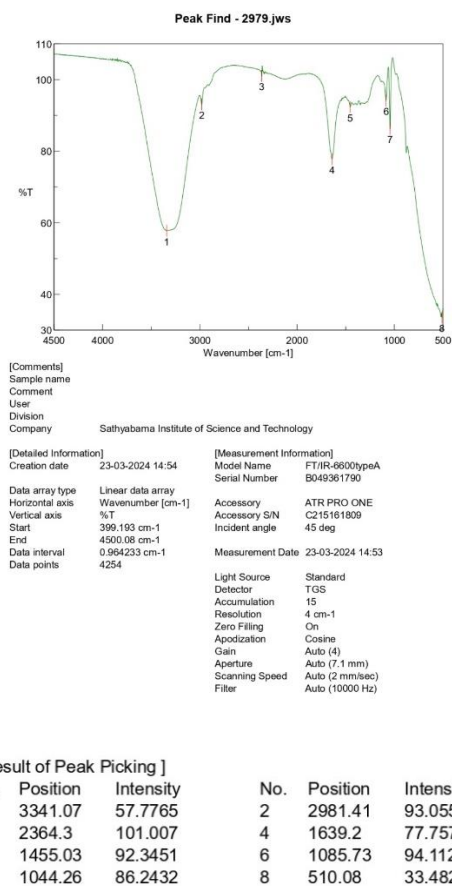


Fig 5.5: FTIR spectroscopy analysis of the extract of bacterial (P1) culture media and FTIR Spectra Chart showing functional groups present in the extract of bacterial culture media.

Table 5.2: Below table is of the interpretation of our samples through FTIR results.

Sl. No.	Wave number [cm-1]	Bond Type	Functional Group
1.	3341.07	N-H	Primary amines
2.	2981	-C-H	Alkanes
3.	2364	P	Phosphine
4.	1639	-C=O	Amide
5.	1455	C=O	Carboxylic acid
6.	1085	-C-O	Secondary alcohol
7.	1044	-C-O	Ether
8.	510	C-C-N (or) C-C	Nitriles (or) Cycloalkanes



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CHAPTER 6: SUMMARY AND CONCLUSION

6.1 SUMMARY AND CONCLUSION:

In the study conducted at Sathyabama Institute of Science and Technology, samples were collected from different locations around the campus, including the boys' hostel, girls' hostel, and a dumping site. These water samples underwent serial dilution at factors of 10^{-3} and 10^{-6} , and were labelled according to their dilution units (DU). Following dilution, the samples were left at room temperature overnight. Samples were inoculated on nutrient agar supplemented with 0.5g, 1.0g, and 1.5g of polyethylene glycol (PEG), respectively, while the remaining without supplementation acted as controls. After two weeks, obtained bacterial colonies were sub-cultured into petri dishes containing different concentrations of PEG. Observations revealed thick white colonies on the media after the incubation period of 15 days, indicating bacterial growth. Notably, no growth was observed in the dish containing 0.5g of PEG, while growth was evident in dishes with 1.0g and 1.5g of PEG. This outcome suggested that the bacteria grew by utilizing PEG as a nutrient source in the absence of other nutrient supplies. Further sub-culturing of bacterial colonies onto petri dishes containing various concentrations and combinations of media confirmed the adaptability of the bacteria to utilize different nutrient sources for growth. Following the growth of bacterial cultures with varying concentrations of PEG (2g, 2.5g, and 3g) as a nutrient source along with yeast extract for 14 days at 37°C , observations revealed that the 2g culture plate recorded the highest bacterial growth, while the 3g culture plate exhibited the lowest concentration of colonies. This suggests that under the given conditions, the minimum inhibitory concentration (MIC) for this particular bacteria is 3g of PEG.

Subsequent DNA sequencing tests identified the bacterial samples as belonging to the genus *Paenibacillus*. The match to *Paenibacillus thiaminolyticus* yielded the highest percentage of precision and a score of 1149. In addition to *Paenibacillus thiaminolyticus*, other species matches were found,

including *Paenibacillus popilliae* ATCC 14706 (score 1136), *P. apiarius* (score 1131), *P. dendritiformis*, *P. profundus*, among others.

Hence, the study at Sathyabama Institute of Science and Technology concludes that bacterial species from the genus *Paenibacillus* display adaptability by utilizing polyethylene glycol (PEG) as a nutrient source. Therefore, these findings highlight the potential ecological roles and applications of PEG-utilizing bacteria in various environmental and biotechnological contexts.

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