SCREENING OF BACTERIA SHOWING BIODEGRADATION OF POLYETHYLENE (LDPE) ISOLATED FROM POLLUTED PLASTIC SAMPLE COLLECTED FROM PADASHEKARAM PLOT, EDAKOCHI, ERNAKULAM DISTRICT

A dissertation submitted in partial fulfilment of the requirement for the degree of

"Master of Science in Botany"

BY

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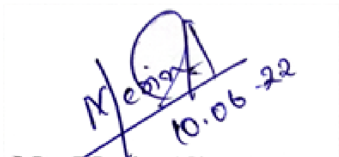
June 2022

CERTIFICATE

This is to certify that the dissertation titled, **“SCREENING OF BACTERIA SHOWING BIODEGRADATION OF POLYETHYLENE (LDPE) ISOLATED FROM POLLUTED PLASTIC SAMPLE COLLECTED FROM PADASHEKARAM PLOT, EDAKOCHI, ERNAKULAM DISTRICT”** submitted in partial fulfilment of the requirement for the Master’s Degree of Science in Botany is an authentic record of work carried out by DOMNICA LIYAN JOHNSON (AM20BOT008) under the supervision and guidance of Ms. MERIN ALICE GEORGE.



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ACKNOWLEDGEMENT

First and foremost, I bow in profound gratitude to the Almighty for the successful completion of this project. I am extremely grateful to my guide and supervisor Ms. Merin Alice George for her patience, support, continued guidance and understanding that kept me motivated till the end of the project. I take this opportunity to express my gratitude to Dr. Liza Jacob, Head of the Department of Botany, and to all the Faculty members. I would like to extend my deep sense of gratitude to Dr. Alphonsa Vijaya Joseph, Vice principal, St Teresa's College (Autonomous) for her valuable help and interpretations. I would like to give a special thanks to Ms. Glaxy Ezekel, Research Scholar, Department of Botany St. Teresa's College (Autonomous) for her invaluable suggestions and support on the project.

I would like to extend my heartfelt thanks to the lab assistants and non-teaching staff of the department for their help and support. I would also like to thank my parents for their unceasing encouragement, support and attention. I also extend my gratitude to Ms. Emily Sandra Francis and my friends for lending a hand in this work directly or indirectly.

Domnica Liyan Johnson

Place: Ernakulam

Date: 13-06-2022

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INTRODUCTION

Plastic pollution is a pervasive and escalating global environmental problem, named among the most serious environmental issues after climate change. The discovery of single use plastic have been a boon for the 20th century but is a serious threat now and to posterity. Uncontrolled use of the plastics for packaging (e.g. fast food), transportation, industry and agriculture in rural as well as urban areas, has elevated serious issue of plastic waste disposal and its pollution from the last three decades. Light-weight, inertness, durability, strength and low cost are the main advantages of plastic while it has disadvantages such as, it is recalcitrant to biodegradation and difficult to degrade naturally.

The global use of plastic is growing at a rate of 12% per year and around 0.15 billion tonnes of synthetic polymers are produced worldwide every year (Premraj and Doble 2005; Leja and Lewandowicz 2010; Das and Kumar 2014).

Annually, India generates 5.6 million metric tonnes of plastic waste, with Delhi accounting for a shocking 689.5 metric tonnes per day. According to Central Pollution Control Board (CPCB) of India, total plastic waste which is collected and recycled in the country is likely to be 9,205 tonnes per day (approximately 60% of total plastic waste) and 6,137 tonnes remain uncollected and littered. The recent report of the State Pollution Control Board (SPCB) of Kerala estimated plastic waste generation is approximately 1,31,400 TPA (tonnes per Annum) during 2019- 20 (Annual Report 2019-20, Central Pollution Control Board, Delhi). The state ranks third among the highest state-wise per capita plastic waste generation. Complete ban has been imposed on single use plastic items including plastic carry bags irrespective of thickness in the state.

Plastic recycling contributes to the value-added advantages of plastic by reducing its toxicity. Recycled plastic can also be used to make bottles, containers, bags, films, and other packaging products that are environmentally friendly. The reprocessing waste materials and waste plastics is growing owing to the numerous benefits associated with it, including a reduction in the amount of waste transported to landfills and incinerators, conservation of natural resources, and prevention of pollution.

Biodegradable plastics are seen by many as a promising solution to this problem because they are environmentally-friendly. Biodegradable synthetic polymers offer a number of advantages over other materials for developing scaffolds in tissue engineering. Polyhydroxyalkanoates (PHA) and lactic acid (raw materials for PLA) can be produced by fermentative biotechnological processes using agricultural products and microorganisms. The key advantages include the ability to tailor mechanical properties and degradation kinetics to suit various applications. A vast majority of biodegradable polymers studied belongs to the polyester family.

Another source of plastics are natural sources and they are called bio-plastics. It serves sustainable and eco-friendly practices against synthetic plastic production and consumption. It aims to decrease the reliance of petroleum based products. The sources that can be used for bio-plastic production are plant-based raw materials, natural polymers (carbohydrates, proteins, etc.), and other small molecules (sugar, disaccharides, and fatty acids).

It is already known that efficient decomposition of plastic bags takes about 1000 years as it is a synthetic, long chain hydrophobic polymer. Plastic causes pollution and global warming not only because of increase in the problem of waste disposal and land filling but also release CO2 and dioxins due to burning. Commonly used methods for plastic disposal were proved to be inadequate for effective plastic waste management, and hence there is growing concern for use of efficient microorganisms meant for biodegradation of non-degradable synthetic polymer.

However, biodegradation could be a potential alternative solution to plastic specifically polyethylene (PE) pollution. However, its hydrophobic surface and long carbon chains make biodegradation less efficient. Any physical or chemical change in polymer are due to environmental factors such as light, heat, moisture, chemical conditions and biological activity is termed as degradation of plastic. Decomposition or destruction of contaminant molecules by the action of the enzyme secreted by microorganisms is known as biodegradation. Additives, antioxidants and stabilizers used in manufacturing of polymer can slow down the rate of degradation and may be toxic to microorganisms (Arutchelvi *et al.* 2008). Besides all above mentioned factor structural (linearity and branching in polymer, type of bond like C-C, amide & ester), molecular composition and physical form of polymer like powder, films, pellets and fibres may also influences the biodegradability polymer.

Ultimately, the way and rate of polymer degradation depends on the mechanism of degradation and acceleration of process.

This study encompasses the need of an alternative method for reduction of accumulated polyethylene in Kerala apart from the Ban of single use plastics. The area of study Padashekaram plot, a water body is located at south-west part Edakochi of Ernakulam district, Kerala. It is a water body comprising three interconnected plots of *Pokkali* fields which have been refurbished to for Pearl spot fish cultivation. Now-a-days, the plots remain unused for cultivation due to the amount of pollution it is exposed to. Noticeably, this area was chosen because of the evident amount of waste disposal and inefficient waste management in its water breaks. Most of the area, is covered by mangrove trees but also plastics. The households flanked on the side of the interconnected plot somehow manage to utilize some of the resources such as mussel dredging and non-commercial fishing. This area is low lying and often flooded during the high tides. Increased amounts of plastic have been the cause of blocked water breaks and swaying of wastes into houses close to the water body. Hence, this area was chosen to study. The sole purpose of this study is to isolate and screen bacteria showing biodegrading properties and further identify them.

OBJECTIVES

1. Collection of sediment and plastic samples from the area of study
2. Isolation bacteria from collected sediment and plastic samples
3. Screening of bacteria isolated from plastic samples using weight loss method
4. Biochemical analysis of bacteria which showed highest degradation potential
5. Identification and characterization of bacteria showing plastic biodegradation potential

REVIEW OF LITERATURE

Plastics are organic polymers containing molecules composed of long carbon chains back-bone formed through the polymerization (Koushal *et. al*., 2014). They are made of carbon and hydrogen, with nitrogen, sulfur, and other various organic and inorganic materials derived from fossil fuels (Asiandu, 2021). Plastics divided into natural plastics, semi-synthetic plastics, synthetic plastics, thermoplastics, and thermosetting plastics (Kumar S., 2013).

In general, plastics can be classified based on two factors: fossil-based or bio-based, non-biodegradable or biodegradable (European Bioplastics. Fact Sheet, 2018).

Five types of petroleum-based polymers are the most commonly used to make single-use plastic materials, namely low-density polyethylene (LDPE), high density polyethylene (HDPE), polypropylene (PP), polyvinyl chloride (PVC), and polyethylene terephthalate (PET). LDPE, mainly used to make plastic carry bags and food packaging materials, is the most abundant petroleum-polymer on earth, and represents up to 64% of single-use plastics that are discarded within a short period after use, resulting in massive and rapid accumulation in the environment. (Ragaert, 2017). Most of these plastics are non-biodegradable Polyethylene.

Bio-based plastics are further classified into three categories: Modified natural polymers, synthesized bio-based polymers from synthesized bio-based monomers and bio-plastics from waste (Thielen, 2014). Currently, only around 1% of the annual plastic production in the world is bio-plastics (European Bioplastics. Bioplastics Market Data). The largest share goes to starch blends, 21% of the total production in 2019. The share of bioplastics in the market is expected to increase. The main application areas are in the packaging industry, followed by the textile industry, the automotive industry and construction (Alaerts, L., Augustinus, *et. al.,* 2018). Nowadays, bio-plastics are derived from terrestrial crops such as corn and potatoes and thus compete with food supplies (Rahman, A., Miller, 2017). Chlorella species is often used in biomass–polymer blends. According to Zeller et al. (2013), after comparing bio-plastic production from 100% microalgae biomass. There have been several studies conducted to examine the potential of Spirulina. Similar to Chlorella, Spirulina has a small cell size, which makes both of them attractive for bio- plastic blend production (Onen Cinar., 2020). Wheat gluten is widely studied to produce sustainable

bioplastic. Although, it has a brittle structure, the structure of the material can be improved with additives and fillers. Because of wheat gluten´s high protein content, it is promising for many application areas (Ciapponi, *et. al.,*2019; Wretfors, C., 2009).

Low density polyethylene is one of the major sources of environmental pollution. Polyethylene is a polymer made of long chain monomers of ethylene. The worldwide utility of polyethylene is expanding at a rate of 12% annum and approximately 140 million tonnes of synthetic polymers are produced worldwide each year (Shimao, M., 2001). The Figure 1 given below shows the chemical structure of Polyethylene.

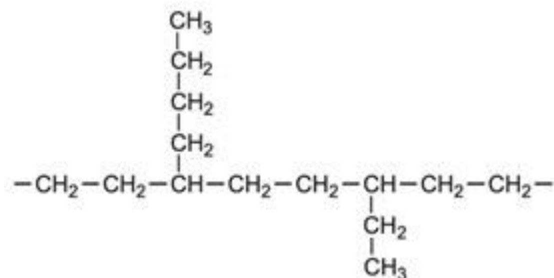


Figure 1

The massive plastics production has begun in the 1950s, which is generally produced for disposable use. Most of the plastics waste is non-biodegradable which takes thousands of years to be decomposed or degraded (UNEP, 2018). In 2010, China was the highest plastic waste producer in the world with 8.8 million tons per year or 27% of the total world plastic waste production. Meanwhile, Indonesia was the second after China as the highest plastic waste-producing country in the world with 3.2 million tons per year or 10% of the total world plastic waste (Jambeck 2015, Geyer R. 2017, UNEP, 2018). In Indonesia, approximately 15% of the individually daily wastes are plastics (Arico, Z., & Jayanthi, S., 2018). Based on the European Plastics in 2018, total world plastic production reaches 335 million tons per year, as much as 60 million of that amount is obtained in Europe. It is estimated that the number of plastic productions will be two times greater in the next 20 years. Meanwhile, plastic bags are the most common form of plastic widely used in daily lives in the world. Although plastic products are reusable, they are still one of the main factors causing environmental pollution (Drzyzga, O., & Prieto, A., 2019).

On solid waste management, the report says that in 2019-20, India generated 3.5 million tonne of plastic waste. Only 12 per cent of this was recycled, and 20 per cent was

burnt, according to the Centre for Science and Environment (CSE). Per capita plastic waste generation has been calculated based on the population of the states which have submitted in their reports. The trend of per capita waste generation for the last five years (2016-20) recorded did observed that the per-capita plastic waste generation has almost doubled. State-wise per capita waste generation for the year 2019- 20. Goa, Delhi & Kerala have reported the highest per capita plastic waste generation and Nagaland, Sikkim & Tripura have reported the lowest per capita plastic waste generation (CPCB, Delhi 2020)

Kerala imposed a complete ban on single use plastic items including plastic carry bags irrespective of thickness in the state. There are 1266 manufacturers, 82 producers, 1 compostable plastic unit and 99 recyclers registered & no unregistered plastic manufacturing/recycling units in the State. Detail of Plastic waste utilization is as given as: recycling: 3.12 TPD (tonnes per day); Road Construction: 1552 MT (metric tonnes); Co-processing: 1551 MT (metric tonnes). Currently, there are only 4 manufacturing Units registered for compostable plastic with the total Capacity of 5.19 TPD. (SPCB Report to CPCB Delhi, 2020)

The uncontrolled plastics use started several decades ago, have caused many environmental problems related to the disposal and pollutions of plastics waste. The decomposition process of plastic polymers takes thousands of years. People usually burn plastics waste to overcome the accumulation of plastics waste in the environment yet the burning of plastics waste leads to air pollution. It releases toxic compounds, CO2, and dioxins, into the air. Those released gases cause lung diseases and cancer (Kale, S.K. *et. al*, 2015). As plastics waste is a pollutant polluting the land, air, and water ecosystem (Soud, S.A.,2019), threatening various living things (Soumya *et. al.*,,2015), therefore the appropriate processing method of plastic waste is necessarily needed to be carried out. The application of reuse, reduce, and recycle is now widely applied to prevent the problem caused by plastics waste. However, this method is less effective, especially for plastics waste that has been mixed with other types of waste (Drzyzga, O., & Prieto, A., 2019). Also, landfill plastics waste processing requires large space, and incineration plastics waste processing can produce toxic gases into the environment (Kumar *et. al.*, 2017). Thus, a more effective and environmentally safe processing plastics waste method is needed.

Polyethylene degradation can take place by different molecular mechanisms; chemical, thermal, photo and bio-degradation (Shah *et. al.*, 2008). As a part of secondary

metabolism, microorganisms have a natural ability to transform or accumulate a sundry of compounds including hydrocarbons (PAHs), pharmaceutical substances and metals. Plastic is the hydrolyzed by enzymes to create functional groups for improvement of hydrophilicity, then the main chains of polymer are degraded resulting in polymer of low molecular weight and weak mechanical properties, thus, making it more accessible for further microbial assimilation (Shah et al. 2009).

The potential of polyethylene degrading microbes had been a curiosity since 1961 when Fuhs (1961) reported that several microorganisms can consume paraffin as a carbon source. Plastic degrading microbes were isolated from a variety of resources such as rhizosphere soil of mangroves, polythene buried in the soil, marine water, plastic and soil at the dumping sites. Bacteria, fungi and algae are the biological factor that degrades plastic naturally (Rutkowska *et. al*. 2002). It is also observed that the enzymatic degradability decreases with increasing time. The higher order structure properties like crystallinity and modulus of elasticity, suppresses the polymer degradability (Tokiwa and Calabia 2004).

Biodegradation is an effective, profitable, and economically valuable plastics waste processing method. The ability of many microorganisms to break down plastic polymers is an advantage that can be used in dealing with problems arising from the increasing accumulation of plastics waste every day. Some microorganisms produce various kinds of enzymes, both intracellular and extracellular, catalyze plastic polymers degradation into safe smaller fragments (Okomoto et.al 2007; Agarwal, Singh, 2016). The utilization of microbial cells directly to degrade plastic C-C bonds is considered more effective (Wei, Zimmermann, 2017). Biodegradation is a specific enzymatic process. Certain enzymes break down certain substrates (Adamcová D, Vaverková M, 2014).

Microorganisms are able to degrade the plastic waste with help of different physical factor such as temperature, moisture, pressure cause deterioration to the polymers in a process called biodegradation (Kumar, S., *et. al.,*, 2013). Microorganisms that able to degrade the common of the organic and inorganic materials as carbon source. It produces many of degrading enzymes that cause cleavage of the polymer chains into monomers and oligomers. Plastic wastes are absorbed by microbial cell. Aerobic metabolism produces carbon dioxide and water and anaerobic metabolism produces carbon dioxide, water, and methane as end products (Usha, R., *et. al.*, 2011). Many microorganism are able to degraded plastic compounds such as fungi, algae, (Kim, Y., *et. al*, 2005), bacteria, eg. *Bacillus, Pseudomonas,*

Klebsiella, Mycobacterium, Rhodococcus, Flavobacterium, Escherichia, Nocardia, and Azotobacter (Gautamr, R., *et. al*., 2007), that able to degradation natural and synthetic plastics

The plastic waste biodegradation process occurs through several stages, including bio-deterioration, de-polymerization, and assimilation. Bio-deterioration is a cooperation between several microbes and abiotic factors that breaks down polymers into smaller ones. This process will be continued with de-polymerization. De-polymerization occurs in which microbes secrete catalytic compounds in the form of enzymes and free radicals to form biofilms helping them to break the polymer chains progressively (Marjayandari *et. al*., 2015). Bio-deterioration is a process of changing or modifying plastic polymers carried out by some microorganisms on the plastic surface. The changes include chemical, physical, and mechanical (Helbling *et. al.,* 2006). This process will be accelerated by biofilms formed by microorganisms on the plastic surface. When microorganisms adhere to the plastic surfaces, they will start trying to use those polymers as their carbon source (Deepika, Madhuri, 2015). A biofilm is a form of living things community. Microbes attach themselves and colonize the surface of an object to form biofilms assisted by an extracellular compound produced by them. In the form of biofilms, microbial cells attach one to another in a polymer matrix containing polysaccharides and proteins (Kumar *et. al*., 2017). Extracellular polymeric substances (EPS) produced by microorganisms help them to break down the plastic surface (Bonhomme *et. al*, 2003 and Sharma B *et. al.,* 2017). EPS consists of polysaccharides, proteins, and nucleic acids (Gilan I, 2013).

Bacillus spp. are potential LPDE degrading agents. By using agar minerals incubated for two months, *B. carbonipphilus* was able to degrade LDPE about 34.55%. Meanwhile, *B. sporothermo-durans* degraded the sample about 36.54%, *B. sporothermodurans* degraded it about 36.54%, *B. coagulans* degraded the sample about 18.37%, *B. neidei* decreased the plastic's weight about 36.07%, *B .smithii* degraded it about 16.0%, and *B. megaterium* degraded it about 34.48%. In Mineral Broth media, B.carbonipphilus degraded LDPE about 25%, B.sporothermodurans 21%, *B. coagulans* 16%, *B. neidei* 14%, *B. smithii* 8%, and *B. megaterium* 21% (Shresta *et. al.,*, 2019). The other LDPE degrading bacteria are *Bacillus weihenstephanensis*, *Burkholderia cepacia*, and *Escherichia coli*. Within six months, *B. weihenstephanensis* was able to reduce the weight of thick LDPE plastic bags around 32.61% and thin plastic bags about 35.64%. *B. cepacia* can reduce the weight of thick plastic bags

about 31.43%, and 36.34% for thin plastic bags. Whereas *E.coli* reduced 23.72% of thick plastic bags weight and 23.57% for thin plastic bags (Mukherjee and Chatterjee, 2014).

Though there are lots of reports demonstrating the potential of plastic degrading microbes, but none of them found to have practical application, thus there is a strong need to screen efficient organisms and developing technologies capable of degrading plastic efficiently without affecting the ecosystem and environment.

METHODOLOGY

1. SAMPLE COLLECTION

Sediment and plastic samples were collected for the isolation of bacteria to test its biodegradation capacity in synthetic media. The samples from the selected site in Edakochi, Kerala, India were transported to the laboratory using aseptic carry bags.

2. GLASSWARE

All the apparatus were washed with soap and water and placed in the hot air oven (dry heat) at 180°C for 2-3 hrs. This is done to kill spores of microorganisms. Agar filled culture vessels are placed in autoclave for about 20-30 minutes in 15lbs under 120°C.

3. REFRIGERATION

All reagents and bacterial cultures were stored in a refrigerator at 4°C and 18°C respectively.

4. MICROBIOLOGICAL ANALYSIS

I. ISOLATION OF BACTERIA

The sediment and plastic samples were subjected to serial dilution to isolate the bacterial colonies from the sample and later purified by spread plate and are later streaked into individual colony cultures. All procedures were carried out in aseptically in the laminar air flow chamber. These cultures were stored at 18°C.

Serial Dilution

It is a simple procedure for the isolation of bacteria. The specimen is serially diluted in a sterile liquid.

- **Materials:**
  Conical flask, test tubes, distilled water, autoclave, cotton, pipette, samples

- **Procedure:**
  The sample is initially diluted in a conical flask and five test tubes each with 9ml of sterile diluents, which can either be distilled water or 0.9% saline, are taken. Using a sterile pipette 1ml of properly mixed sample/ culture is drawn and added into the first tube to make the total volume of 10ml. This provides an initial

dilution of 10^{-1}. The dilution is thoroughly mixed by emptying and filling the pipette several times. The pipette tip is discarded and using a new pipette tip 1ml of mixture is taken from the 10^{-1} dilution and is emptied into the second tube to obtain the total dilution factor of 10^{-2}. The same process is repeated for the remaining tubes, taking 1 ml from the preceding test tubes and adding into the next 9ml diluents. As five tubes are used, the final dilution for the bacteria or the cell will be 10^{-5} (1 in 1,00,000)

Nutrient agar medium preparation

- **Materials:**
  Conical flask (250ml), glass rod, spatula, measuring cylinder, sterilized petri dishes, bacterial agar, nutrient broth, distilled water, microwave oven

- **Procedure:**
  To a conical flask, 100 ml distilled water is added along with 2g of nutrient agar and 1.3 g of nutrient broth. It is the mixed thoroughly and heated in the oven to dissolve the agar. Heat the mixture till it just start to boil. The In a laminar air flow chamber, this mixture is then poured into sterile petri plate and are kept undisturbed till it cools and solidifies.

A. Spread plate

- **Materials:**
  Bacterial culture, nutrient agar plates, measuring cylinder, L- rod, spirit lamp, absolute alcohol, sterile pipette.

- **Procedure:**
  5ml of each of the serially diluted bacterial culture was inoculated into the surface of agar plate using a sterile pipette. The plates were placed on a turn table. The L-rod was sterilized by alcohol and heat sterilization and allowed to cool. The plate was placed on the turn table and using an L-shaped glass rod the culture was spread evenly by gently stroking back and forth. When the turn table stops spinning, the lid was put over the petri plate. The L-rod was sterilized after each inoculation. The petri plate was incubated at 30°C for 24 hrs. The plates were observed after incubation.

B. Quadrat streak

- **Materials:**

  Sterile petri plates, inoculating loop, distilled water, Bunsen burner, bacterial cultures

- **Procedure:**

  Heat sterilize the inoculation loop. Take a loop from a bacterial colony observed earlier on the spread plate and streak it on the sterilized solid medium in one direction from a common point of inoculation. While re-flaming the loop close the plate and turn it approximately 45° sideways and streak to spread the original inoculum over more of the agar. Repeat this two times. Label the plate and incubate it inverted at 30°C for 24hrs.

C. Slant agar preparation and inoculation

- **Materials:**

  Nutrient broth, bacterial agar, distilled water, conical flask, culture tubes, glass rod, measuring cylinder, test tube stand, cultured bacteria

- **Procedure:**

  Weigh 2g of bacterial agar and 1.3g of nutrient broth and add it into conical flask containing 100 ml distilled water. The contents are then gently heated with slight agitation to dissolve the ingredients. 5 ml of molten agar is dispensed into each test tubes using pipettes. The mouth of the tubes and plugged with cotton plugs containing hot agar. The amount of medium maybe adjusted depending on the volume of the culture tube. The tubes are then sterilized at 121°C for 15 minutes with plugs loosely on. While the medium is still hot, tilt the rack of tubes to a solid support to get a slant with a narrow bottom. Allow the medium to harden in this position by keeping it undisturbed for cooling. After cooling of agar slants, the bacteria is inoculated one by one in each of the slant accordingly using inoculation loop. The tubes are incubated for 24hrs and later stored in the refrigerator.

I. SCREENING OF BACTERIA: – WEIGHT - LOSS TECHNIQUE

The method is carried out to test the polythene degrading bacteria from the isolates obtained.

- **Materials:**

  Sterile 100ml conical flasks, 1000 ml conical flask, glass rod, measuring cylinder, sterile nutrient broth (50ml each), distilled water, cotton plugs, pre-weighed polyethylene pieces, incubator

- **Methods:**

  Weigh 7.15g of nutrient broth is added to conical flask containing 550 ml of distilled water and autoclaved. It is then transferred to 100ml conical flask and plugged with cotton loosely. After the broth is cooled, a loop full of the purified bacterial isolates are inoculated into each culture vessel and labelled uniquely. The plastic pieces were pre-weighed around 8.5 milligrams and were transferred to each conical flask aseptically. It is incubated at 37°C for 28 days. The vessels were shaken at regular intervals initially for even distribution in the medium. After 28 days, the polythene pieces were removed, washed with distilled water, air dried and weighed to note the final weight.

II. BIOCHEMICAL TESTS

a) Grams staining

- **Principle:**

  To differentiate Gram positive and Gram negative bacteria

- **Materials:**

  Bacterial culture, Crystal violet, Gram's iodine, 95% ethanol, safranin, distilled water, glass slide, inoculating loop, spirit lamp, microscope

- **Procedure:**

  Prepare a smear of bacteria using inoculating loop on a clean glass slide and heat fix it. Flood the slide with crystal violet staining reagent for 1 minute. Rinse the reagent off gently using distilled water. Flood the slide with Gram's iodine for 1 min. Wash with a gentle indirect stream of distilled water for 2 seconds. Flood the smear with ethanol for 5 seconds. Blot dry the smear and counter stain with safranin for one minute. Rinse off the stain, air dry and keep it undisturbed

b) IMVIC TEST

1. Indole test

- **Principle**:

  Detects the ability of bacteria to decompose amino acid tryptophan by an enzyme tryptophanase and produce indole. Indole reacts with paramethyldiaminobenzaldehyde (Kovac's reagent) and gives colour.

- **Materials**:

  (a) Composition of medium per litre –

Tryptone	1.5 g
NaCl	20g
pH	7.2+/- 0.3

  (b) Kovac's reagent

Paradimethylaminobenzaldehyde	5g
Amyl or isoamyl alcohol	75ml
Concentrated HCl	25ml

  (c) Bacterial culture (screened)
  (d) Inoculating loop
  (e) Culture tubes
  (f) Incubator

- **Procedure**:

  Inoculate the tube of tryptone water with a loop full of 24hr culture growth and is incubated for 48-96 hrs at 37°C. After incubation, 0.5ml Kovac's reagent is added to each tube without mixing and is held for 2-5mins. Appearance of dark red ring indicates positive result.

2. Methyl Red (MR) Test

- **Principle**:

  Detects mixed acids during fermentation of glucose and maintenance of pH below 4.5 in old culture.

- **Materials**:

  (a) glucose-phosphate peptone water medium

Glucose	5g
K_2HPO_4	5g
Peptone	5g
pH	neutral

  (b) methyl red indicator

  Dissolve 0.1g methyl red in 300ml 95% ethylalcohol in standard flask dilute to 500ml with distilled water.

  (c) Bacterial culture (screened)

  (d) Inoculating loop

  (e) Culture tubes

  (f) Incubator

- **Procedure**:

  A loop full of bacteria is inoculate in liquid medium (glucose-phosphate broth) incubate at 37°C for 2-5 days. At post incubation, add 5 drops of 0.04 solution of methyl red along the sides of the test tube. The results were read immediately. Positive test shoes red colored broth.

3. Voges- Proskauer (VP) Test

- **Principle**:

  Detects Acetoin production, and intermediate of fermentation pathway that leads to production of 2, 3- butandiol

- **Materials**:

a) Bacterial culture (screened)

b) Baritts reagent A and B

c) Inoculating loop

d) Culture tubes

e) Incubator

- **Procedure**:

A loop full of screened bacteria is inoculated in glucose-phosphate broth and incubated at 37°C or 30°C for 48 hrs only. After which, equal amounts of Baritts A and B is added. Positive test result in color change of the medium to red.

4. Citrate Test

- **Principle**:

To test the ability of the organism to utilize citrate as sole carbon source and energy for growth.

- **Materials**:

(a) Simmon's citrate agar medium

Sodium citrate	2.0g
$MgSO_4$	0.2g
$(NH_4)H_2PO_4$	1.0g
K_2PO_4	1.0g
NaCl	5.0g
Bromothymol blue	0.08g
Agar	15.0g
Distilled water	1L
pH	7.0

(b) Bacterial culture (screened)

(c) Inoculating loop

(d) Culture tubes
(e) Incubator

- **Procedure**:
  Prepare a few slants of Simmon's citrate agar medium. a loop full of bacteria is inoculated onto the slants by stabbing to the base of the slant. Thereafter, streak the surface. The tubes are then kept at 37°C for 48 hrs for incubation. Examine the tubes, for culture growth, which causes blue color of the medium due to change in pH and is recorded as positive.

5. DNA EXTRACTION AND PCR AMPLIFICATION

a) Isolation of Genomic DNA

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions.

A part of culture was taken in a microcentrifuge tube and to it 180 µl of T1 buffer and 25 µl of proteinase K were added. The contents were incubated at 56 ºC in a water bath until it was completely lysed. After lysis, 5 µl of RNase A (100 mg/ml) was added and incubated at room temperature for 5 minutes. To this mixture, 200 µl of B3 buffer was added and incubated at 70ºC for 10 minutes and then 210 µl of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into NucleoSpin® Tissue column placed in a 2 ml collection tube and centrifuged at 11000 x g for 1 minute. The NucleoSpin® Tissue column was transferred to a new 2 ml tube and washed with 500 µl of BW buffer and again rinsed using 600 µl of B5 buffer. After washing, the NucleoSpin® Tissue column was placed in a clean 1.5 ml tube and DNA was eluted out using 50 µl of BE buffer.

b) PCR Amplification

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the 16S rRNA gene. The amplification profile consisted of the initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 40 sec followed by a final

extension at 72°C for 8 min. The PCR product was stored at 4°C.

c) SEQUENCING USING BIGDYE TERMINATOR V3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufactures protocol.

The Sequencing PCR mix consisted of the following components:

D/W	6.6µL
5X Sequencing Buffer	1.9µL
Forward Primer	0.3µL
Reverse Primer	0.3µL
Sequencing Mix	0.2µL
Exosap treated PCR product	1µL

Sequencing PCR amplification profile

The amplification profile consisted of the initial denaturation at 96ºC for 2min followed by 30 cycles of denaturation at 96°C for 30 sec, annealing at 50°C for 40 sec followed by a final extension at 60°C for 4 min. The PCR product was stored at 4°C.

Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond et al., 2010). The number of sequences obtained were analysed using NCBI BLAST.

OBSERVATION

➢ SAMPLE COLLECTION

Figure 1 shows the site from which sediment and plastic samples were collected and Figure 2 displays the satellite image of the selected site.

Figure 1

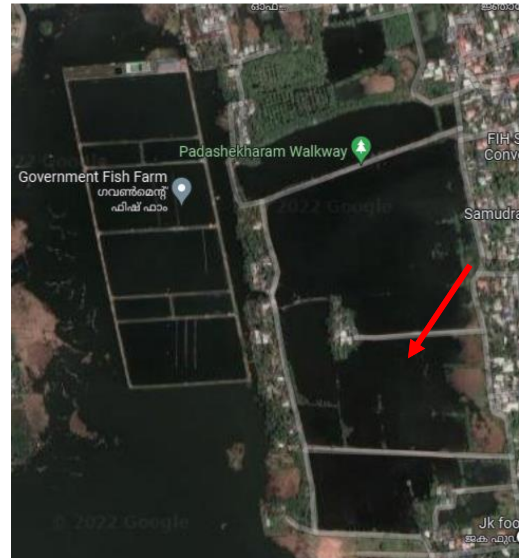


Figure 2

I. <u>ISOLATION OF BACTERIA</u>

A. SERIAL DILUTION - SPREAD PLATE

Sediment sample

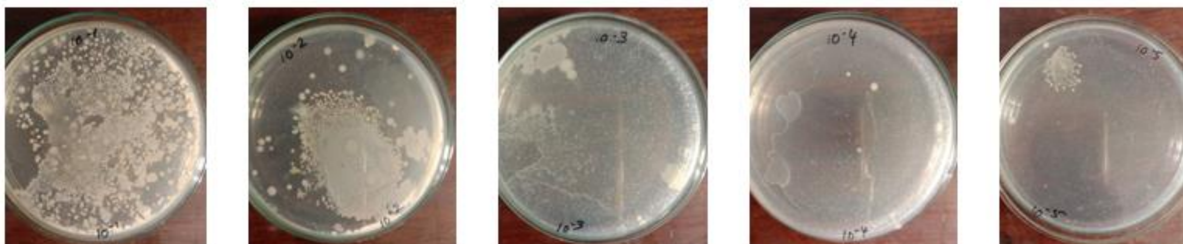


Figure 3

Plastic sample

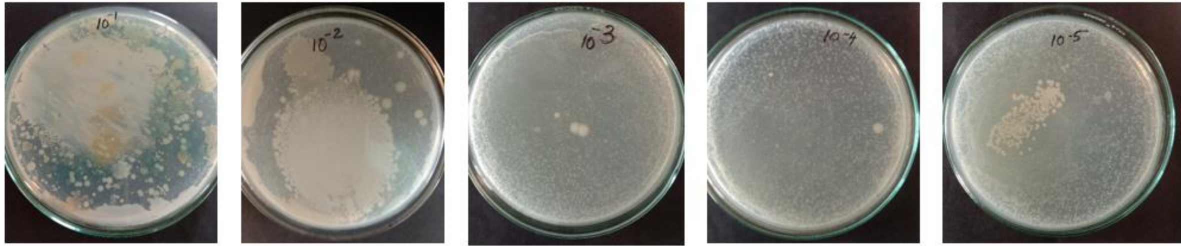


Figure 4

B. STREAK PLATE – QUADRAT STREAK

The following figures shows the bacterial streaks of both sediment and plastic sample.

Sediment sample

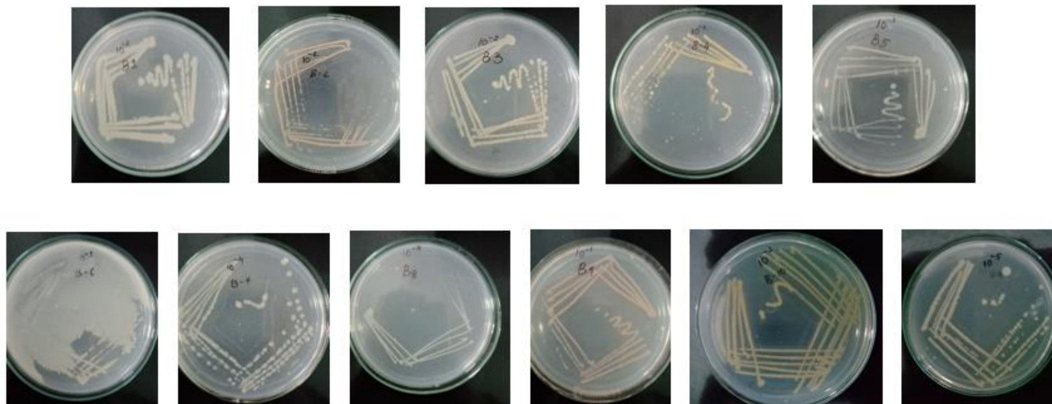


Figure 5

Plastic sample

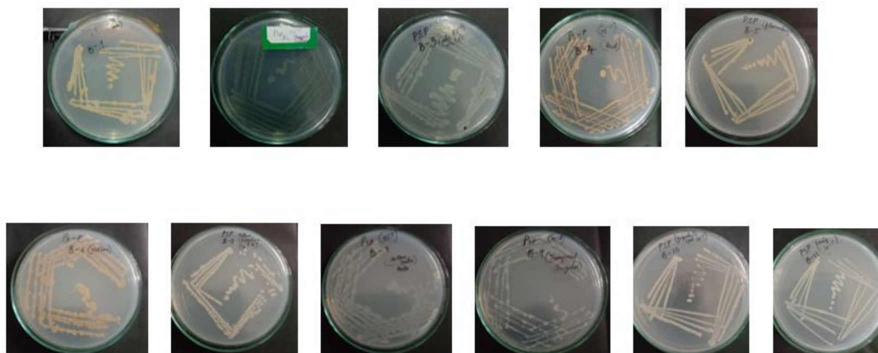


Figure 6

C. SLANT PREPARATION

For ease of storage and refrigeration slant cultures were prepared as is shown in Fig 7 and Fig 8.

Sediment sample

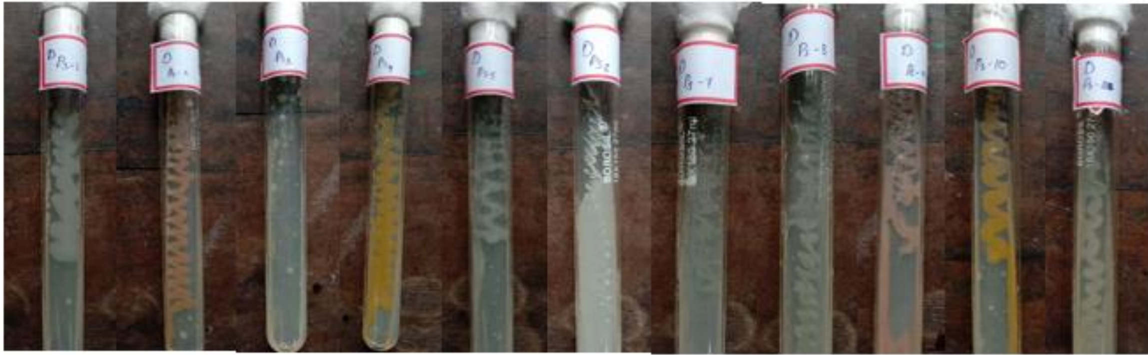


Figure 7

Plastic sample

Figure 8

II. SCREENING OF BACTERIA

The following pictures shows the nature of the cultures during incubation for screening the bacteria of its Degradation potential. The drying of plastic strips is shown in Fig 13.

Figure 9

Figure 10

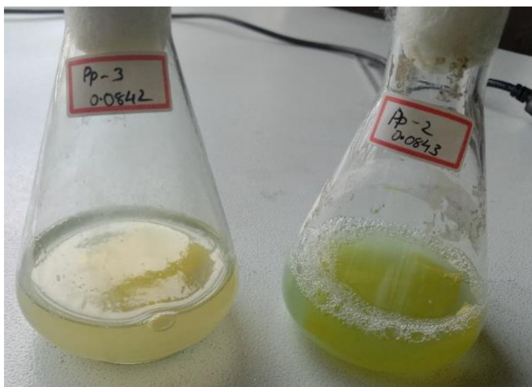


Figure 11

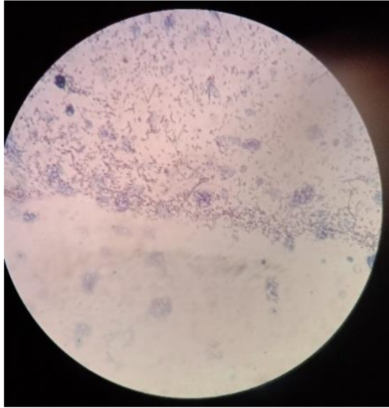
Figure 12

Figure 13

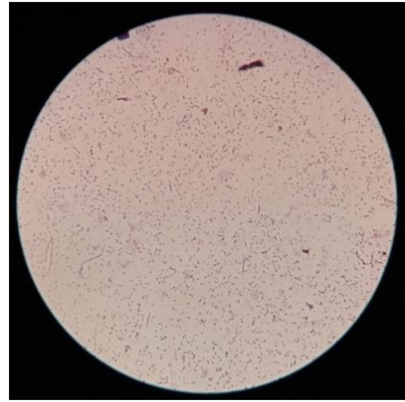
III. BIOCHEMICAL TESTS

a) Gram's Staining

Both the isolates were observed to be Gram positive and Rod – shape cells as shown in Fig 14 and Fig 15.



ISOLATE - 6 [Figure 14]



ISOLATE – 10 [Figure 15]

b) IMViC TEST

Figure 16

I. DNA SEQUENCE

Isolate 10

The sequence analysis of the 16S rRNA gene of Isolate 10 showed that it belongs to the class Bacceliaceae, genus *Priestia* and has a high level of similarity (~99.84%) with the *Priestia aryabhattai*

RESULTS

I. ISOLATION OF BACTERIA FROM SAMPLES

Colony morphology of collected samples

The bacteria were isolated from samples by serial dilution and observed for peculiar morphological characters in their colony which are shown below:

Table 1

SERIAL DILUTION	ISOLATE NO.	COLONY MORPHOLOGY OF SEDIMENT SAMPLE
10^{-1}	PS – 9	Light red round
10^{-2}	PS -1	White irregular
	PS - 2	Crimson round
	PS – 3	Cream round
10^{-3}	PS – 4	Yellow round
	PS – 5	Small dotted
	PS – 6	Branched colony
	PS – 10	Bright yellow round
10^{-4}	PS – 7	Transparent core
	PS – 8	Tiny spots
10^{-5}	PS - 11	Pale white Irregular outline

Table 2

SERIAL DILUTION	ISOLATE NO.	COLONY MORPHOLOGY OF PLASTIC SAMPLE
10^{-1}	PP - 2	Irregular yellow
	PP - 4	Crimson red round
10^{-2}	PP - 1	Pale yellow round
	PP – 3	Pale white
10^{-3}	PP – 8	Transparent core pale white
	PP – 9	Transparent irregular
	PP – 5	Branched irregular
	PP – 6	Yellow – orange irregular
10^{-4}	PP – 7	Yellow irregular outline
10^{-5}	PP - 10	Bright white spots
	PP - 11	Tiny white

II. Screening of bacteria from plastic sample– Polyethylene degradation capacity by weight loss method

The following table depicts the amount of weight lost in the polyethylene when subjected to bacterial degradation in synthetic media. Certain isolates showed significant biodegrading potential out of which two isolates were selectively screened for biochemical analysis.

Table 3

BACTERIAL ISOLATE FROM PLASTIC	CODE	INITIAL PLASTIC WEIGHT (mg)	FINAL PLASTIC WEIGHT (mg)	WEIGHT LOSS (mg)	Weight loss/ month (in %)
Isolate 1	PP 1	85.9	85.3	0.6	0.6 %
Isolate 2	PP 2	84.3	83.8	0.5	0.5%
Isolate 3	PP 3	84.2	82.0	2.2	2.2%
Isolate 4	PP 4	84	82.2	1.8	1.8%
Isolate 5	PP 5	84.5	82.7	1.8	1.8%
Isolate 6	PP 6	85.2	82.5	2.7	2.7%
Isolate 7	PP 7	85.7	84.7	1.0	1.0%
Isolate 8	PP 8	84	81.5	2.5	2.5%
Isolate 9	PP 9	85.5	83.2	2.3	2.3%
Isolate 10	PP 10	85.2	82.5	2.7	2.7%
Isolate 11	PP 11	84.7	82.2	2.5	2.5%

III. BIOCHEMICAL ANALYSIS

a) GRAM'S STAINING AND MOTILITY TEST

SL NO.	TEST	Isolate 6	Isolate 10
1.	Gram's staining	Positive (rod)	Positive (rod)
2.	Motility test	Non- motile	Non- motile

Table 4

b) IMViC TEST

SL NO.	TEST	Isolate 6	Isolate 10
1.	Indole Test	Positive	Positive
2.	Methyl red Test	Negative	Negative
3.	Voges Proskauer Test	Negative	Negative
4.	Citrate Test	Negative	Negative

Table 5

➢ **DNA sequence Analysis**

A. Isolate 10 – PP10

The isolate was identified as a Bacillus sp. *Priestia arybhattai*. It is predominantly soil dwelling Gram positive bacteria and rod shaped cells in chains. The following Table 6 shows the taxonomic position of the Bacteria.

Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Bacillaceae
Genus	*Priestia*
Species	*Priestia aryabhattai*

Table 6

The sequence is as given below

CACTGGGATAACTTCGGGAAACCGAAGCTAATACCGGATAGGATCTTCTCCTTCATGGGAGATGATTGAAAG
ATGGTTTCGGCTATCACTTACAGATGGGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC
AACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCT
TTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACGAGAGTAACTGCTCGTACCTTGACGGTACCTAA
CCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTAT
TGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCAT
TGGAAACTGGGGAACTTGAGTGCAGAAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGAT
GTGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACG

DISCUSSION

It is evident from this study that enzymatic degradation is an emerging alternative to the various modes of plastic degradation. Enzymes extracted from various sources of microbes are been studied by scientists and in search for new and improved mechanisms in enzymatic degradation. Similar to the studies by Amobonye *et al*., 2021, this small study related to the enzymatic degradation of plastic proves the possibility of its degradation that pollutes our environment. It lead to an increased interest towards the basic mechanism by which microorganism are able to degrade this very tough polymer.

In contrast to the most of the previous studies on microbial degradation methods, this study solely aimed at isolating bacteria directly from the plastic sample and incubating it in normal conditions without providing much stress on the growth of the bacteria on the plastic strip. The isolated bacteria was native to the site of polyethylene disposal and exhibited biodegradation in laboratory conditions on synthetic media.

Biodegradation is influenced by many factors (Asiandu *et.al.,* 2020). Optimization of proper environmental factors is the main factor to enhance the ability of bacteria to degrade plastics waste. In spite of considering all the parameters influencing the process, this study aimed at maintaining environmental and biological factors. The optimum temperature (25± 2°C with adequate nutrients for its growth were provided. As per the studies shown by Tokiwa & Suzuki 1977, low molecular weight of the plastic of choice was subjected to the biodegradation process.

As mentioned by Kaushal *et.al.,*2021, the screening of pure breeding colonies of the bacteria increased the time to degrade the plastic. Hence out of the 11 Isolates cultured in the nutrient broth, two isolates – ISOLATE 06 (PP-6) AND ISOLATE 10 (PP-10) resulted in remarkable degradation of the low density polyethylene.

According to Soud, S. A. (2019), measurement of dry weight of plastic were carried out and weight loss in percentage was recorded. Similarly, a total of 11 bacterial isolates were obtained from plastic sample from which Isolate -10 showed accountable degradation capacity.

The sequence analysis of the bacteria showed similarities with *Priestia aryabhattai*. This bacterial strain belongs to the Bacillus sp. The percentage of similarity was recorded to be ~99.84%.

The microscopic view of isolate showed Gram-positive, rod-shaped bacteria and are non-motile. The colony of isolates showed different morphological characteristics of which the screened isolated colony color was bright white and round resemble the species strain broadly studied by Esikova *et.al.,*2021.

CONCLUSION

Plastic waste production around the world is increasing, which leads to global plastic waste pollution. The need for an innovative solution to reduce this pollution is inevitable. Increased recycling of plastic waste alone is not a comprehensive solution. Furthermore, decreasing fossil-based plastic usage is an important aspect of sustainability.

It is obvious that without plastic we can't meet our day to day life needs, but in view of its detrimental effect it is required to develop competent process for its safe disposal and explore alternative.

The bacterial strain isolated displayed accountable percentage of weight loss within a month's time. The bacterial Isolate 10 was identified by sequence analysis. It is known in the scientific name *Priestia aryabhattai* showed the highest degradation potential. It belongs to Bacillus genus where recent studies have proved its use in soil and agriculture as an anti-microbial agent. The Bacillus sp. such as *B. subtilis* is already known to show efficient degradation percentage through various studies.

This study as well as the recent studies prove that biodegradation or enzymatic deterioration of plastics leaves no side effects that pollute the environment. This enzymatic process breaks down the recalcitrant plastic polymers into microbial biomass and other environmentally safe compounds throughout several steps, including biodeterioration, depolymerization, assimilation, and mineralization. Optimization of proper environmental factors is the main factor to enhance the ability of bacteria to degrade plastics waste. Hence, there should be an increasing demand to screen bacteria showing the degradation from non-biodegradable waste materials. Biodegradation of plastic waste using plastics degrading bacteria is a valuable plastic waste treatment that must be implemented to maintain the environment quality of the problems caused by plastic waste. The results of the study portrays that along with efficient technologies biodegradation of plastics can be the solution to waste management problem.

Furthermore, a considerable amount of knowledge is to be gained on the organism screened in this study and its behaviour in the synthetic media. So far, additives such as minerals and benzoates were not used. Studies can be conducted on this particular organism apart from Beef- extract medium to detect the presence of other possible intermediates during degradation.

According to Tatiana et.al, (2021), *Priestia aryabhattai* is shows anti-microbial nature in agriculture and is soil dwelling and use various substrates provided with appropriate culture conditions. The organism is predicted to degrade aromatic compounds with its enzymes. This leads to the versatile properties to be discovered on the context of degradation using various substrates and conditions provided.

The biofilm formation of this bacteria, its growth and its relevance can be further studied in context to finding out the mechanism of extracellular enzymatic action. The bacterium can be assumed to have properties common to Bacillus sps., but further research should be intended for its elucidation. Recent researches on the enzymatic degradation studies proves the need for this process to be commercialized for waste management in the area of study. Segregation of collected waste and subjecting LDPE or low molecular weight plastic bags to biodegradation can be the initial phase. Standardization of the enzymes produced is also another demanding area, biodegradation techniques can be made efficient only if the organism is used to screen such bacteria in a short duration of time is standardized for its action.

REFERENCES

1. Adamcová, D., & Vaverková, M. (2014). Degradation of Biodegradable/Degradable Plastics in Municipal Solid-Waste Landfill. *Polish Journal of Environmental Studies*, *23*(4).
2. Agrawal, P., & Singh, R. K. (2016). Breaking down of polyethylene by Pseudomonas species. *International Journal of Scientific&Engineering Research*, *7*(3), 124-7.
3. Alaerts, L., Augustinus, M., & Van Acker, K. (2018). Impact of bio-based plastics on current recycling of plastics. *Sustainability*, *10*(5), 1487.
4. Amobonye, A., Bhagwat, P., Singh, S., & Pillai, S. (2021). Plastic biodegradation: Frontline microbes and their enzymes. *Science of The Total Environment*, *759*, 143536.
5. Arico,Z., & Jayanthi S. (2018). Processing Plastic Waste into Products Creative as an Economic Improvement of Coastal Communities. *Martabe: Journal of Community Service*, 1(1), 1-6
6. Arutchelvi, J., Sudhakar, M., Arkatkar, A., Doble, M., Bhaduri, S., & Uppara, P. V. (2008). *Biodegradation of polyethylene and polypropylene*. 7: 9-22.
7. Asiandu, A. P., Wahyudi, A., & Sari, S. W. (2021). A review: plastics waste biodegradation using plastics-degrading bacteria. *Journal of Environmental Treatment Techniques*, *9*, 148-157.
8. Bonhomme, S., Cuer, A., Delort, A. M., Lemaire, J., Sancelme, M., & Scott, G. (2003). Environmental biodegradation of polyethylene. *Polymer degradation and Stability*, *81*(3), 441-452.
9. Central Pollution and Control Board. Delhi, 2020. *Annual Report 2019-20 on Implementation of Plastic Waste Management Rules, 2016.* 8279/2021/UPC-II-HO.. *https://cpcb.nic.in/status-of-implementation-of-plastic-waste/*
10. Ciapponi, R., Turri, S., & Levi, M. (2019). Mechanical reinforcement by microalgal biofiller in novel thermoplastic biocompounds from plasticized gluten. *Materials*, *12*(9), 1476.
11. Deepika, S., & Jaya, M. R. (2015). Biodegradation of low density polyethylene by microorganisms from garbage soil. *J Exp Biol Agric Sci*, *3*, 1-5.

12. Drzyzga, O., & Prieto, A. (2019). Plastic waste management, a matter for the ‘community’. *Microbial biotechnology*, *12*(1), 66.
13. Esikova, T. Z., Anokhina, T. O., Abashina, T. N., Suzina, N. E., & Solyanikova, I. P. (2021). Characterization of soil bacteria with potential to degrade benzoate and antagonistic to fungal and bacterial phytopathogens. *Microorganisms*, *9*(4), 755.
14. Gautamr, R., Bassi A. S. and Yanful E. K. (2007). A review of biodegradation of synthetic plastic and foams.Appl. Biochem Biotechnol. 141: 85-108
15. Geyer, R., Jambeck, J. R., & Law, K. L. (2017). Production, use, and fate of all plastics ever made. *Science advances*, *3*(7), e1700782.
16. Gilan, I., & Sivan, A. (2013). Extracellular DNA plays an important structural role in the biofilm of the plastic degrading actinomycete Rhodo-coccus ruber. *Advances in Microbiology*, *2013*.
17. Gironi, F., & Piemonte, V. (2011). European Bioplastics. Bioplastics market data 2018. *Energy sources, Part A: recovery, utilization and environmental effects, https://www. european-bioplastics. org/new-market-data-the-positive-trend-for-the-bioplastics-industry-remains-stable*, 1949-1959. (accessed on 1 April 2020).
18. Gunatillake, P. A., Adhikari, R., & Gadegaard, N. (2003). Biodegradable synthetic polymers for tissue engineering. *Eur Cell Mater*, *5*(1), 1-16.
19. Helbling, C., Abanilla, M., Lee, L., & Karbhari, V. M. (2006). Issues of variability and durability under synergistic exposure conditions related to advanced polymer composites in the civil infrastructure. *Composites Part A: Applied Science and Manufacturing*, *37*(8), 1102-1110.
20. Jambeck, J. R., Geyer, R., Wilcox, C., Siegler, T. R., Perryman, M., Andrady, A., & Law, K. L. (2015). Plastic waste inputs from land into the ocean. *Science*, *347*(6223), 768-771.
21. Kale, S. K., Deshmukh, A. G., Dudhare, M. S., & Patil, V. B. (2015). Microbial degradation of plastic: a review. *Journal of Biochemical Technology*, *6*(2), 952-961.
22. Kale, S. K., Deshmukh, A. G., Dudhare, M. S., & Patil, V. B. (2015). Microbial degradation of plastic: a review. *Journal of Biochemical Technology*, *6*(2), 952-961.
23. Kaushal, J., Khatri, M., & Arya, S. K. (2021). Recent insight into enzymatic degradation of plastics prevalent in the environment: A mini-review. *Cleaner Engineering and Technology*, *2*, 100083.

24. Kim, Y., Yeo, S., Kum, J., Song, H. G. and Choi, H.T. (2005). Cloning of a manganese peroxidase Cdna gene repressed by manganese in Trametes versicolor. J. Microbiol. 43: 569-71.
25. Koushal, V., Sharma, R., Sharma, M., Sharma, R., & Sharma, V. (2014). Plastics: issues challenges and remediation. *International Journal of Waste Resources*, *4*, 1-6.
26. Kumar, R. V., Kanna, G. R., & Elumalai, S. (2017). Biodegradation of polyethylene by green photosynthetic microalgae. *J Bioremediat Biodegrad*, *8*(381), 2.
27. Kumar, S., Das, M. P., Rebecca, L. J., & Sharmila, S. (2013). Isolation and identification of LDPE degrading fungi from municipal solid waste. *Journal of chemical and pharmaceutical research*, *5*(3), 78-81.
28. Montazer, Z., Habibi Najafi, M. B., & Levin, D. B. (2020). Challenges with verifying microbial degradation of polyethylene. *Polymers*, *12*(1), 123. Crossref, https://doi.org/10.3390/polym12010123
29. Mukherjee, S., & Chatterjee, S. (2014). A comparative study of commercially available plastic carry bag biodegradation by microorganisms isolated from hydrocarbon effluent enriched soil. *International Journal of Current Microbiology and Applied Sciences*, *3*(5), 318-325.
30. Okamoto, K., Izawa, M., & Yanase, H. (2003). Isolation and application of a styrene-degrading strain of Pseudomonas putida to biofiltration. *Journal of bioscience and bioengineering*, *95*(6), 633-636.
31. Onen Cinar, S., Chong, Z. K., Kucuker, M. A., Wieczorek, N., Cengiz, U., & Kuchta, K. (2020). Bioplastic production from microalgae: a review. *International journal of environmental research and public health*, *17*(11), 3842. https://doi.org/10.3390/ijerph17113842
32. Phelan, A. A., Meissner, K., Humphrey, J., & Ross, H. (2022). Plastic pollution and packaging: Corporate commitments and actions from the food and beverage sector. *Journal of Cleaner Production*, *331*, 129827.
33. Premraj, R., & Doble, M. (2005). Biodegradation of polymers. *Indian Journal of Biotechnology*, *4*(2), 186-193.
34. Rahman, A., & Miller, C. D. (2017). Microalgae as a source of bioplastics. In *Algal green chemistry* (pp. 121-138). Elsevier.
35. Ru, Jiakang, Yixin Huo, and Yu Yang. "Microbial degradation and valorization of plastic wastes." *Frontiers in microbiology* 11 (2020): 442.

36. Shah, A. A., Hasan, F., Hameed, A., & Ahmed, S. (2008). Biological degradation of plastics: a comprehensive review. *Biotechnology advances*, *26*(3), 246-265.
37. Sharma, B., Rawat, H., & Sharma, P. R. (2017). Bioremediation—a progressive approach toward reducing plastic wastes. *International Journal of Current Microbiology and Applied Sciences*, *6*(12), 1116-1131.
38. Shimao, M. (2001). Biodegradation of plastics. *Current opinion in biotechnology*, *12*(3), 242-247.
39. Shovitri, Maya, and Lisa Marjayandari.(2015) "The Potential of Bacillus SP. Bacteria in Degrading Plastics." *ITS Journal of Science and Arts* 4.2: 15617.
40. Shrestha, J. K., Joshi, D. R., Regmi, P., & Badahit, G. (2019). Isolation and Identification of Low Density Polyethylene (LDPE) Degrading Bacillus spp. from a Soil of Landfill Site. *Acta scientific microbiology*, *2*(4), 30-34.
41. Soud, S. A. (2019). Biodegradation of Polyethylene LDPE plastic waste using Locally Isolated Streptomyces sp. *Journal of Pharmaceutical Sciences and Research*, *11*(4), 1333-1339.
42. Sowmya, H. V., Krishnappa, M., & Thippeswamy, B. (2015). Degradation of polyethylene by Penicillium simplicissimum isolated from local dumpsite of Shivamogga district. *Environment, development and sustainability*, *17*(4), 731-745.
43. Thielen, M. (2014). Bioplastics MAGAZINE In: Bioplastics–Plants and crops, raw materials. Published in Berlin: Fachagentur Nachwachsende Rohstoffe eV (FNR). *Order*, (237).
44. Tokiwa, Y., & Calabia, B. P. (2008). Biological production of functional chemicals from renewable resources. *Canadian Journal of Chemistry*, *86*(6), 548-555.
45. Tokiwa, Y., Calabia, B. P., Ugwu, C. U., & Aiba, S. (2009). Biodegradability of plastics. *International journal of molecular sciences*, *10*(9), 3722-3742.
46. United Nations Environment Program (UNEP) (2018) Single-use plastics: a roadmap for sustainability. https://www.unenvironment.org/resources/report/single-use plastics-roadmap-sustainability. Accessed 3 Apr 2021
47. Usha, R., Sangeetha, T. and Palaniswamy, M. (2011). Screening of Polyethylene Degrading Microorganisms from Garbage Soil Libyan Agriculture Res. Cen. J. Internat. 2 (4): 200-204.
48. Wei, R., & Zimmermann, W. (2017). Microbial enzymes for the recycling of recalcitrant petroleum-based plastics: how far are we?. *Microbial biotechnology*, *10*(6), 1308-1322.

49. Wretfors, C., Cho, S. W., Hedenqvist, M. S., Marttila, S., Nimmermark, S., & Johansson, E. (2009). Use of industrial hemp fibers to reinforce wheat gluten plastics. *Journal of Polymers and the Environment*, *17*(4), 259-266.
50. Zacharia, P. U., Haritha, J., Shamiya, H., Rojith, G., Sharon, B., Dhanya, J., Roshen, G.N., Sajna, V.H., Kaladharan, P., Sipson, A & Padua, S. (2020). Preliminary assessment, restoration and aquaculture support for a small wetland. *Marine Fisheries Information Service; Technical and Extension Series*, (243), 16-20.