ISOLATION AND CHARACTERIZATION OF LOW-DENSITY POLYETHYLENE DEGRADING BACTERIA FROM LANDFILL SOIL

A thesis submitted to

Department of Environmental Science, Faculty of Science and Technology, Bangladesh University of Professionals

For partial fulfillment of the requirements for the Degree of BSc in Environmental Science

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DEDICATION

I dedicate this thesis to my family for caring for me with compassion and love and for their unwavering support for all my achievements in life, as well as to all my hard- working and esteemed teachers.

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This thesis is the result of my hard work. However, it would not have been possible without the generous support of my supervisor, faculty members and the assistance of many others. A heartfelt debt of gratitude is due to everyone involved.

DECLARATION

I hereby declare that the research work entitled “Isolation and characterization of low-density polyethylene degrading bacteria from landfill soil” has been carried out under the Department of Environmental Science, Faculty of Science and Technology, Bangladesh University of Professionals in fulfillment of the requirement for the Degree of BSc in Environmental Science. I have composed this thesis based on original research findings from experiments acquired by me along with references from published literature. This has not been submitted in part or full for any other institution for any other degree. I also certify that there is no plagiarized content in this thesis (Maximum 25%).

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CERTIFICATE OF THE SUPERVISOR

This is to certify that Sun Jarin Juthi carried out her thesis under my guidelines and supervision, and hence prepared the thesis entitled "Isolation and characterization of low-density polyethylene degrading bacteria from landfill soil". So far as I am aware, the researcher duly acknowledged the other researchers' materials and sources used in this work. Further, the thesis was not submitted to any other Universities or institutions for any other degree or diplomas.

It is thus recommended that the thesis be submitted to the Department of Environmental Science, Faculty of Science and Technology, Bangladesh University of Professionals, in fulfilment of the requirements for the award of the degree of BSc in Environmental Science. I also certify that there is no plagiarized content in this thesis (Maximum 25%).

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Abstract

Low-density polyethylene (LDPE) poses environmental challenges due to its non-biodegradable nature. The aim of this study was to isolate the bacteria from the soil of landfill and to evaluate microbial degradation of the sheet of low-density polythene (LDPE). To find the microbes that can degrade polythene, samples were collected from Aminbazar landfill soil, Dhaka. Screening of polythene degrading bacteria was performed by analyzing the growth in Low Density Polyethylene (LDPE) powder. Eight potential bacterial isolates were obtained using mineral salt media containing LDPE powder as sole carbon source. These isolates were found to be polythene degrading through weight loss of polythene in a 30 days of incubation period. These bacterial isolates were characterized both morphologically and biochemically. The Gram staining test revealed all the isolates were Gram positive. The dry cell weight (g/100 L) was measured at 0.1% concentrations of LDPE powder; the biomass was increased for all the bacterial isolates. Isolates 2 and 7 had the highest cell weight values of 0.9 g and 0.8 g, respectively. The weight loss in LDPE sheet by isolate strain 3 and strain 2 was 41% and 33%, respectively. Thus, the potential isolates could be used as LDPE degrading bacteria. This environmentally friendly approach harnesses the power of microbial agents to mitigate LDPE pollution, promoting a more sustainable and natural means of microplastic management.

Keywords: LDPE, Bioremediation, Plastic, Isolation

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Chapter Ⅰ

Introduction

1.1 Background of the study:

A polymer is a macromolecule composed of repeating structural units connected by covalent bonds. Examples of polymers include plastics, starch, and proteins. The term plastic is derived from the Greek word “plastikos‟ which means “capable of being shaped or molded”. Organic and inorganic raw materials such as carbon, silicon, hydrogen, nitrogen oxygen and chloride are used for the manufacture of plastic and are in use today. Plasticity is the property of any material by which the material can irreversibly deform without breaking (Divalaskshmi and Suvashini, 2022). Plastics and their use have become a part of all sectors of the economy. Infrastructure such as agriculture, telecommunication, building and construction, consumer goods, packaging, health and medical are all high growth areas that ensure present demand for plastics (Pooja, 2021). In 1988, the Society of the Plastics Industry introduced the Resin Identification Code (RIC) system which divided plastic resins into 7 different categories (Fig.1). (Such as: PET, HDPE, LDPE, PP, PS, PVC, and others (Roy et al., 2023).

Fig. 1: Types of plastics (Nina and Isabel, 2021)

The first low density polyethylene (LDPE) was created using the high-pressure polymerization of ethylene. Its low density is due to the existence of a modest degree of branching in the chain (on around 2% of the carbon atoms). LDPE is chemically inert at ambient temperature, however it is progressively damaged by strong oxidizing agents and some solvents cause softening or swelling. It can be used at temperatures as high as 95 °C for brief periods of time and as low as 80 °C continually. Low-density polyethylene is an incompletely crystalline solid with a crystallinity of 50-60%, which results in opacity, tensile strength, tear strength, rigidity and chemical resistance, and flexibility even at low temperatures (Ferreira et al., 2005).

Low-density polyethylene (LDPE) is a widely used thermoplastic known for its flexibility and durability. It is a type of polyethylene with a lower density and is commonly used in various applications, including packaging, toys, and medical devices. LDPE's unique properties, such as high chemical resistance and ease of processing, make it a popular choice in the manufacturing industry (Pino et al., 1995).

Low-density polyethylene (LDPE) presents a significant environmental challenge due to its non-biodegradable nature and widespread use. LDPE products, such as plastic bags, transportation, food, clothes, medicine, recreation, fishing nets, packaging, and the food industry contribute significantly to plastic pollution. These items take hundreds of years to decompose, filling landfills and polluting oceans, harming marine life and ecosystems (Jenna et al., 2015). The study emphasized the alarming amount of plastic, including LDPE, entering oceans annually, posing a severe threat to aquatic life and the environment. LDPE also contributes to greenhouse gas emissions during its production and incineration, exacerbating climate change. 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 tons of synthetic polymers are produced worldwide each year (Shimao, 2001). With such a huge amount of polyethylene accumulated in the environment, their disposal evokes a big ecological issue. It takes thousands of years for their efficient degradation.

A LDPE object will take three pathways once it has served its purpose (Fig.2). The first one is recycling, which refers to the reuse of materials. This is considered as one of the environmentally friendly alternatives to produce new plastic items. About 70% of all plastic has been used only once and discarded, while only about 6% of the total has been recycled. This is because each plastic has different material properties and not all the types can be recycled (Vatseldutt and Anbuselvi, 2014). The second one is burning plastics to recover their energy content is another option but doing so frequently results in the release of toxic chemicals like dioxins and furans, which are harmful greenhouse gases that contribute significantly to the ozone layer's depletion. Dioxins cause significant issues with the activity of human endocrine hormones, which makes them a serious threat to human health (Pilz et al., 2010). The left-over plastic waste goes to landfills, where it remains buried (Francis et al., 2010).

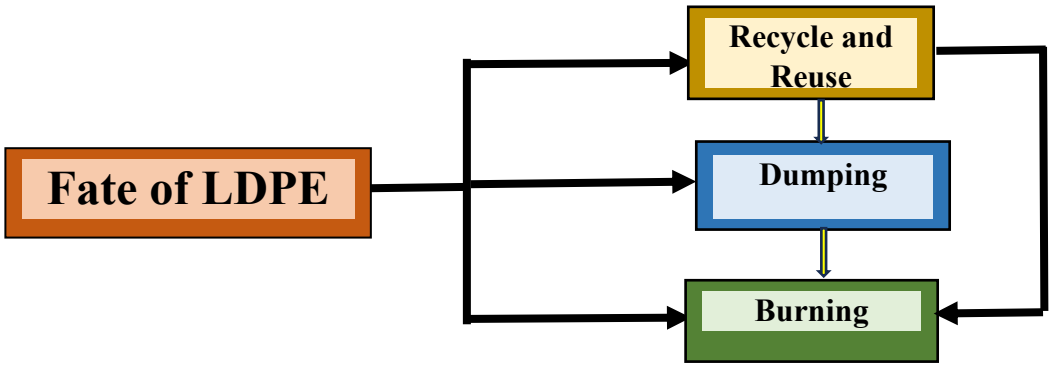


Fig. 2: Fate of LDPE

Low-density polyethylene (LDPE) is a widely used non-biodegradable thermoplastic. To deal with this environmental problem related to non-biodegradable thermoplastics, research to modify non-biodegradable thermoplastics to biodegradable materials is of great interest (Zheng et a., 2005). Furthermore, these synthetic polymers are normally not biodegradable until they are degraded into low molecular mass fragments that can be assimilated by microorganisms (Francis et al., 2010).

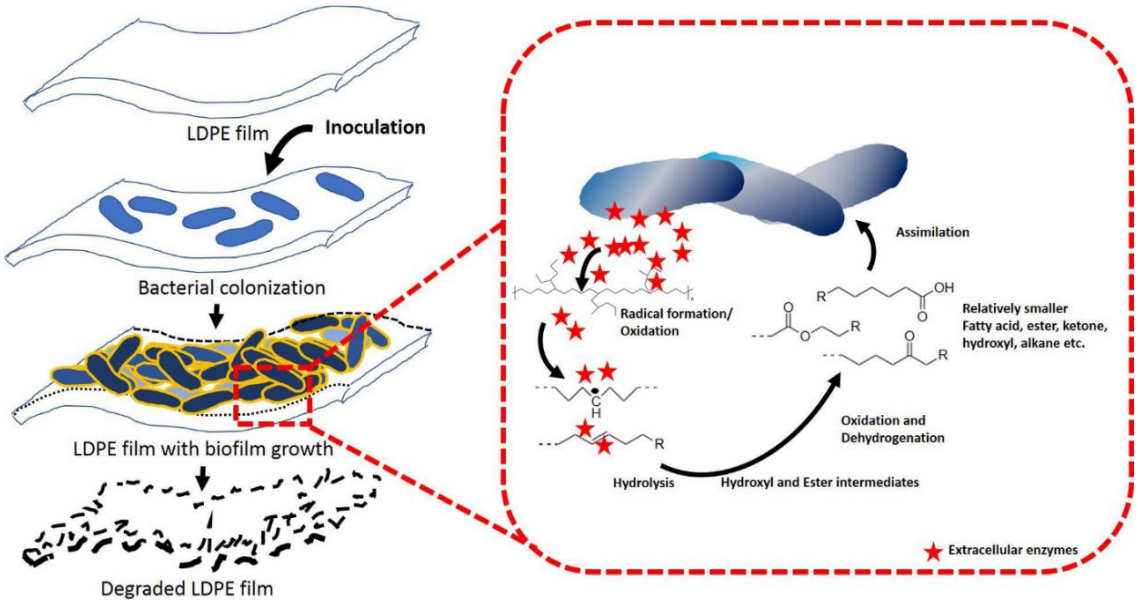


Fig. 3: Biodegradation of LDPE (Anindya et al., 2020)

Microbial biodegradation is widely accepted and is still underway for its enhanced efficiency. Recently several microorganisms have been reported to produce degrading enzymes. Microbial species are associated with degrading materials. Microbial degradation of plastics is caused by certain enzymatic activities that lead to a chain cleavage of the polymer into oligomers and monomers (Fig.3). These water soluble enzymatically cleaved products are further absorbed

by the microbial cells where they are metabolized. Aerobic metabolism results in carbon dioxide and water (Starnecker and Menner,1996), and anaerobic metabolism results in the production of carbon dioxide, water and methane and are called end products, respectively (Gu et al., 2000). The degradation leads to breaking down of polymers to monomers creating an ease of accumulation by the microbial cells for further degradation.

Bacteria (*Pseudomonas, Strep-tococcus, Staphylococcus, Micrococcus, Moraxella*), fungi (*Aspergillus niger, Aspergillus glaucus), Actinomycetes sp.*, and *Saccharomonospora* genus were found as being connected with the degrading polymers (Swift, 1997). Biodegradation is a natural process in the microbial world in which polymers can be utilized as carbon and energy sources for their growth, and it plays an important part in the recycling of these materials in the natural environment (Albertsson et al., 1987). Certain enzymatic activities drive microbial degradation of plastics, resulting in polymer chain cleavage into oligomers and monomers. These water soluble enzymatically cleaved products are further absorbed by the microbial cells where they are metabolized. Aerobic metabolism results in carbon dioxide and water (Starnecker and Menner, 1996), whereas anaerobic metabolism results in carbon dioxide, water, and methane as the end products, respectively (Gu et al., 2000). The aim of this research was to study the biodegradation of low-density polyethylene using various techniques in vitro by selected and potent microorganism isolated from municipal solid waste.

1.2. Problem statement:

There are a variety of ways in which plastic trash harms ecosystems. Because plastics stay so long in the ecosystem, it pollutes, takes up space, and contaminate habitats. This massive amount of plastic is still floating in the water and may be seen on top of and underneath the earth. It's become a serious issue because of the harm it does to people, wildlife, and their habitats. All the trash that didn't get recycled became a hazard for marine life. Discarded fishing gear and nets pose a significant threat to marine life, including birds, fish, turtles, seals, and whales. The ocean is littered with trash. When marine organisms ingest it, it's a happy accident. This plastic has a negative impact on their health.

Micro plastics, for instance, have been detected in the organs of marine mammals, including the liver, the stomach, and the kidneys. Furthermore, soil animals that ingest micro plastics exhibit abnormalities in biochemical responses, including reduced immune responses and aberrations in gene appearance, and this is all before they even begin to experience the more obvious effects of micro plastics, such as energy scarcity, decreased growth and reproduction,

intestinal obstruction, and so on (Jaoa et al., 2016). Large amounts of plastic are also buried underground. It's bad news for the plant kingdom. The plastic trash disrupted the water collection processes of these animals. The widespread use of plastics creates a barrier that prevents rainwater from percolating all the way down to the soil, which has serious consequences for plant life. In the past, plastics' creation took a very long time to decompose. Because of this, there is an ever-increasing problem with soil pollution caused by trash plastics. Contamination of the soil led to a decline in crop yields and ultimately poisoned the food supply. Humans and animals fed the tainted food quickly grew ill.

Fig. 4: Plastic pollution in Bangladesh (Akhter, 2021)

Plastics are being burnt openly in several nations, leading to the formation of photochemical smog. Harmful gases including soot, nitrogen oxides, carbon monoxide, and phosgene are produced in large quantities by this cremation method. These chemicals were not only responsible for ozone depletion, but also for health issues associated with breathing them in. Ashes and smog are released into the air, where they float and eventually settle on the leaves of plants. A coating of plastic pollution formed on the leaves, preventing them from absorbing.

So, in this era of massive plastic pollution bioremediation is an ecological friendly and promising approach. There are numerous studies about LDPE degradation by bacteria and fungi community, yet it is needed to assess more to build up a strong bioremediation technique.

1.3. Rationale of the study:

Dhaka, the capital of Bangladesh, grapples with a significant environmental challenge: the proliferation of Low-Density Polyethylene (LDPE) plastic waste. The city's rapid urbanization

and population growth have led to an alarming increase in plastic consumption, especially single-use LDPE products. Dhaka's inadequate waste management infrastructure and lack of awareness among the populace exacerbate this issue.

The LDPE problem in Dhaka manifests in several ways. First and foremost, the city's drainage system is severely affected. Discarded LDPE bags and wrappers clog drains and sewer lines, leading to waterlogging during monsoons (Hossain et al., 2020). Consequently, this waterlogging disrupts transportation, damages roads, and creates breeding grounds for disease-carrying mosquitoes, posing public health risks. The stagnant water also infiltrates into groundwater reservoirs, contaminating the city's water supply. This not only impacts the city's tourism potential but also poses a threat to the diverse flora and fauna within the city (Shakoor et al., 2019).

The environmental consequences of LDPE pollution extend beyond the city's boundaries. Improper disposal and the lack of recycling facilities mean that a considerable portion of plastic waste ends up in rivers, ultimately reaching the Bay of Bengal. This pollution harms marine life, disrupts ecosystems, and negatively affects fisheries, impacting the livelihoods of coastal communities (Jambeck et al., 2015).

Accumulation of plastic waste is a serious environmental issue. Biodegradation of plastics can be viewed as one of the strategic studies to overcome this problem. The microbes release extracellular enzymes such as lignin peroxidase and manganese peroxidase to degrade the polythene and this study will help us identify the potential isolates for bioremediation of plastic substances from soil and will enable us to develop a comparative analysis of microbial degrader communities present in plastic contaminated soil at different location in Bangladesh

1.3. Research questions:

- ✓ Is the soil microbe potential to degrade the plastic substances in plastic contaminated soil in Bangladesh?

✓ Which plastic degrader species are abundant in such contaminated soil?

✓ Which are the most efficient plastic degraders among all the microorganisms in the collected samples?

1.4. Research objectives:

Broad objective:

To Isolate and characterize the low-density polyethylene (LDPE) degrading soil bacteria from plastic contaminated soil.

Specific objectives

- To isolate the potential bacteria which can degrade LDPE.
- To determine the morphological and biochemical characteristics of potential bacteria.
- To determine plastic degradation efficacy of soil bacteria, present in plastic contaminated site.
- To determine the weight loss of LDPE sheet by potential isolated bacterial strains.

1.5. Research gap

Although there are numerous studies on determination of the plastic degradation efficacy of soil microbes all around the world, but there is yet to be assessed on the identification of potential plastic degrader microorganism and their degradation rate or efficacy isolated from plastic contaminated soil at different location in Bangladesh. There are several research on identification and management of plastic, alternatives of plastics and the threat of plastic on environment in Bangladesh, while it is essential to detect the potential degraders to establish strong bioremediation technique.

1.6. Limitations of the study:

- ✓ Only a few biochemical tests were conducted which are not enough for identification of bacteria.
- ✓ The 16s RNA technique for obtaining genomic identification of each isolate was not conducted.
- ✓ The CO_2 evolution test and FTIR analysis were not performed due to time constraints.

Chapter Ⅱ

Literature Review

Some important works relevant to present thesis paper are reviewed and briefly cited below:

2.1. Plastics

Plastics are a kind of synthetic or semisynthetic polymer that are made up of long chains of carbon atoms, and they may also have oxygen, nitrogen, or sulfur atoms attached to them. From the Greek plastikos, this means "moldable into diverse forms and sizes," we get the English term "plastic" (Joel, 2022). Synthetic polymers derived from petroleum amount to around 140 million metric tons yearly, and a huge proportion of them are released into the environment as byproducts of industrial processes (Shimao, 2023). Products including food, medicine, cosmetics, detergents, and chemicals all use packaging made of synthetic polymers. About 30 percent of all plastics manufactured are used in packaging. There is still rapid growth in use, which stands around 12% annually (Pooja, 2021).

It has revolutionized modern society with its remarkable versatility and wide-ranging applications. Beginning in the early 20th century, the development of plastics accelerated, leading to a myriad of uses in packaging, construction, transportation, healthcare, and electronics. The inception of this material can be traced back to the groundbreaking work of Leo Baekeland, who invented Bakelite, the world's first synthetic plastic, in 1907 (Abir et al., 2021). Since then, plastics have undergone continuous innovation and development. Researchers like Carothers (2015) explored polymerization techniques, paving the way for nylon, while Ziegler and Natta (2022) revolutionized the field with their work on polyethylene and polypropylene.

Plastics are synonymous with "environmentally resistant materials" since their stability and durability have been steadily enhanced throughout time. Due to plastics' relatively brief lifespan in nature, evolution has not had time to create enzyme structures capable of breaking down the manmade polymers (Mueller, 2006). The dramatic increase in production and lack of biodegradability of commercial polymers, primarily commodity plastics used in packaging (e.g., fast food), industry, and agriculture, has attracted public attention as a potentially enormous environmental accumulation and pollution problem that could persist for centuries (Albertsson et al., 1987). There are several methods for getting rid of plastic trash, including burying it, burning it, and recycling it. Furthermore, the combustion of polyvinylchloride (PVC) polymers releases furans and dioxins, two persistent organic pollutants (POPs)

(Jayasekara et al., 2005). Chemical, photo, thermal and biological methods are also involved in the current polymer degradation policies. But for environmental, economic and health advantages; the practice of biodegradable plastics are the attractive options. (Sumaira et al., 2015)

2.2. Types of plastics:

Plastics are a diverse group of materials with a wide range of properties and applications, classified into several types based on their molecular structure and polymerization process.

Table 1: Types of plastics

Type	Properties and use
Polyethylene terephthalate (PET)	Polyethylene terephthalate (PET) is a versatile thermoplastic polymer known for its excellent mechanical, thermal, and chemical properties. It is commonly used in beverage bottles, food packaging, and textile fibers due to its unique characteristics. PET exhibits high tensile strength, making it suitable for applications requiring robust materials (Whinfield & Dickson, 1941). Its transparency, coupled with high gloss, makes it ideal for clear packaging materials, enhancing product visibility. Moreover, PET offers good resistance to moisture, chemicals, and abrasion, ensuring durability in various environments (Ochigus & Kawai, 1982). Additionally, PET has excellent gas barrier properties, preserving the freshness and quality of packaged goods, especially in the food and beverage industry (Kyriakos, 2013). It also possesses good thermal stability, enabling it to withstand high temperatures during processing and sterilization (Chang & Chang, 1996). These exceptional properties have established PET as a preferred choice in the packaging industry, contributing to its widespread use worldwide.
High density polyethylene (HDPE)	High density polyethylene (HDPE) is known for its high tensile strength, stiffness, and excellent impact resistance, making it suitable for applications such as pipes, containers, and toys (Mark et al., 2012). High-density polyethylene (HDPE) is a thermoplastic

	polymer known for its excellent physical and chemical properties. It is characterized by its high strength-to-density ratio, making it a robust and lightweight material. HDPE is resistant to moisture, chemicals, and UV rays, ensuring durability in various environmental conditions. Its excellent impact resistance and flexibility make it suitable for applications requiring toughness and ease of processing. Additionally, HDPE is recyclable, contributing to its eco-friendly profile. These properties have made HDPE a popular choice in packaging, pipes, toys, and other consumer products. (Moore et al., 2006)
Low density polyethylene (LDPE)	Low density polyethylene (LDPE), on the other hand, offers flexibility and excellent resistance to moisture, making it ideal for packaging materials, squeeze bottles, and agricultural films (López et al., 2002). Low-density polyethylene (LDPE) is a versatile thermoplastic known for its unique set of properties. LDPE exhibits excellent flexibility, making it highly resistant to impact and allowing it to conform to various shapes. Its low density grants it lightweight characteristics, contributing to its widespread use in packaging materials, plastic bags, and films. LDPE also boasts high chemical resistance, making it suitable for containers and piping systems handling a variety of substances. Furthermore, LDPE has good electrical insulating properties, making it valuable in cable and wire applications. This combination of flexibility, low density, chemical resistance, and electrical insulation renders LDPE indispensable in numerous industrial and consumer applications (Michael and Ralph, 2003).
Polypropylene (PP)	Polypropylene (PP) is a thermoplastic polymer characterized by its exceptional durability, high melting point, and resistance to chemical solvents. It possesses remarkable mechanical properties, including high tensile strength and impact resistance, making it ideal for a wide array of applications such as packaging, textiles, automotive

	components, and medical devices. PP is also known for its excellent thermal stability, allowing it to withstand high temperatures without significant deformation. Additionally, it is relatively lightweight, making it easy to handle and transport. Its resistance to moisture and most chemicals further enhances its usability. The unique combination of these properties makes PP a popular choice in various industries (Natta et al., 1995).
Polystyrene (PS)	Polystyrene (PS) is a versatile thermoplastic known for its lightweight and rigid properties, making it ideal for a variety of applications. It possesses excellent electrical insulation, making it valuable in the electronics industry (Carraher, 2016). PS exhibits clarity and can be transparent, making it suitable for applications where visibility is important. Additionally, it has a low water absorption rate and good resistance to acids and bases, enhancing its durability in different environments. PS can be molded into intricate shapes and has a relatively low melting point, facilitating easy processing in manufacturing (Carraher, 2016). Its insulating properties also make it popular in the construction industry, particularly in insulation materials. However, it is worth noting that PS is susceptible to degradation from ultraviolet (UV) light exposure. These characteristics underline the diverse utility of polystyrene in everyday items, ranging from packaging materials to disposable tableware and insulation products, contributing significantly to various industries (Carraher, 2016).
Polyvinyl chloride (PVC)	Polyvinyl chloride (PVC) is a versatile plastic renowned for its exceptional properties. PVC is durable, chemically resistant, and has excellent fire-retardant qualities, making it ideal for various applications. It can be rigid or flexible, depending on the additives used during its production, allowing for a wide range of uses in construction, healthcare, automotive, and electrical industries. PVC also possesses good electrical insulation properties, enhancing its usability in electrical wiring and cable applications. Additionally, PVC is lightweight and easy to process, making it a popular choice

	in manufacturing. Its unique combination of properties, including durability, chemical resistance, and flexibility, has contributed to its widespread adoption (Barnes, 2005).

2.2. Brief history of LDPE:

Low-Density Polyethylene (LDPE) stands as a testament to the innovative spirit of polymer science, transforming the landscape of modern materials since its discovery. The story of LDPE begins in the early 1930s when two scientists, Reginald Gibson, and Eric Fawcett, working at Imperial Chemical Industries (ICI) in the United Kingdom, stumbled upon a substance that displayed peculiar properties. The researchers, intrigued by the waxy material formed during an ethylene gas experiment, delved deeper. In 1933, Michael Perrin, an ICI researcher, identified the substance as a new type of polyethylene, characterized by its low density. This marked the birth of LDPE, a material that would soon revolutionize various industries.

In the following decades, significant advancements were made to refine LDPE's production process. In 1939, an accidental discovery by researchers Ralph Wiley and Ralph Shaw at DuPont led to the development of high-pressure free-radical polymerization techniques, a breakthrough that enabled the mass production of LDPE. This pioneering work laid the foundation for LDPE's commercial production and widespread applications.

During the 1950s and 1960s, LDPE found its way into diverse applications, ranging from packaging materials to agricultural films. Its flexibility, chemical resistance, and ease of processing made it a popular choice for manufacturers. In 1953, Karl Ziegler and Giulio Natta received the Nobel Prize in Chemistry for their revolutionary work on polymerization, including the development of polyethylene variants, contributing significantly to the understanding of LDPE's molecular structure.

The 1970s witnessed the introduction of low-density polyethylene into new territories, such as the healthcare industry. Its use in medical devices and packaging materials highlighted LDPE's biocompatibility and versatility. Concurrently, research efforts were directed towards enhancing LDPE's properties, leading to the discovery of copolymers and blends, expanding its range of applications.

In the late 20th century, environmental concerns prompted researchers to explore sustainable alternatives and methods for recycling LDPE. Studies by Thompson, Moore, and Saal (2009)

shed light on plastic pollution, emphasizing the need for responsible disposal and recycling practices. These findings ignited global initiatives to mitigate plastic waste, prompting researchers to develop biodegradable LDPE formulations (Tharanathan, 2003), aiming to reduce the material's environmental impact.

In the 21st century, research continued to push the boundaries of LDPE applications. Scientists focused on enhancing its mechanical strength (Plackett & Andersen, 2010), making it suitable for demanding engineering applications. Additionally, advancements in nanotechnology enabled the development of LDPE nanocomposites with superior properties (Rahman et al., 2015), opening new avenues in materials engineering.

Today, LDPE remains a cornerstone of the plastic industry, finding applications in packaging, agriculture, healthcare, and beyond. Its rich history of innovation, coupled with ongoing research, underscores LDPE's enduring significance in shaping the modern world.

2.3. Environmental contamination by LDPE:

Environmental contamination by Low-Density Polyethylene (LDPE) is a critical issue globally. Improper disposal and slow degradation lead to the accumulation of LDPE waste, polluting ecosystems, water bodies, and soil. This contamination disrupts natural habitats, harms wildlife, and poses health risks to humans through the food chain. LDPE particles have been found in various environments, indicating the widespread nature of this problem (Wright et al., 2013). Microplastics, including LDPE fragments, persist in the environment, raising concerns about their long-term impact on ecosystems and human health (Gigault et al., 2018).

2.3.1. Soil contamination by LDPE:

Soil contamination by LDPE (Low-Density Polyethylene) is a significant environmental concern. LDPE can persist in the soil for hundreds of years, negatively impacting soil health and ecosystems. As LDPE degrades, it can release potentially harmful additives and microplastics into the soil, leading to issues like reduced soil fertility and disrupted nutrient cycling (Silva et al., 2020). LDPE fragments can also be ingested by soil-dwelling organisms, potentially affecting their health and the entire soil food web.

2.3.2. Water pollution by LDPE:

Water contamination by LDPE (Low-Density Polyethylene) is a major environmental concern and disposal of polyethene bag into ocean, river and seas has been increasing rapidly. LDPE,

a common plastic used in packaging and containers, leaches potentially harmful chemicals into water sources over time, posing risks to aquatic ecosystems and human health (Smith, 2018). The release of phthalates and other additives from LDPE can disrupt endocrine systems in aquatic organisms, leading to adverse effects on reproduction and development (Jones & Johnson, 2019). Moreover, LDPE debris in aquatic environments can accumulate toxins and serve as vectors for the spread of pollutants, compounding contamination issues (Brown et al., 2020). Effective mitigation strategies and increased awareness are needed to address this pervasive environmental challenge.

2.3.3. Air pollution by LDPE:

Air contamination by LDPE (Low-Density Polyethylene) is a concerning environmental issue. LDPE, commonly used in plastic manufacturing, can degrade under sunlight, releasing microplastics into the atmosphere (Thompson, 2019). These airborne microplastics may act as carriers for harmful chemicals and become deposited in various environments, potentially affecting air quality and human respiratory health (Wright et al., 2020). The inhalation of LDPE-derived particles has raised concerns about their potential toxicity and long-term health impacts.

2.3.4. Effects of LDPE on public health:

The effects of LDPE (Low-Density Polyethylene) on public health are a growing concern. LDPE, commonly used in plastic products, can release chemicals and microplastics that may contaminate food and water sources, potentially leading to human exposure (Gallo et al., 2018). Ingested microplastics may act as vectors for harmful pollutants, raising questions about the long-term health impacts, including potential carcinogenic and endocrine-disrupting effects (Koelmans et al., 2019). Moreover, LDPE pollution in the environment can exacerbate respiratory problems through the release of airborne microplastics (Wright et al., 2020).

2.4. Biodegradation of LDPE:

While biodegradation shows potential, optimizing the process and understanding its ecological implications is ongoing research. LDPE biodegradation offers a sustainable avenue for plastic waste reduction, aligning with global environmental goals. As microorganisms possess different characteristics, the degradation varies from one microorganism to another (Bhardwaj et al., 2012). Recently, several microorganisms have been reported for degradation of plastics.

The bacterial species 25 identified from the polyethylene bags tested were *Bacillus sp., Staphylococcus sp., Streptococcus sp., Diplococcus sp., Micrococcus sp., Pseudomonas sp.* and *Moraxella sp.* Among the fungal species identified, *Aspergillus niger, Amauroclopius ornatus, Aspergillus nidulans, Janibacter cremeus, Aspergillus flavus, Aspergillus candidus 26* and *Aspergillus glaucus* were the predominant species (Kathiresen et al., 2003). *Brevibacillus borstelensis* strain isolated from soil, a thermophilic bacterium, recovered for the degradation of branched low-density polyethylene by utilizing it as the sole carbon source and energy source. The incubation of polyethylene film with B. borstelensis revealed the reduction in molecular weight of polyethylene by 30% (Hadad et al., 2005).

2.4.1. Biodegradation of LDPE by bacteria:

Biodegradation of LDPE (Low-Density Polyethylene) by bacteria is an environmentally promising approach to tackle plastic pollution. Some bacterial species, such as *Pseudomonas*, *Bacillus*, and *Ideonella sakaiensis*, have been identified as LDPE degraders (Yoshida et al., 2016; Hadad et al., 2018). These microbes produce enzymes like PETase and MHETase that break down LDPE's complex polymer chains into biodegradable byproducts.

One of the significant studies in this field was conducted by Shah et al. (2008), who isolated a bacterial strain, *Pseudomonas citronellolis*, capable of degrading LDPE. The researchers identified the enzymatic pathways involved in LDPE degradation and highlighted the potential of this strain for bioremediation purposes. Similarly, Hadad et al. (2005, 2007) explored the biodegradation of LDPE by different bacterial strains, emphasizing the role of microbial consortia in breaking down this persistent plastic.

The process of LDPE biodegradation by bacteria involves several steps (Fig.5). First, the bacteria produce extracellular enzymes, such as lipases and esterases, which hydrolyze the LDPE polymer chains into smaller molecules. These smaller molecules are then taken up by the bacterial cells and metabolized through various metabolic pathways, ultimately leading to the complete degradation of LDPE into harmless byproducts such as carbon dioxide and water (Mohee et al., 2008). Factors such as temperature, pH, and the presence of co-substrates significantly influence the biodegradation rate. Studies by Mohee et al. (2008) and Kyaw et al. (2012) focused on optimizing these parameters to improve the efficiency of LDPE biodegradation.

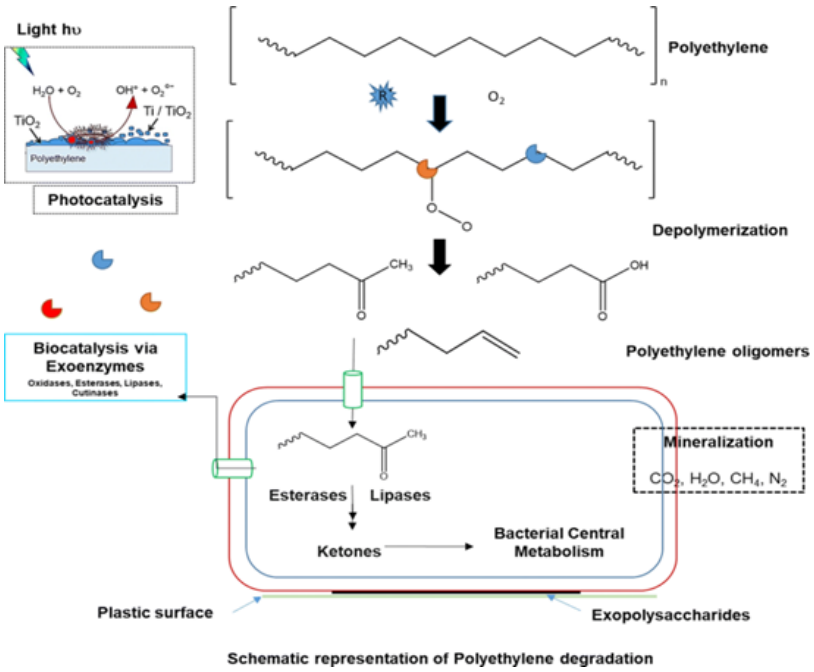


Fig. 5: Biodegradation of polyethylene by bacteria (Sunil et al., 2020)

Moreover, the use of microbial consortia, where multiple bacterial strains work together synergistically, has shown promising results. These consortia often involve bacteria with complementary enzymatic activities, allowing for the efficient breakdown of LDPE (Shah et al., 2016). Research by Urbanek et al. (2017) highlighted the potential of microbial consortia in degrading LDPE, emphasizing the importance of biodiversity in enhancing biodegradation processes.

In recent years, genetic engineering techniques have been employed to enhance the biodegradation capabilities of bacteria. Scientists have modified bacteria to produce novel enzymes capable of efficiently breaking down LDPE (Yang et al., 2014). These genetically modified bacteria hold great promise for accelerating the biodegradation of LDPE and reducing plastic pollution in the environment.

The biodegradation of LDPE by bacterial strains not only offers a solution to plastic pollution but also presents opportunities for various applications. Biodegraded LDPE can be utilized in the production of biodegradable plastics, reducing the dependency on fossil fuels and mitigating the environmental impact of plastic production (Arutchelvi et al., 2008). Additionally, the byproducts of LDPE biodegradation, such as organic acids, can be used in industrial processes, contributing to the development of a circular economy (Chamas et al., 2020).

However, challenges remain in scaling up LDPE biodegradation processes for large-scale applications. Factors such as the availability of suitable bacterial strains, cost-effectiveness, and environmental impact assessments need to be carefully considered. Continued research and collaboration between scientists, policymakers, and industry stakeholders are essential to overcome these challenges and implement effective LDPE biodegradation solutions on a global scale.Genetic engineering techniques further enhance the efficiency of this process, paving the way for innovative solutions to plastic pollution. By harnessing the natural capabilities of microorganisms, we can move towards a more sustainable future, where plastic waste is biodegraded, reducing its environmental impact and contributing to a cleaner, healthier planet.

Table 2: Bacterial strains associated with polyethene biodegradation:

Genus	Species	References
Pseudomonas	*Ps. aeruginosa*	Tribedi et al., 2013
	Ps. fluorescens	Rajandas et al., 2012
Paenibacillus	*Pa. macerans*	Nowak et al., 2011
Rahnella	*Rahnella aquatilis*	Nowak et al., 2011
Ralstonia	*Ralstonia sp*	Nowak et al., 2011
Rhodococcus	*Rhodo. erythropolis*	Koutny et al., 2009
	Rhodo. rhodochrous	Koutny et al., 2009
	Rhodo. ruber	Fonlanella et al., 2010
Staphylococcus	*Staphylo. cohnii*	Santo et al., 2012
	Staphylo. epidermidis	Chattarjee et al., 2010
	Staphylo. xylosus	Nowak et al., 2011
Stenotrophomonas	*Steno. sp*	Koutny et al., 2009
Streptomyces	*Strepto. badius*	Pometto et al., 1992
	Strepto. setonii	Pometto et al., 1992
	Strepto. viridosporus	Pometto et al., 1992
Bacillus	*B. amyloliquefaciens*	Walanbee et al., 2009
	B. brevies	Nowak et al., 2011
	B. cereus	Walanbee et al., 2009
	B. circulans	Ferreira et al., 2005
	B. halodenitrificans	Nowak et al., 2011

	B. mycoides	Nowak et al., 2011
	B. pumilus	Sudhakar et al., 2008
	B. sphericus	Nowak et al., 2011
	B. thuringiensis	Hadad et al., 2005
Brevibacillus	*Brevi.borstelensis*	Koutny et al., 2009
Delftia	*Delftia. acidovorans*	Koutny et al., 2009
Flavobacterium	*Flavo. sp*	Nowak et al., 2011
Micrococcus	*Micro. luteos*	Nowak et al., 2011
	Micro. lylae	Rajandas et al., 2012
Microbacterium	*Micro. paraoxydans*	Fonlanell et al., 2010
Nocardia	*N. asteroids*	Nowak et al., 2011
Acinetobacter	*Acineto. baumannii*	Balashuvaramanian et al., 2010
Arthobacter	*Artho. sp*	Albertson et al., 2010
	Artho. paraffineus	Nowak et al., 2011
	Artho. viscosus	Albertson et al., 2010

2.4.2. Biodegradation by fungi:

Although several microorganisms are involved in degradation of LDPE, it remains a challenging task to obtain a strain for commercial and eco-friendly degradation of LDPE. Over the years, culturable, LDPE degrading microbes have been isolated from a wide variety of sources such as soil, wastewater, compost, garbage, etc. Moreover, efficient screening techniques are a prerequisite for isolation of novel strains. Fungi offers potential for biodegradation of LDPE (Low-Density Polyethylene), addressing plastic pollution concerns. Species like *Aspergillus, Penicillium,* and *Fusarium* have shown LDPE-degrading capabilities (Akhtar et al., 2014; Shah et al., 2018). These fungi secrete enzymes that break down LDPE's polymer structure into smaller, more biodegradable components. However, optimizing the process for efficiency and scale remains a challenge. LDPE biodegradation by fungi presents an ecologically sound solution for plastic waste reduction, contributing to a more sustainable and environmentally friendly future.

Kumar et al. (2010) reported the techniques for isolation and screening of potential fungal strains to degrade LDPE in-vitro. Identification of microorganisms is based on their cellular

fatty acid methyl ester (FAME) profiles. Paul Das and Kumar (2014) isolated four Aspergillus sp. (FSM-3, 5, 6, 8) and one Fusarium sp. (FSM-10) from soil samples by spread plate technique using mineral salt medium (g/l: K_2HPO4 1.0, KH_2PO_4 0.2, NaCl 1.0, $CaCl_2 \cdot 2H_2O$ 0.002, H_3BO_3 0.005, NH_4 $(SO_4)^2$ 1.0, $MgSO_4$-$7H_2O$ 0.5, $CuSO_4.5H_2O$ 0.001, $ZnSO_4.H_2O$ 0.001, $MnSO_4.H_2O$ 0.001, $Fe_2(SO_4)$-$3.6H_20$ 0.01, Agar 15) supplemented with 3% LDPE powder as carbon source. The developed fungal mats were subcultured on Saboraud's dextrose agar to get pure culture and preserved in slant at 4°C. The identification of the fungal isolate was performed by recognizing the diagnostic morphological features of genera using macroscopic and microscopic examinations (A. Esmaeili et al., 2013). In addition, the molecular identification methods using PCR to amplify a segment of the rRNA operon encompassing the 5.8S rRNA gene and flanking internal transcribed spacers (ITS) is now in progress at the Iranian Biological Resource Center (IBRC).

Esmaeili et al. (2013) isolated *A. niger* from landfill soils (in which PE wastes had been deposited for varying amounts of time) on mineral medium with PE powder (5% ethylene oligomer) as the sole carbon source. Based on microscopic examination and morphological characteristics, Mishra et al. (2013) isolated *Chrysonilia, Aspergillus,* and *Penicillium* using synthetic medium. Gilman (2012) used the "Manual of Soil Fungi" to identify the fungus strain. This taxonomic identification was further validated by the Agharkar Research Institute in Pune. More fungal forms were identified in the second series of experiments using the same synthetic media but with plastic as the only carbon source instead of glucose. About 4 different forms were found growing on powder of PVC and granules of LDPE and HDPE. These forms were species of genus, *Aspergillus, Penicillium. Fusarium and Chaetomium.*

LDPE pieces buried in soil mixed with sewage sludge were examined microscopically after 10 months, fungal attachment was found on the surface of the plastic, indicating possible utilization of plastic as a source of nutrient (Shah, 2007). The isolated fungal strains were identified as Fusarium sp. AF4, Aspergillus terreus AF5 and Penicillum sp. AF6. The ability of the fungal strains to form a biofilm on polyethylene was attributed to the gradual decrease in hydrophobicity of its surface (Orr et al., 2004).

Table 3: Fungal strains associated with polyethylene biodegradation

Genus	Species	References
Aspergillus	*A. niger*	Manzur et al., 2004

	A. versicolor	Karlsson et al., 1988
	A. flavus	Koutny et al., 2006
Cladosporium	*Cl. cladosporiodes*	Koutny et al., 2006
Chaetomium	*Ch. sp.*	Sowmya et al., 2002
Chaetomium	*CH. sp.*	Koutny et al., 2006
Fusarium	*F. redolens*	Manzur et al., 2004
Glioclodium	*G. virens*	Framila et al., 2011
Mucor	*M. circinrlloides*	Yamada et al., 2001
Penicillum	*P. simplicissimum*	Manzur et al., 2004
	P. pinophilum	Seneviratne et al., 2006
	P. frequentas	Manzur et al., 2004
Phanerochaete	*Ph. Chrysosporium*	Manzur et al., 2004
Verticillium	*V. lecanii*	Seneviratne et al., 2006

2.5. Mechanism of LDPE degradation by microorganisms:

The mechanisms of biodegradation for polyethylene can be studied from three different perspectives: colonization of the polymer by microorganisms; chemical/biochemical reactive pathways; and the impact of macromolecular structure of the polymer on microbial usage. Biofilms are sessile communities of microorganisms developed on a surface that can be composed of individuals from the same or different species (Donlan, 2002). Complex biofilm communities comprised of different microorganisms have been detected on polyethylene surfaces once they were exposed to different biotic environments (Siven et al., 2006).

Studies on microorganism attachment to polyethylene have identified that the main limitation of the colonization process is the relatively high hydrophobicity of the polymer in contrast to the regularly hydrophilic surfaces of most microorganisms (Gilan et al., 2004; Tribedi and Sil, 2013). It has been proposed that strains with more hydrophobic surfaces can play an important role in the initial colonization of the polymer (Karlsson et al., 1988; Tribedi and Sil, 2013).

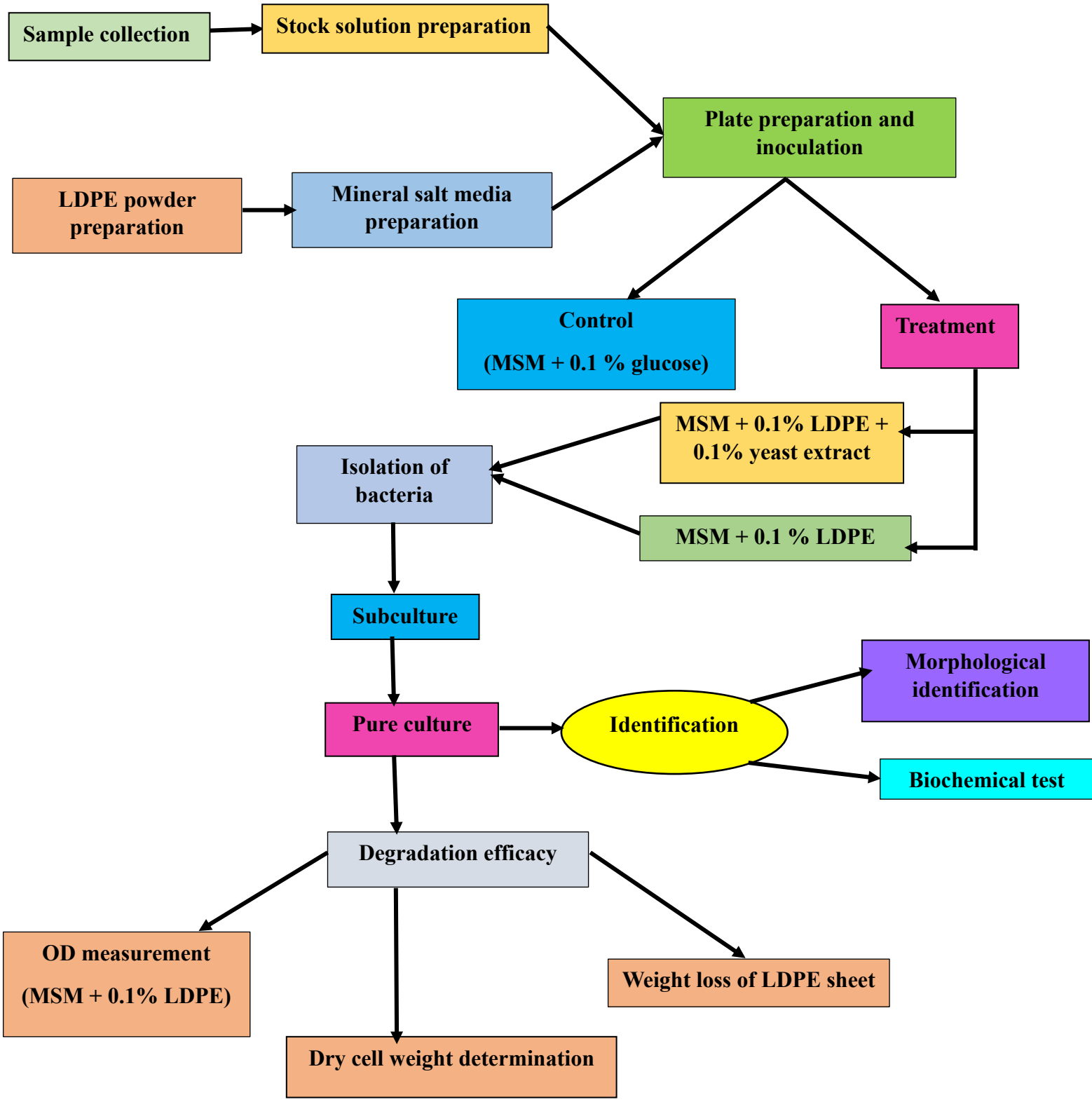
Theoretically, polyethylene can be used as a carbon source for microorganisms like many other hydrocarbons; however, its high molecular weight limits its use as a substrate for enzymatic reactions to take place. In terms of the chemical/biochemical processes involved in polyethylene biodegradation it can be stated that there are two key reactions, the first one being

the reduction of its molecular weight and the second being the oxidation of the molecules. Reduction of molecular weight is required for two reasons, firstly to enable transport of molecules through the cell membrane, and secondly because enzymatic systems present in the microorganisms are only able to attack certain molecular weights, usually in the range of 10e50 carbons, though there has been a report of enzymatic activity up to 2000 carbons (Yoon et al., 2012).

Once the size of the molecule is reduced, oxidation is required to transform the hydrocarbon into a carboxylic acid that can be metabolized by means of b-oxidation and the Krebs cycle (Albertsson et al., 1987). Both oxidation and molecular weight reduction during the biodegradation process are a result of synergistic effects between biotic and abiotic factors (photooxidation or heat treatment).

The biotic factor is determined by groups of enzymes able to degrade oxidized or reduced polyethylene molecules. Breaking down large polyethylene molecules can be accomplished by enzymatic action, as proven by Santo et al. (2012), who found that by incubation with the enzyme laccase the molecular weight of a polyethylene was reduced, and its keto carbonyl index increased. Fungi secrete extracellular enzymes like esterases, lipases, and cutinases. These enzymes cleave the ester bonds and hydrophobic regions of LDPE, creating smaller fragments. The hydrophilic and hydrophobic parts of the enzymes interact with the polymer, facilitating degradation (Ronkvist et al., 2009). Bacteria like *Pseudomonas*, *Bacillus*, and *Ideonella sakaiensis* and fungi like *Aspergillus* and *Penicillium* produce enzymes, such as PETase and MHETase, which further break down the polymer (Yoshida et al., 2016; Shah et al., 2018). These enzymes hydrolyze LDPE into monomers and other intermediates. The smaller LDPE fragments serve as a carbon source for microbial growth.

Conceptual Framework



Chapter Ⅲ

Methodology

3.1. Materials:

3.1.1. Area of Study

Soil samples were collected from two different sites at Amin bazar Landfill (2_o 05'29" S 0_o 30'41" E and 2_o 07'10" S 0_o 29'58" E) at Amin bazar (Fig. 6) considering two depths; from topsoil and the depth at 10 cm.

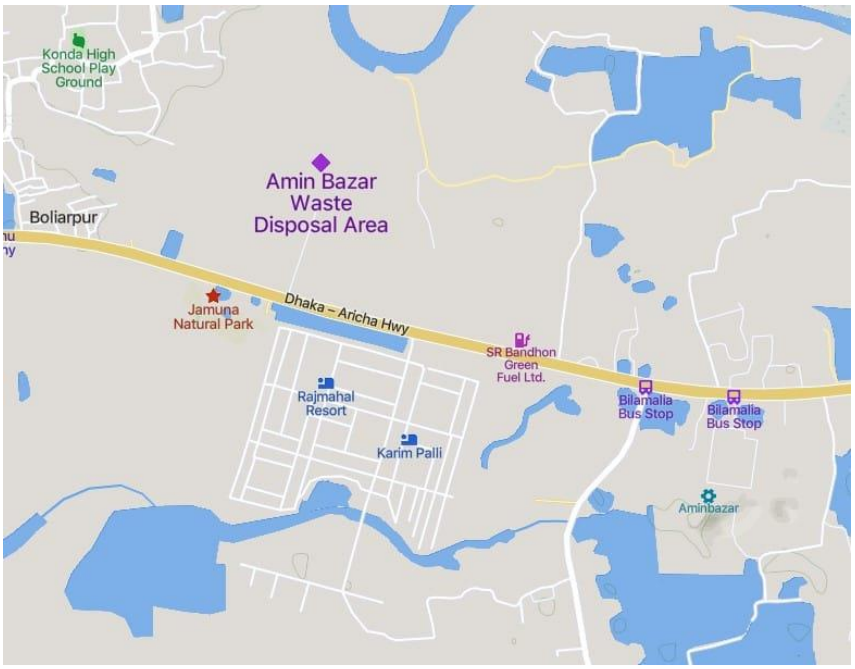


Fig. 6: Map of study area

3.1.2. Sample collection:

In this study, soil sample was collected from Amin bazar landfill as shown in Fig.7. Two different sites were selected for sampling. One set of soil samples was collected from the topsoil and another set of samples was collected from below the surface. The collected samples were stored in sterile polybag and brought to the laboratory as soon as possible.

Fig. 7: Sample collection from Aminbazar landfill

3.2. Method

3.2.1. Instruments

The instruments used in the present study were as follows:

- Incubator (Binder, Model -L1B150M, Germany)
- Oven (Memmert, Model TV 10-802407, Germany)
- Balance (Mettler-H51, Model no.604569, Switzerland)
- Vortex (Heidoeph, Type: Reax 2000, Germany)
- Magnetic stirrer (Cat no.3, England)
- pH Meter (, Switzerland)
- Autoclave (Gallen Kamp, England)
- Laminar Airflow Cabinet (Lam systems, Model-CAH1800, Russia)
- Distilled water machine (Model-WSB/4, Serial no.5583)
- Fluorescent microscope (Nikon, Microplot, UFX-IIA, Japan)
- UV-Visible (SHIMADZU, UV-1601, Japan)
- Orbital shaker (DK-SI030, Daiki Scientific company, Korea)

3.2.2. Sterilization of glassware and media:

All the glassware used in this study were sterilized in hot air oven (Memmert, Model TV 10802407, Germany) at 181°C for one hour. Media and solutions were sterilized in an autoclave under 15 lb. psi for 20 minutes at 121°C.

3.2.3. Mineral salt media preparation:

The mineral salt media (Fig. 8) was (MSM) prepared by the following chemical ingredients:

Chemical constituents of MSM:

Chemicals	Quantity (per liter)
NH_4NO^3	1.0 g
$MgSO_4.7H_20$	0.2g
K_2HPO_4	1.0 g
$CaCl_2.2H_2O$	0.15g
KCl	0.15 mg
$FeSO_4.6H_20$	1.0 mg
$ZnSO_4.7H_2O$	1.0 mg
$MnSO_4$	1.0 mg
Agar	15

The media was supplemented with LDPE powder as the sole carbon source for bacterial growth. LDPE powder (1.0 g/L) was added to the medium after sterilization at 121°C and 15 lbs. pressure for 15 minutes.

(a) **(b)**

Fig. 8:
pH measurement of Mineral salt media (a), Media ready for sterilization (b)

3.2.4. LDPE powder preparation:

LDPE sheets were bought from a local shop and were shredded with sharp scissors. These small pieces were dissolved by immersing these in xylene and heated at the temperature between 250° C to 300° C. The whole process was conducted in a fume hood to prevent the release of hazardous substances into the general laboratory space by controlling and then exhausting hazardous and/or odorous chemicals (Fig. 9). The crushed residue of LDPE was kept in a hot air oven at 45° C overnight and then stored in a closed container at room temperature.

Fig. 9: LDPE preparation

3.2.5. Isolation and maintenance of microbes:

Isolation involves separating microbes from a sample, cultivating them in a controlled environment. Maintenance ensures their survival by providing optimal conditions.

3.2.5.1. Stock solution preparation:

The soil solution was prepared by adding 1 g Soil into 100 ml of water. 0.5 g of topsoil and 0.5 g of soil at the depth of 10 cm were mixed in 100 ml water. The pH was found to be 6.95 for both locations (Fig. 10). For measuring soil pH, it is recommended that the soil must resemble the soil water ratio as it is in field condition at field capacity. That is the reason, 1: 2 ratio was considered while measuring the pH of the sample solution.

Fig. 10: pH measurement of Stock solution

3.2.5.2. Enumeration by serial dilution technique:

Diluting a sample in microbiology is a common technique to reduce the concentration of microorganisms, making it easier to work with and facilitating accurate counting or analysis. Here's a general procedure for serial dilution:

Selection of Diluent: Sterile water was chosen as dilute.

Labeling Tubes: A series of tubes were labeled as D3, D4, D5 etc. to indicate the dilution factor for each.

Transferring Sample: 0.5 ml volume of the stock solution was taken using a pipette and transferred it into the first tube. This was the initial dilution. The contents of the first tube were mixed thoroughly to ensure an even distribution of microorganisms.

Transfer to Next Tube: Using a clean pipette, 0.5 ml sample solution was transferred from the first tube to the second tube. This created the next level of dilution.

Repeat Dilutions: The process was repeated for subsequent tubes, creating a series of dilutions. The dilution factor was multiplied at each step (Fig. 11).

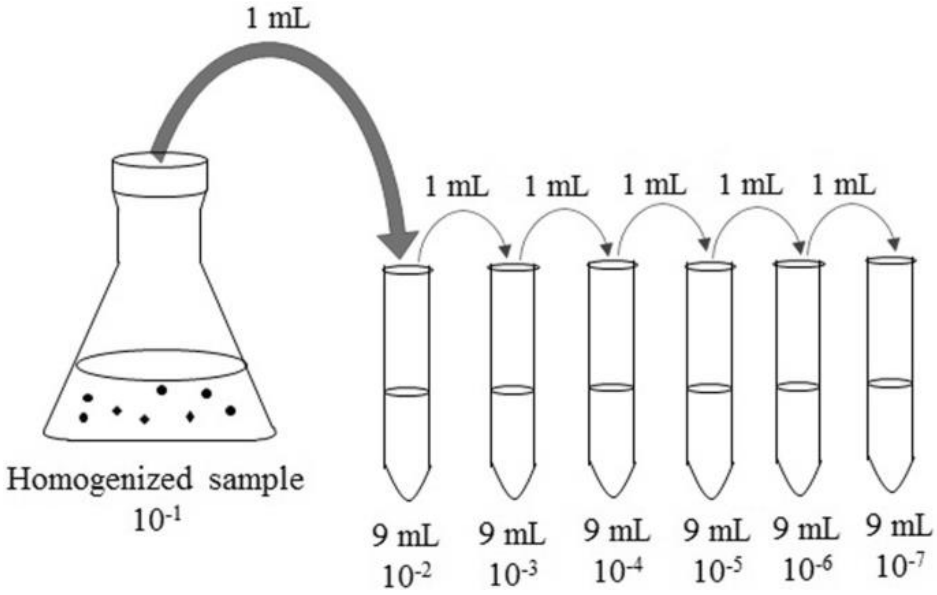


Fig. 11: Schematic representation of serial dilution technique (Cinthia and Karina, 2021)

3.2.5.3. Plate preparation and inoculation:

- MSM media was poured into petri dishes under aseptic condition to create a solid agar surface. The agar was allowed to cool and solidify (Fig. 12).
- The dilution level of 10^{-4}, 10^{-5}, 10^{-6} were considered and for each dilution three replicates were made.
- Control was prepared by adding glucose instead of LDPE and for each dilution two control replicates were prepared.
- 0.1 ml of soil sample were inoculated in each Petridis by spreading plate technique.
- The plates were incubated at 30° C temperature.

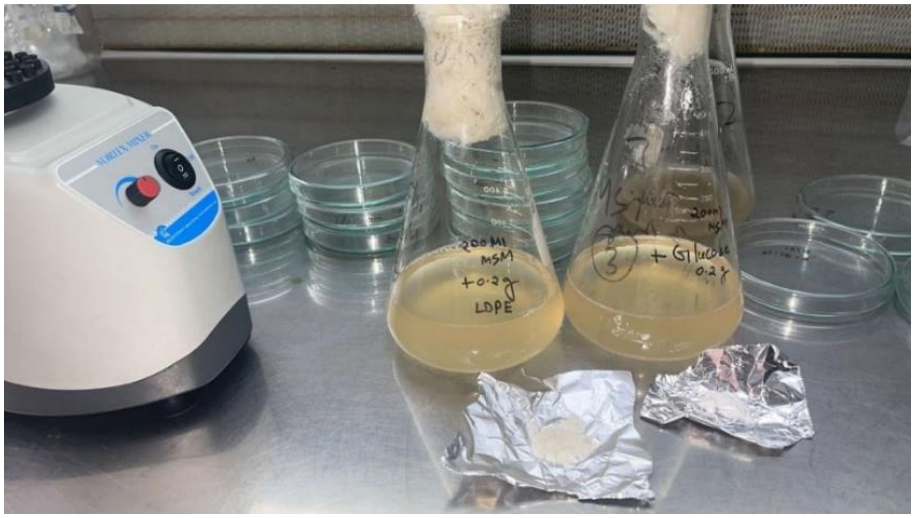


Fig. 12: Plate preparation and inoculation

The whole procedure was repeated to observe the growth enhancement by adding 0.1% yeast extract (Fig.13). In this case, the dilution level of 10^{-4}, 10^{-5}, 10^{-6} were considered. Yeast extract contains a variety of essential nutrients such as amino acids, vitamins, minerals, and nitrogenous compounds. These nutrients provide a rich growth medium for the cultivation of microorganisms. Yeast extract also helps induce the production of specific enzymes in microorganisms.

Fig. 13: Yeast extract

3.2.5.4. Isolation of bacterial colony:

The colonies were isolated from the petri dishes which were inoculated with micro-organisms in both MSM with 0.1% LDPE and MSM with 0.1% LDPE, 0.1% yeast extract. The isolates

were picked from colonies showing different morphology. Nutrient agar was used to isolate bacterial cultures by spread plate method.

3.2.5.5. Pure Culture Preparation:

Preparing pure cultures from microbial isolates is a fundamental technique in microbiology, essential for accurate characterization and research purposes. The process involves streaking the isolated microorganism onto a solid medium to obtain isolated colonies of the same species. Sub culturing was done three times to obtain purity of cultures and preserved at 4° C until use (Fig. 13).

Sterilization: Begin by sterilizing the inoculation loop or needle in a flame. This step ensures that no contaminants are introduced during the process (Madigan et al., 2014).

Streaking: Aseptically the isolated microorganism was streaked onto a solid agar medium using the sterilized loop. Streaking involves spreading the microorganisms thinly over the surface of the agar, promoting the growth of isolated colonies (Brock et al., 2018).

Incubation: The agar plates were incubated at the appropriate temperature and conditions suitable for the microorganism's growth. This step allows the colonies to develop over time (Tortora et al., 2017).

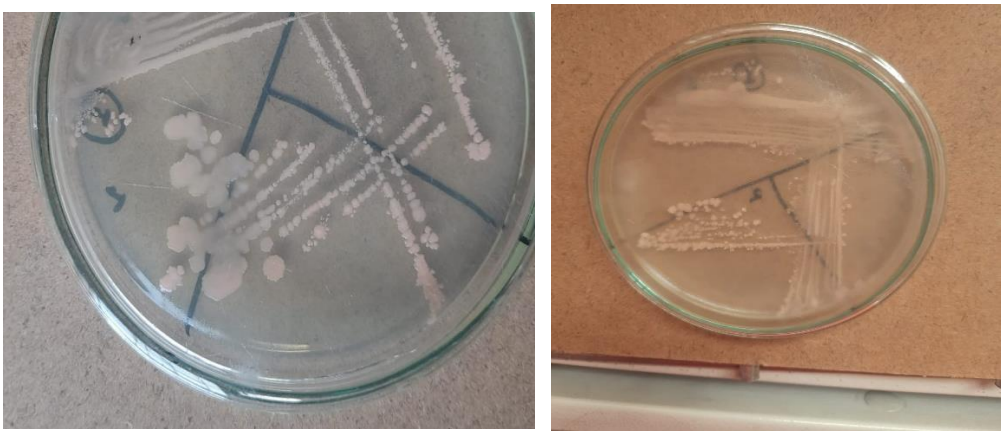


Fig. 13: Pure colony

Colonial Selection: After incubation, a well-isolated colony was from the streaked plate. A sterilized loop was used to the selected colony onto a new agar plate to obtain a pure culture (Willey et al., 2019).

Confirmation: To confirm the purity of the culture, Gram staining, biochemical tests, etc. were conducted. (Prescott et al., 2008).

3.2.6. Characterization of bacteria

Characterization of bacteria in this study involved gram staining, determining colony morphology and biochemical properties.

3.2.6.1. Colony morphology:

Colony morphology analysis focuses on the visible characteristics of bacterial colonies grown on solid media. Parameters such as size, shape, color, texture, and elevation are observed, providing preliminary information about the microorganism's identity and behavior (Talaro & Chess, 2017). Colony morphology was observed by growing purified colonies on MSM plates. Isolated colonies were characterized by the following manner as shown in Fig. 14

Shape: circular, irregular, rhizoid, punctiform.

Pigmentation: creamy white, light yellow, yellowish, or orange color.

Margin: entire, lobate, or undulate

Elevation: convex, flat, raised.

Opacity: opaque, translucent, or transparent.

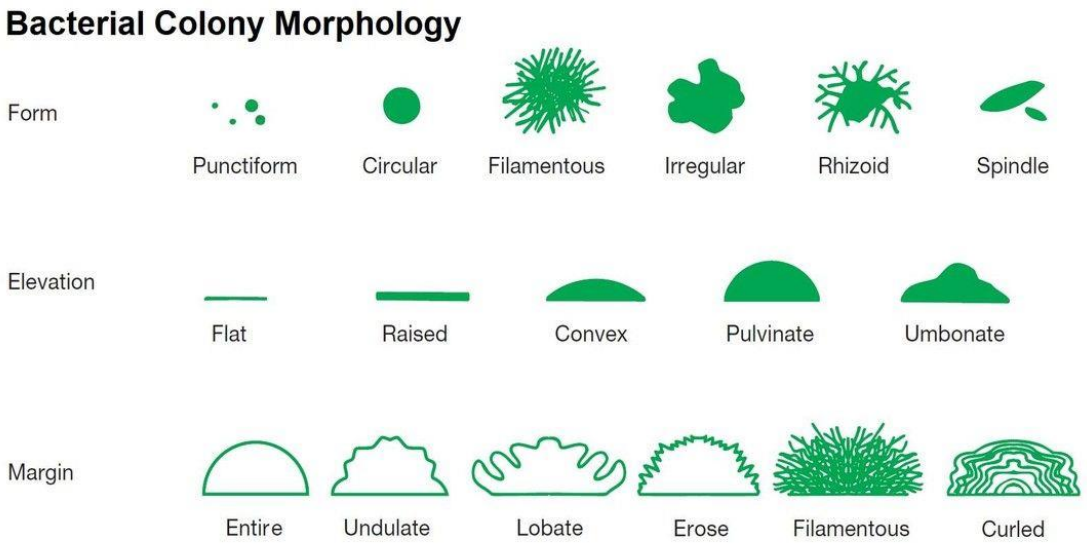


Fig. 14: Colony morphology (Talaro & Chess, 2017)

3.2.6.2. Gram staining:

Gram staining is a microbiological technique used to differentiate bacteria based on cell wall characteristics. In the process, bacterial cells were initially stained with crystal violet, followed by iodine, which formed a complex within the cell. After a decolorization step with alcohol or acetone, Gram-positive bacteria retained the violet stain due to their thick peptidoglycan layer, while Gram-negative bacteria lost the stain due to their thinner peptidoglycan layer and outer membrane. The final step involved counterstaining with safranin, highlighting Gram-negative bacteria in a contrasting color.

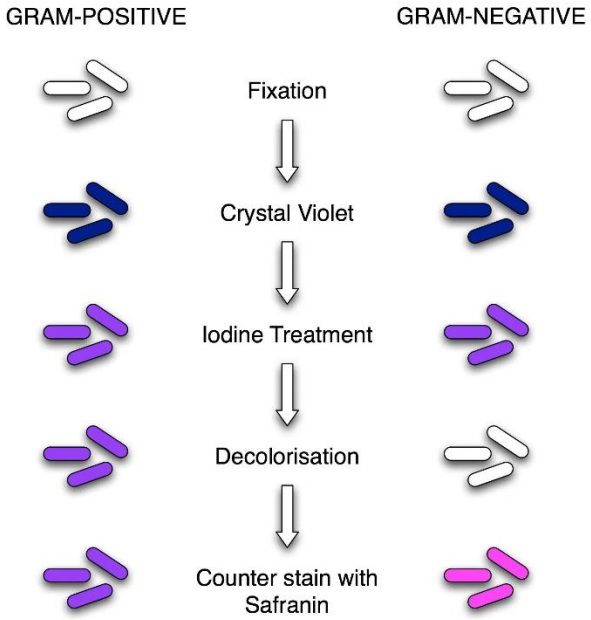


Fig. 15: Gram staining (Sagar, 2022)

3.2.6.3. Biochemical characterization:

Biochemical tests are invaluable tools in identifying microbes based on their metabolic activities. These tests analyze enzymes, sugars, and other substrates to determine specific metabolic pathways and reactions unique to different microorganisms. Biochemical tests are used to identify microorganisms based on their metabolic activities. By exposing bacteria to specific conditions and measuring the resulting metabolic changes, different bacteria can be differentiated based on their unique metabolic profiles Some commonly used biochemical tests were conducted in this study including IMViC tests (Indole test, Methyl red and Voges-Proskauer tests and Citrate utilization test), citrate utilization, catalase test and oxidase test (Fig. 16).

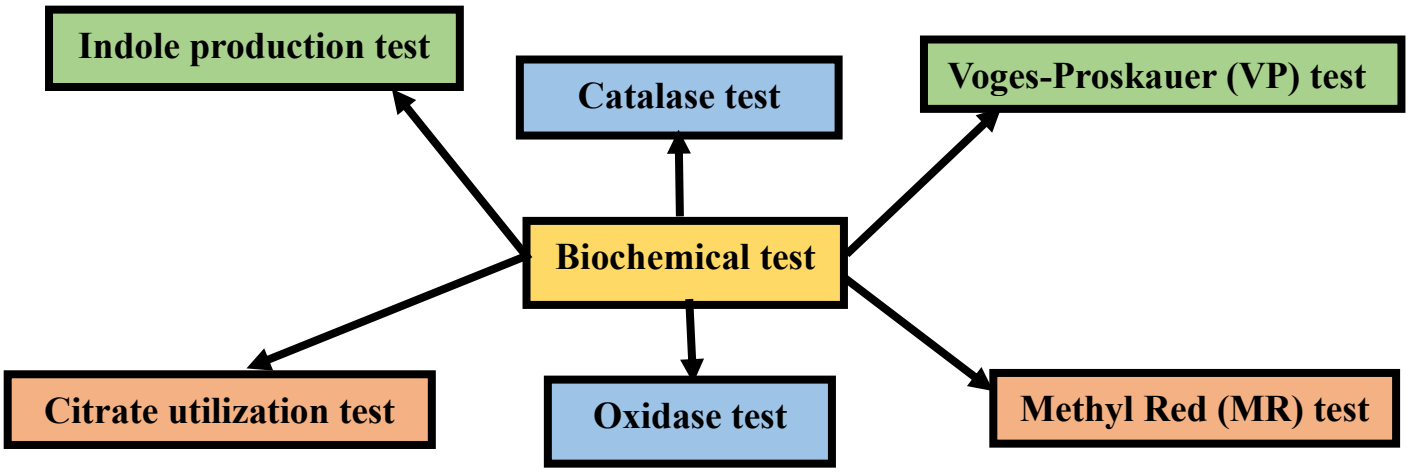


Fig. 16: Flowchart of biochemical test

3.2.6.3.1. Indole Production Test

This test was for the ability or inability of bacteria to form indole from the amino acid tryptophan. By the enzyme 'tryptophanase' Test for indole production was tryptophane splits into indole. The test for indole production was performed by using 1% tryptone broth following Ehrlich and Kovac's (1928) method. Indole production was detected with a reagent called Para dimethyl Kovac's (1928) method amino (p-DMAB) with concentrated hydrochloric acid (HCI).

In Ehrlich's method, a few drops of xylene was added to the culture and was shaken well. Then a few drops of solution containing (p-DMAB), ethyl alcohol and HCL was added down the side of the tube.

In Kovac's method, a mixture of acid was used. The inoculated tubes were incubated at 37°C and results were recorded after 12 and 4 days. The test was positive when acidified p-DMAB reacted with indole and formed a red-violet dye called rosindole which colored the whole broth (SAB 1957)

3.2.6.3.2. Methyl Red (MR) Test

The methyl red test is a microbiological test used to determine the metabolic pathway used by bacteria to produce energy from glucose. MR-VP medium was inoculated with a loop full of 24 hours of old culture of the test organism was incubated for 24-48 hours at 37°C. After incubation, a few drops of pH indicator methyl red solution were added in each tube. A distinct

red color indicated methyl red positive meant that the bacteria are using mixed-acid fermentation to produce energy and yellow color indicated methyl red negative reaction.

3.2.6.3.3. Voges-Proskauer (VP) Test

The Voges-Proskauer (VP) test is used to identify the presence of the bacterium *Klebsiella pneumoniae* and other microorganisms that produce acetoin as a metabolic byproduct. A loopful of 24 hours old culture of the test organism was used to inoculate. MR-VP medium and was incubated for 24-48 hours at 37° C. Following incubation, 3 mL of alpha naphthol solution and 1 mL of potassium hydroxide-creatine solution were added to each test tube. For two minutes, the tubes were shaken ferociously. Pink color appearance indicated a successful outcome.

3.2.6.3.4. Citrate Utilization Test

In this experiment, test culture that was 24-48 hours old was inoculated into citrate medium (Simmon's Citrate Agar). By inserting an inoculating needle containing an isolate's culture into the agar butt and streaking the slant in a wavy pattern, the citrate medium tube was inoculated. It was then incubated for 18 to 24 hours at 37 °C. Citrate-positive cultures might be distinguished by the development of growth on the slant's surface along with blue coloring. Citrate negative medium will stay green.

3.2.6.3.5. Catalase Test

To find aerobic species that produce the catalase enzyme, the catalase test was utilized. On a slide with the appropriate markings, a few drops of a solution of three percent hydrogen peroxide were added. A clean colony was selected using a sterile loop, introduced to the solution, and thoroughly mixed. The appearance of bubbles signified the presence of catalytic enzymes.

Reaction: $2H_2O_2 + \text{Catalase} \rightarrow 2H_2O + O_2$

3.2.6.3.6. Oxidase Test:

This test was carried out as per the method given by cappuccino & Sherman (1992) to distinguish between groups of bacteria on the base of oxidase activity. Cytochrome oxidase catalyzes the oxidation of a reduced cytochrome by molecular oxygen, resulting in the formation of H2O2 or H2O by aerobic bacteria which exhibits oxidase activity. For this

purpose, 2/3 drops of 1% was added to filter paper. Culture was smeared across the filter paper with a glass rod. The formation of a purple color indicates the positive & yellowish color indicate the negative reaction.

3.2.7. Utilization of LDPE as sole carbon source in shake flask:

The LDPE powder was added to determine if the potential isolates could utilize the LDPE as carbon source. The control was prepared without inoculating isolates.

3.2.7.1. Optical density measurement:

- For measuring the optical density, 1000 ml liquid mineral salt media was prepared without adding agar. The pH of the media was 7.65. 100 ml of prepared MSM was poured into ten 250 ml conical flasks.
- After sterilization, 0.05 g (0.1%) LDPE was added to each flask and potential isolates were inoculated in 9 flasks, while one was kept for control without inoculating microbes (Fig. 17).
- The flasks were kept in rotary flask shaker at 180 rpm.

Fig. 17: Optical density measurement

3.2.7.2. Dry cell weight measurement:

Cell weight measurement in microbiology is crucial for understanding microbial physiology, growth kinetics, and biotechnological applications. Precise determination of cell mass provides insights into cell composition, metabolic activities, and biomass production. Common techniques include using a centrifuge to separate cells from the growth medium, followed by washing, drying, and weighing the cell pellet.

One common method for measuring cell weight involves centrifugation (Fig.18). In this technique, microbial cultures are harvested and then centrifuged to separate cells from the growth medium. The resulting pellet, containing the cells, is carefully washed, dried, and weighed. The weight of the cell pellet corresponds to the biomass produced during the culture period, offering valuable data for various applications, including bioprocess optimization and environmental studies (Glick, 2014).

Fig. 18: Centrifugal method for cell weight determination

3.2.8. LDPE degradation experiment using LDPE sheet in MSM broth:

Microbial degradation was characterized by determining the weight loss of LDPE sheet, after 30 days of incubation.

3.2.8.1. Pre-treatment of polythene samples

Polythene samples (LDPE) were cut into approximately (3 cm × 3 cm) square pieces. The cut pieces were soaked in 70% ethanol solution for 30 min and washed with sterile distilled water. Subsequently, polythene pieces were kept inside laminar airflow chamber until surface moisture was removed.

3.2.8.2. Incubation of bacteria with treated polythene

Pre-weighed LDPE sheet of 1-cm diameter and 3 cm * 3 cm area prepared from polythene bags were aseptically transferred to the conical flask containing 50 ml of culture broth medium, inoculated with different bacterial species (Fig.19). Control was maintained with plastic discs

in the microbe-free medium. Different flasks were maintained for each treatment and left in a shaker. After one month of shaking, the plastic discs were collected, washed thoroughly using distilled water, shade-dried and then weighed for final weight. From the data collected, weight loss of the plastics was calculated.

Fig. 19: LDPE sheet inoculation in MSM media

Chapter Ⅳ

Data analysis

4.1. Measurement of Optical density:

After inoculating the potential isolates into mineral salt broth media, the optical density was recorded for each 7 days interval up to 35 days.

Table 4: Optical density of Isolates

Isolate No	OD (nm) 14 days	OD (nm) 21days	OD (nm) 28 days	OD (nm) 35 days
1	0.178	0.192	0.187	0.153
2	0.308	0.166	0.168	0.159
3	0.219	0.217	0.226	0.213
4	0.252	0.183	0.165	0.160
5	0.144	0.102	0.110	0.104
6	0.214	0.249	0.226	0.221
7	0.253	0.317	0.247	0.230
8	0.202	0.219	0.297	0.283

From the table we can see that all the isolates have an increasing trend in OD up to 21 days, it describes that the population had been rapidly increasing. Though only Isolate no 8 began to decline after 14 days. After 21 days, the OD for each population kept declining because of the deterioration of the culture condition, which was no longer suitable for their rapid growth.

Table 5: Comparative analysis of optical density

Isolate No	OD (nm) 14 days	OD (nm) 21days	OD (nm) 28 days	OD (nm) 35 days
1	0.178 ± 0.001	0.192± 0.015	0.187± 0.010	0.153± 0.024
2	0.308± 0.1	0.166 ± 0.034	0.168 ± 0.032	0.159 ± 0.041
3	0.219 ± 0.001	0.217 ± 0.001	0.226 ± 0.008	0.213 ± 0.003
4	0.252 ± 0.062	0.183 ± 0.007	0.165 ± 0.025	0.160 ± 0.03
5	0.144 ± 0.029	0.102 ± 0.013	0.110 ± 0.005	0.104 ± 0.011

6	0.214 ± 0.013	0.249 ± 0.22	0.226 ± 0.001	0.221 ± 0.006
7	0.253 ± 0.059	0.317 ± 0.056	0.247 ± 0.014	0.230 ± 0.031
8	0.202 ± 0.048	0.219 ± 0.031	0.297 ± 0.047	0.283 ± 0.033

4.2. Determination of cell weight:

After inoculating the potential isolates into mineral salt broth media, the cell weight was recorded for each 7 days interval up to 35 days. The weight of the empty tube was subtracted from the weight of the tube with the cell pellet to get the net weight of the cells. This net weight represents the mass of the harvested cells. As the weight of the cell was measured for 1 ml of the culture media, the net weight of the cell was multiplied with 100 to get the total harvested cell of 100 ml of MSM broth media.

∴Net weight of cell (ΔW) = The weight of the tube with cell pellet(W2) – the weight of the empty tube (W1)

Table 6: Cell weight of Isolates (After 14 days)

Isolate No	W1 (gm)	W2 (gm)	ΔW (1 ml) (gm)	ΔW (100 ml) (gm)
1	1.074	1.076	0.002	0.2
2	1.062	1.064	0.002	0.2
3	1.063	1.064	0.001	0.1
4	1.054	1.056	0.002	0.2
5	1.092	1.094	0.002	0.2
6	1.071	1.074	0.003	0.3
7	1.062	1.063	0.001	0.1
8	1.092	1.093	0.001	0.1

Table 7: Cell weight of Isolates (After 21 days)

Isolate No	W1 (gm)	W2 (gm)	ΔW (1 ml)	ΔW (100 ml)

			(gm)	(gm)
1	1.074	1.078	0.004	0.4
2	1.062	1.069	0.007	0.7
3	1.063	1.064	0.001	0.1
4	1.054	1.059	0.005	0.5
5	1.092	1.094	0.002	0.2
6	1.071	1.078	0.007	0.7
7	1.062	1.066	0.004	0.4
8	1.092	1.093	0.001	0.1

Table 8: Cell weight of Isolates (After 28 days)

Isolate No	W1 (gm)	W2 (gm)	ΔW (1 ml) (gm)	ΔW (100 ml) (gm)
1	1.074	1.078	0.004	0.4
2	1.062	1.070	0.008	0.8
3	1.063	1.066	0.003	0.3
4	1.054	1.059	0.005	0.5
5	1.092	1.095	0.003	0.3
6	1.071	1.078	0.007	0.7
7	1.062	1.067	0.005	0.5
8	1.092	1.094	0.002	0.2

Table 9: Cell weight of Isolates (After 35 days)

Isolate No	W1 (gm)	W2 (gm)	ΔW (1 ml) (gm)	ΔW (100 ml) (gm)
1	1.074	1.080	0.006	0.6
2	1.062	1.071	0.009	0.9
3	1.063	1.066	0.003	0.3
4	1.054	1.061	0.007	0.7
5	1.092	1.096	0.004	0.4
6	1.071	1.078	0.007	0.7

7	1.062	1.070	0.008	0.8
11	1.092	1.095	0.003	0.3

4.3. Determination of weight loss:

The weight loss experiment of LDPE was conducted to determine the biodegradation ability of each isolate. The weight was measured after 15 days, 30 days and then the initial weight was substituted to get the weight loss.

∴ Weight loss of LPDE sheet = (Initial weight – Final weight) / Initial weight × 100%

Table 10: Weight loss after 15 days

Isolate No	Initial weight	Final weight	Difference	Weight loss in month
1	10	No loss	0	0%
2	10	7.4	2.6	26%
3	10	6.2	3.8	38%
4	10	9.4	0.6	6%
5	10	9.1	0.9	9%
6	10	No loss	0	0%
7	10	8.3	1.7	17%
8	10	No loss	0	0%

Table 11: Weight loss after 30 days

Isolate No	Initial weight	Final weight	Difference	Weight loss in month
1	10	9.5	0.5	5%
2	10	6.7	3.3	33%

3	10	5.9	4.1	41%
4	10	8.7	1.3	13%
5	10	7.9	2.1	21%
6	10	No loss	0	0%
7	10	7.5	2.5	25%
8	10	No loss	0	0

Chapter Ⅴ

Result and Discussion

5.1. Isolation of LDPE degrading microbes:

To isolate the potential microbes, LDPE powder was prepared in a laboratory condition which was then added to mineral salt media at 0.1% (w/v) concentration. For control media, glucose was added to MSM, instead of LDPE. The number of colonies in the treatment was less than in the control media. In D3 plates, the number of colonies was found from only 1×10^4 cfu/g to 2×10^4 cfu/g, where the colony count in the control media was from 25×10^4 cfu/g to 30×10^4 cfu/g after three days of incubation. No growth was observed in D5 and D6 plates containing treatment media. In control D5 plates the average colony count was 4×10^5 cfu/g, but no growth was observed in control D6 plates. The observed result of colony counts, and morphology after three days are mentioned in Table 12.

Total heterotrophic count:

C.F.U. /g= Number of colonies/ inoculum size (ml) × dilution factor

Table 12: Microbial growth using LDPE and Glucose after three days of incubation:

Dilution No	Treatment (MSM + 0.1% LDPE)		Control (MSM + 0.1% Glucose)	
	Colony count	Colony Morphology	Colony count	Colony Morphology
10^{-4}	R1 =1 × 10^4 cfu/g	White, circle, smooth, opaque, translucent	R1= 30 × 10^4 cfu/g	White, circle, smooth, opaque, translucent
	R2 =2 × 10^4 cfu/g	White, circle, smooth, opaque, translucent	R2=27 × 10^4 cfu/g	White, circle, smooth, opaque, translucent
	R3=1 × 10^4 cfu/g	White, circle, smooth, opaque, pinpoint	R3= 25 × 10^4 cfu/g	White, circle, smooth, opaque, translucent
10^{-5}	No growth	-	R1 = 7 × 10^5 cfu/g	White, circle, smooth, opaque, translucent
			R2 = 2 ×10^5 cfu/g	White, circle, smooth, opaque
10^{-6}	No growth	-	No growth	-

In D3 plates the number of colonies was from only 4 × 10^4 cfu/g to 5 × 10^4 cfu/g, where the colony count in the control media was from 32× 10^4 cfu/g to 45× 10^4 cfu/g after six days of incubation. No growth was observed in D5 and D6 plates containing treatment media. In control D5 plates the average colony count was 9 × 10^5 cfu/g, but no growth was observed in control D6 plates. The observed result of colony counts, and morphology after six days are mentioned in the Table 13.

Table 13: Microbial growth using LDPE and Glucose after six days of incubation:

Dilution No	Treatment (MSM + 0.1% LDPE)		Control (MSM + 0.1% Glucose)	
	Colony count	Colony Morphology	Colony count	Colony Morphology
10^{-4}	R1= 4×10^4 cfu/g	White, circle, smooth, opaque, translucent	R1= 45×10^4 cfu/g	White, circle, smooth, opaque, translucent
	R2= 4×10^4 cfu/g	White, circle, smooth, opaque, translucent	R2= 35×10^4 cfu/g	White, circle, smooth, opaque, translucent
	R3= 5×10^4 cfu/g	White, circle, smooth, opaque, pinpoint	R3= 32×10^5 cfu/g	White, circle, smooth, opaque, translucent
10^{-5}	No growth	-	R1= 12×10^5cfu/g	White, circle, smooth, opaque, translucent
			R2= 6×10^5 cfu/g	White, circle, smooth, opaque
10^{-6}	No growth	-	No growth	-

The colonial count in the treatment media was very less compared to the control media (Fig. 20). To confirm that the bacteria were not depending on the inorganic molecules of the MSM media, the growth of bacteria was also studied without any carbon source. The microbial growth in the MSM media without any carbon source was negligible and only one colony was observed after 8 days in D3 plate (Dilution no: 10^{-3}).

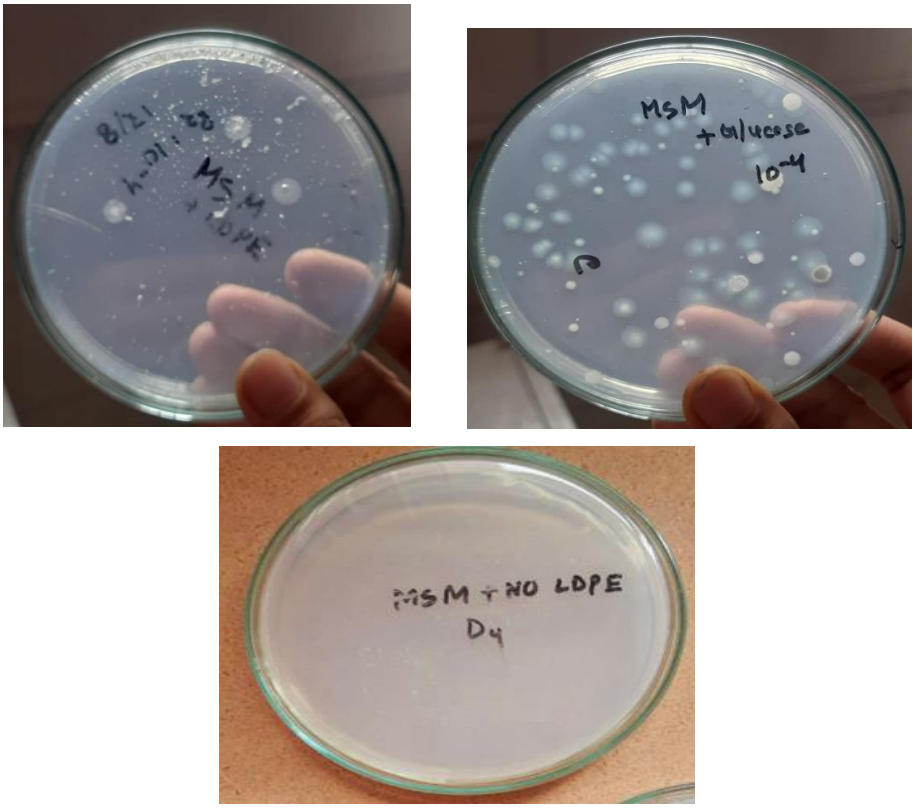


Fig. 20: Growth of the microbes in treatment and control media

The experiment of "isolation of potential microbes" was conducted again by adding 0.1% to yeast extract to enhance the growth of microbes. This was helpful in identifying variety of colonies and more isolation of LDPE degrading microbes. The growth after three days has been shown in the Table 14.

Table 14: Growth enhancement using yeast extract with LDPE:

Dilution No	MSM+ 0.1% LDPE + 0.1% Yeast	
	Colony Count	**Colony Morphology**
10^{-4}	R1 = 26 * 10^4 cfu/g	White, circle, smooth, opaque, translucent, pinpoint
	R2 = 19* 10^4 cfu/g	White, circle, smooth, opaque, translucent, pinpoint
	R3 = 27* 10^4 cfu/g	White, circle, smooth, opaque, pinpoint
10^{-5}	R1 = 6* 10^5 cfu/g	White, circle, smooth, opaque, translucent, pinpoint
	R2 = 3* 10^5 cfu/g	White, circle, smooth, opaque, translucent, pinpoint
	R3 = 2* 10^5 cfu/g	White, circle, smooth, opaque, pinpoint
10^{-6}	No growth	-

After adding yeast extract the growth increased to a significant level (Fig. 21). By adding this growth enrichment product more different colony were isolated. When bacteria were inoculated only into MSM containing LDPE the growth was also slow compared to the growth rate in yeast extract (0.1%) with LDPE (0.1%) media.

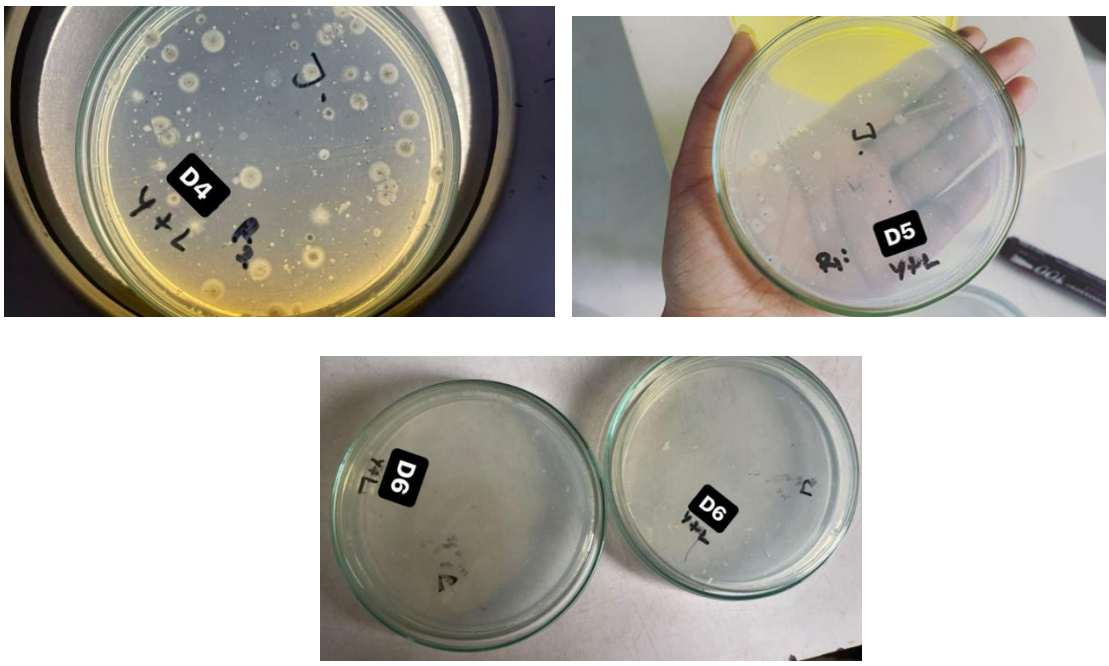


Fig. 21: Growth enhancement using yeast extract (0.1%) with LDPE (0.1%)

Colony with identical morphology was isolated in plates containing MSM media and 0.1% LDPE. After several streaking 8 pure bacterial colonies were preserved in a slant (Fig .22).

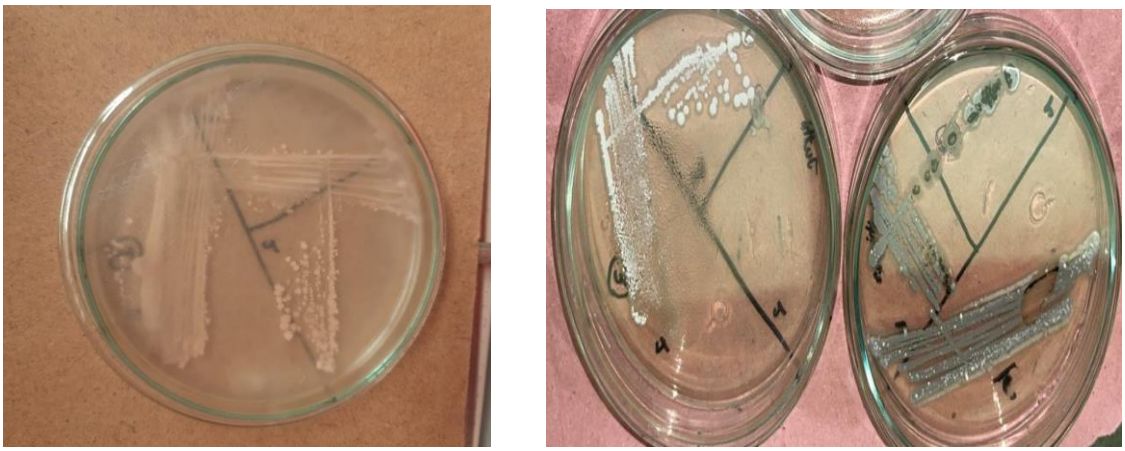


Fig. 22: Streaking to obtain pure culture

5.2. Determination of morphological and biochemical characteristics:

After isolation of the potential bacteria, morphological and biochemical analysis were carried out for determination of cell morphology and other metabolic property of the selected strains.

a) Gram staining:

Each isolate was found to be gram positive. Isolate 4,5 and 8 were in the shape of *Cocci* (Fig. 24). The other isolates were rod shaped or of *Bacillus* strain. All the organisms were found to form chains.

Table 15: Gram staining of isolates

Isolate No	Shape of the organism	Color	Characteristics
1	Rods in chain	Purple	Gram + ve, *Bacillus*
2	Rods in chain	Purple	Gram + ve, *Bacillus*
3	Rods in chain	Purple	Gram + ve, *Bacillus*
4	Coccus in chain	Purple	Gram + ve, *Coccus*
5	Coccus in chain	Purple	Gram + ve, *Coccus*
6	Rods in chain	Purple	Gram + ve, *Bacillus*
7	Rods in chain	Purple	Gram + ve, *Bacillus*
8	Coccus in chain	Purple	Gram + ve, *Coccus*

The percentage composition of isolated bacteria is mentioned in Fig. 21. *Bacillus* is in the highest amount with 62.5%, and then *Coccus* was found to be second highest with a percentage of 37.5% (Fig. 23).

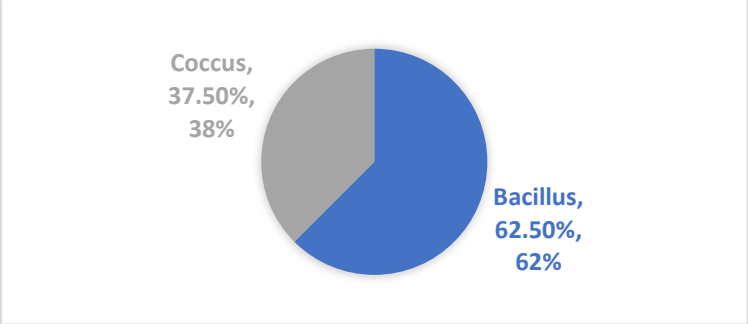
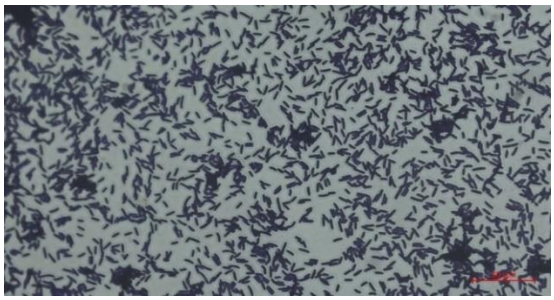
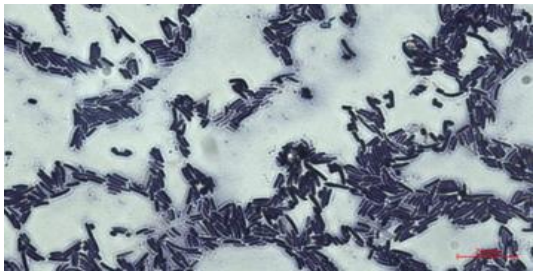


Fig. 23: The percentage composition of isolated bacteria in soil sample

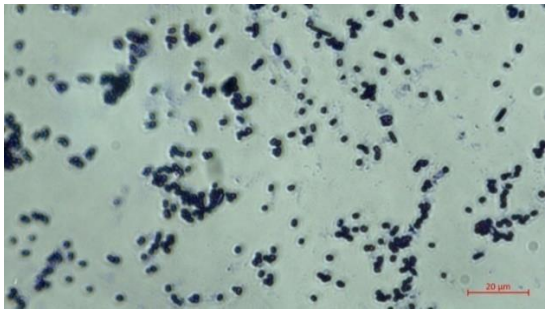
The bacterial strains of isolate no 1, 2, 3, 6 and 7 were in the shape of *Bacillus*. Some of them were found to be induvial colonies while others were found to have formed a chain. Isolate no 4, 5 and 8 were in the shape of *Coccus*. Both individual colony and chain formed *Coccus* were found (Fig. 24).



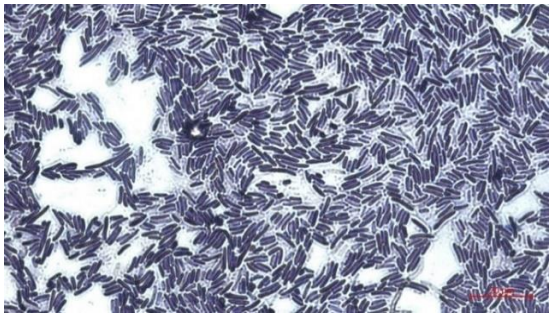
(a)



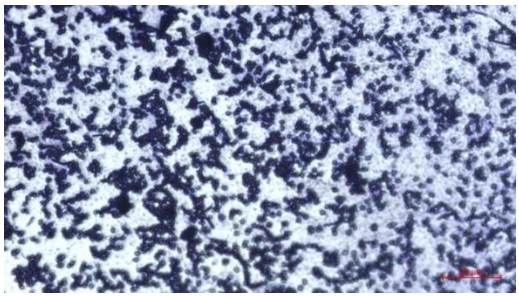
(b)



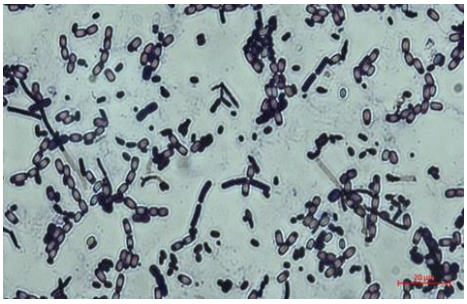
(c)



(d)



(e)



(f)

Fig. 24: Gram staining of the isolates (a, b, d, f: gram positive *Bacillus*, c, e: gram positive *Coccus)*

b) Biochemical characteristics

The result of the biochemical test is shown in Table 16.

Table 16: Biochemical test

Isolate No	Catalase	Oxidase	MR	VP	Citrate utilization	Indole production test
1	+	-	+	-	-	-
2	-	+	+	-	+	+
3	-	-	-	-	-	-
4	+	-	-	-	-	-
5	+	+	+	+	+	+
6	+	+	+	-	-	-
7	-	+	-	+	+	+
8	+	-	+	+	+	+

- Catalase test result (Fig. 25) was found to be positive for isolate no 1, 4, 5, 6 and 8. As the bubbles were produced rapidly upon adding hydrogen peroxide, the test is positive. No bubble indicates negative result.

Fig. 25: Catalase test

- Isolate 2, 5, 6 and 7 show the positive result (Fig. 26) for the oxidase test. The area was observed where the reagent and the bacterial colony came into contact. The color was changed to blue purple within 10-30 seconds indicates a positive oxidase reaction. No color change within the time frame represents a negative result.

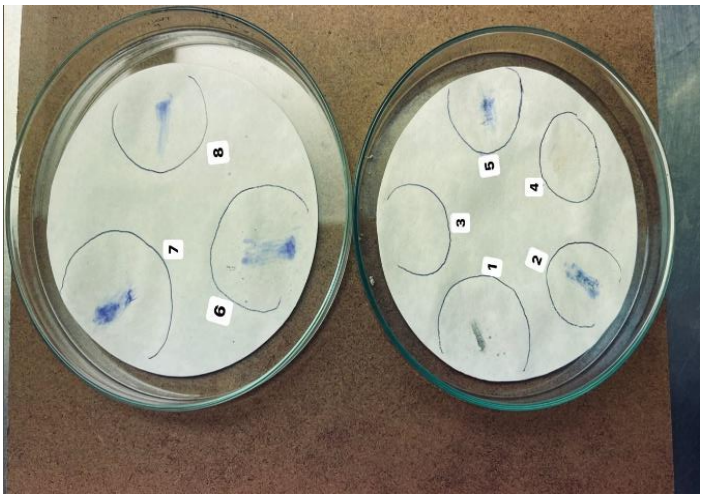


Fig. 26: Oxidase test

- The Methyl Red test of the strains was found positive excluding strain no 3, 4 and 7 (Fig. 27). Bacteria were grown in a broth containing glucose. After incubation, Methyl Red indicator was added to the broth. As the pH of the broth was below 4.4 (indicating stable acid production), the Methyl Red indicator turned red. For the negative result, the color did not change.

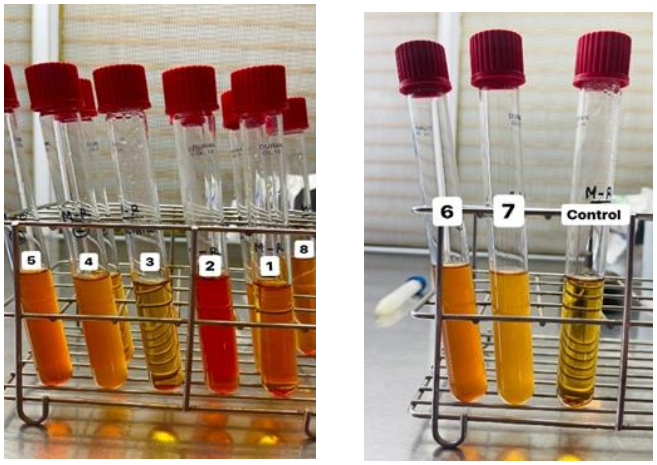


Fig. 27 : Methyl red test

- Voges – Proskauer test of strain no 5, 7 and 8 were found positive and rest of them showed a negative result (Fig. 28). The tubes were observed for the development of a pink red color. A positive VP test resulted in the development of a red color in the medium after the addition of reagents, indicating the presence of acetoin.

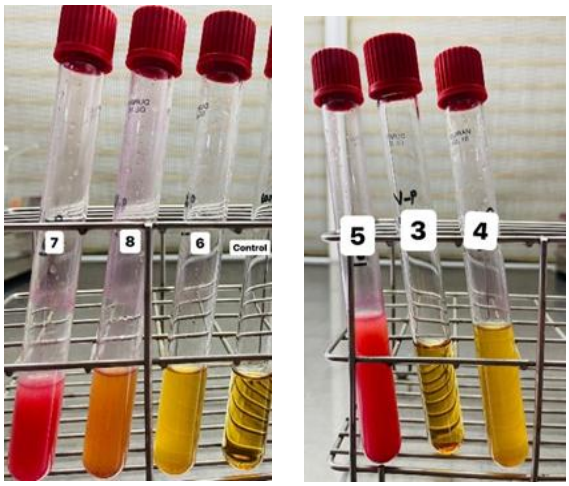


Fig. 28 : Voges – Proskauer test

- The Citrate utilization test of strain no 2, 5, 7 and 8 was found positive and rest of them showed a negative result (Fig. 29). Positive result shows the color change into blue color indication production of alkaline by product.

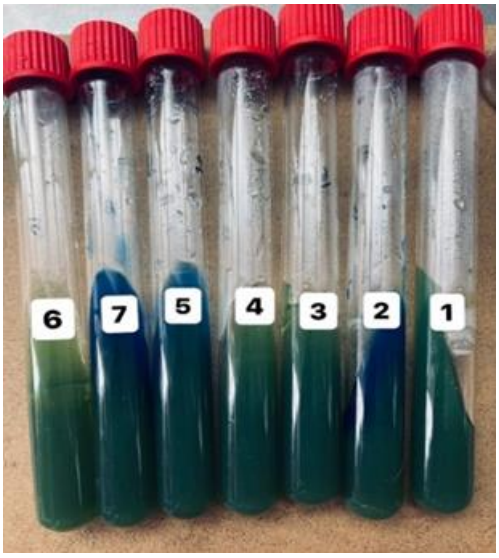


Fig. 29: Citrate utilization test

- The test named Indole production test estimates that strains 2, 5, 7 and 8 showed a positive result (Fig. 30). Red color development after adding Kovac's reagent indicates positive result.

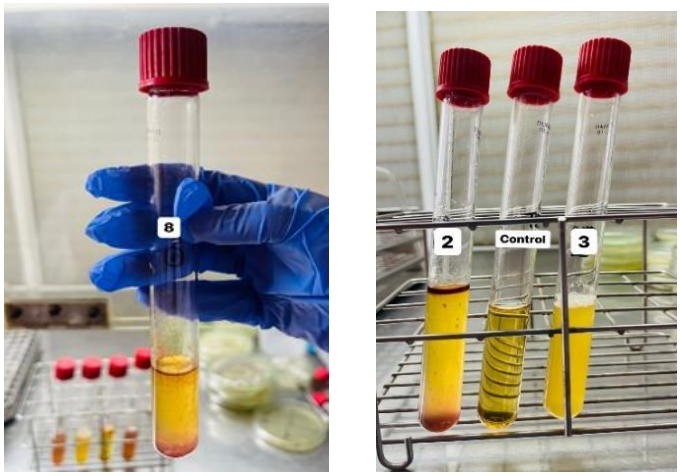


Fig. 30: Indole production test

4.3. Determination of LDPE degradation efficacy of the isolated bacterial strains:

After isolation of the bacterial strains, the LDPE degradation efficacy of the selected isolates was determined according to the optical density of the MSM media, cell wight of the bacterial growth and the weight loss of the LDPE sheet. The MSM broth media was fortified with 0.1%

LDPE and inoculated with potential isolates in separate flask. The OD was measured at 600 nm.

a) Optical density measurement:

The optical density (OD) curve is a graphical representation of microbial growth in a liquid culture medium based on the measurement of light absorbance at a specific wavelength. It is a widely used method in microbiology and biotechnology to monitor the growth of microorganisms over time.

Fig. 31 shows the OD of each isolate. At the beginning of microbial growth curve, the microbes adapt to their new environment, thus the OD value for each isolate was relatively constant up to first 14 days. This phase is called the initial lag phase where organisms synthesize enzymes and other molecules for their survival and rapid growth. Isolate 2 had the highest OD (0.3 nm). The lowest OD in the initial lag phase is 0.1 nm by isolate 1, while other microbial isolates showed value near 0.2 nm.

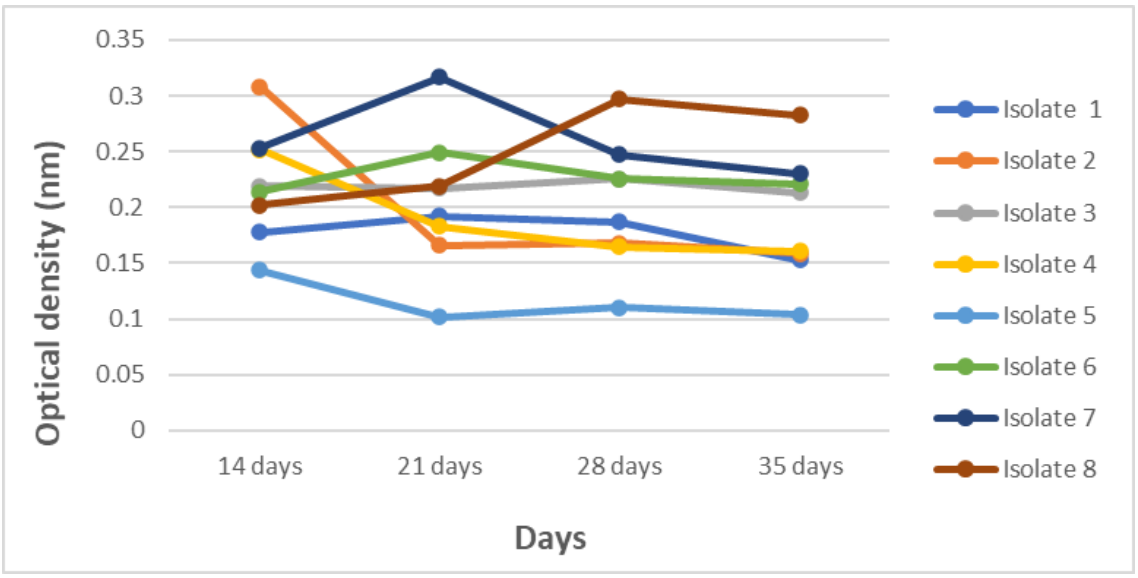


Fig. 31: Graph of optical density (OD) of the MSM with LDPE (0.1%) containing pure bacterial culture

After 14 days, the microbial population starts to grow rapidly. The cells were actively dividing and utilizing available nutrients for reproduction. This phase is known as exponential log phase. As a result, the number of cells in the culture increased exponentially. In the OD curve, this phase is represented by a steep upward slope, indicating a rapid increase in absorbance. The value of OD significantly increased for isolate no 1, 6, 7 and 8. While the value for isolate 3 is

almost the same because of the stationary phase of the growth curve. During this phase, the rate of cell growth equals the rate of cell death. Nutrient depletion, waste accumulation, and other factors lead to a balance between cell division and cell mortality. This stationary phase is represented by a horizontal line, indicating that the absorbance remains relatively constant.

Eventually, the growth rate slowed down and entered death phase for the nutrient depletion in media. From 21 days to 35 days, the microbial growth phase is characterized by a downward slope as absorbance decreased. Unlike the other isolates, only the isolate 2, 4 and 5 entered the death phase after 14 days. During this phase, the number of dying cells exceeded the number of new cells being produced. The number of populations declined in this stage. The final OD was found to be the highest for isolate no 8 (0.3 nm).

The color of the control media without any inoculation of microbes and the color of the treatment media in which bacteria was inoculated was different. The one with isolate had a cloudy and yellowish appearance and the control was comparatively more transparent. The change in color of the media indicates the growth of the microbes (Fig. 30).

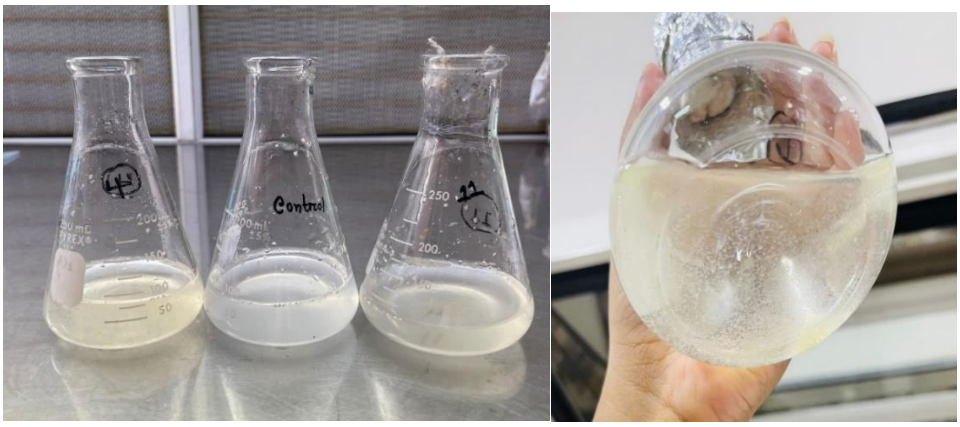


Fig. 30: Difference in color of the treatment and control

b) Dry cell weight:

Dry cell weight is a method used to quantify the biomass concentration in a microbial culture by measuring the weight of cells after these had been harvested, washed, and dried to remove all water and other extracellular components. Fig. 32 shows the dry cell weight of all the bacterial strains after 14, 21, 28 and 35 days of incubation.

Cell weight of the bacterial strains has an increasing trend on the graph. As the microbes grow with time, the cell concentration gradually increases from 14 days to 35 days. Initially the cell mass was 0.1g to 0.3 g. After 21 days, the value raised for maximum of the bacterial isolates,

while the value remains same for the isolate 3, 5 and 8. From 28 days to 35 days the cell concentration gradually expands. Isolate no 2 and 7 have the highest concentration on 35 days.

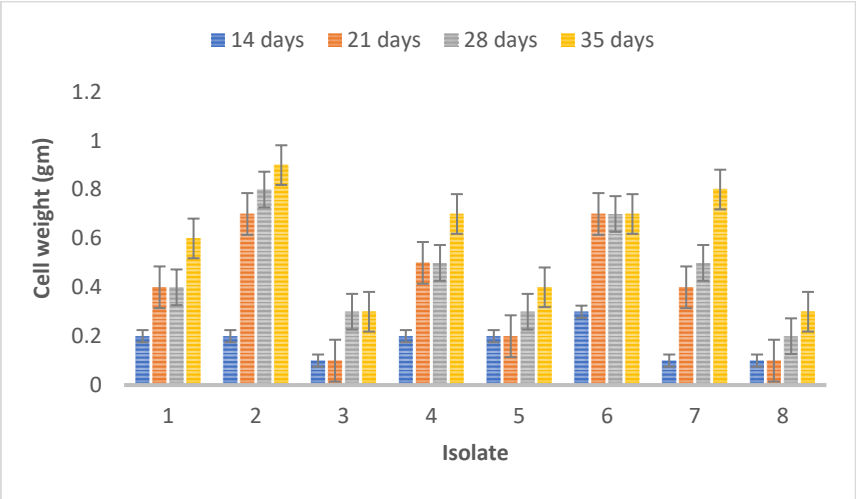


Fig. 32: Cell weight of the isolates

5.4. Weight loss of LDPE by potential isolates:

It is claimed that weight loss is the initial stage of polythene degradation. Weight loss of LDPE polythene was observed in this study after a period of incubation (Fig. 32).

The observation showed significant weight of polythene pieces was reduced to 26% and 38% in 15 days for isolate 2 and 3, respectively. No change in weight of control sample indicated lack of degradation. Again, LDPE pieces weight was reduced by isolate 4, 5 and 7 in 6%, 9% and 17%, respectively within 15 days of incubation. In contrast, no weight loss was calculated for the isolate 1, 6 and 8 (Fig. 33).

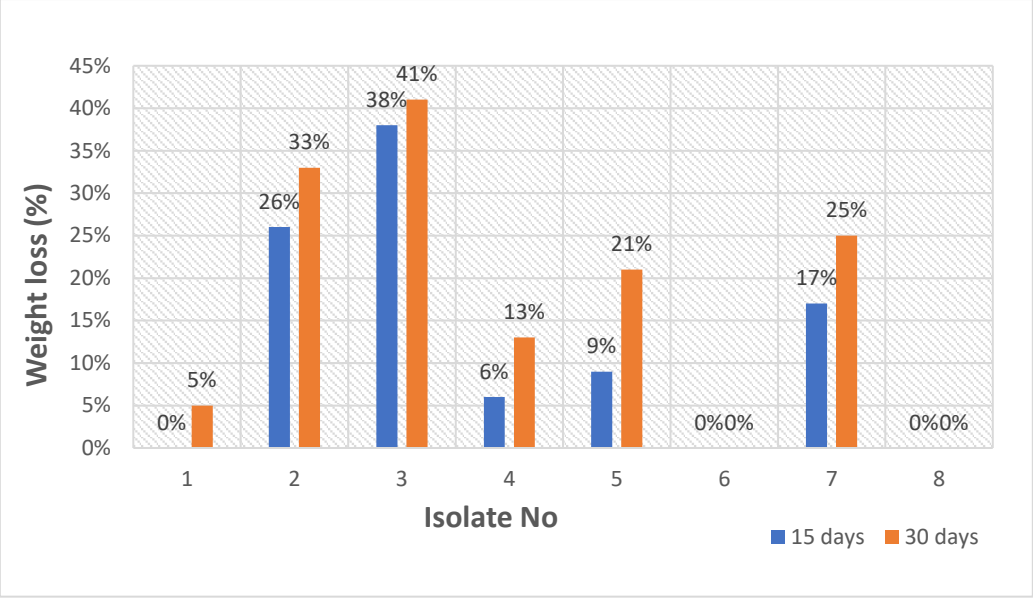


Fig. 33: Weight loss of LDPE sheet by isolated strains

After 30 days of incubation, 41% weight loss was found in LDPE polythene by using isolate 3. The second highest degradation rate was found to be 33% by isolate 2. Isolate 5 shows significantly increase in weight loss in the next 15 days which is from 9% to 21%. The LDPE degradation rate for isolated 1, 4 and 5 was estimated to be 5%, 13% and 21%, respectively. After 30 days of incubation no weight loss was found for control media, isolate 6 and isolate 8. Microorganisms secrete different enzymes that break down the complex molecules into smaller ones and the microorganism membrane can then further absorb the smaller molecules to use as carbon and as an energy source (Singh et al., 2016). Thus, the result indicates that the isolated bacteria could degrade polythene then the resulting molecules used as carbon sources.

5.5. Major findings

- A total of eight bacterial strains were isolated which are LDPE degrading microbes. When the media was supplemented with 0.1% LDPE powder, the selected isolates showed significant growth. After streaking several times, each of the isolates had adopted to the carbon source LDPE in the media and the growth was observed within 24 to 48 hours of incubation. This means that the selected isolates had the enzymatic capability to breakdown the molecular structure of LDPE.
- All bacterial isolates were found to be gram positive, five of them were *Bacillus sp* and the rest of the isolates were *Coccus* sp.
- The value of OD was more for isolate 2, 7 and 8 than the other isolates. Isolate 2 showed the highest OD of 0.3 nm after 14 days of incubation, but the OD started to decline readily later. On the other hand, isolate 7 had the second highest OD of 0.25 nm which increased to 0.317 nm. This indicates the rapid growth rate of this strain and its capability to adjust within this harsh media. The OD of isolate 8 rose to almost 0.3 nm on the day of 28.
- The cell weight increased for isolate 2 and 7 from 14 to 35 days. Isolate 2 and 7 gave the highest value of cell weight which were 0.9 g and 0.8 g respectively isolates.
- The weight loss of LDPE sheet gave different values for different isolates. Isolate 3 could reduce the weight of the sheet by 41%. The second highest weight reduction rate was 33% by isolate 2.

5.6. Discussion

In my study, eight potential bacterial strains were isolated. The LDPE powder was added to the media to determine if the bacteria from the landfill sample could utilize the LDPE as carbon source. Each of the isolates showed significant growth in the media as they have the enzymatic capability to break down the bond of LDPE.

It was identified that microbial strains have the capability to enzymatically break down low-density polyethylene (LDPE) (Shivan et al., 2012). Notably, Yoshida et al. (2016) discovered a bacterium, *Ideonella sakaiensis*, that effectively utilizes two enzymes to degrade LDPE, offering a promising avenue for bioremediation. 20 potential isolates had been identified which survived on the LDPE powder in the media.

Moreover, research conducted by Galli et al. (2019), explored the diversity of microbial communities associated with plastic degradation in various environments. By adding different concentrations of LDPE powder, this study isolated the potential microbes and examined its degradation efficacy. The mechanism of LDPE degradation by bacteria involves the secretion of enzymes, such as lipases and esterases, which initiate the breakdown of polymer chains (Hadad et al., 2005). Additionally, recent studies by Shah et al. (2021) proved the presence of specific enzymes such as PETase and MHETase required for the degradation of LDPE.

In this study, each isolate was found to be gram positive. 62.5% of them were bacillus in shape and 37.5% of the isolates were cocci in shape.

Research indicates that both gram-positive and gram-negative bacteria harbor the potential to enzymatically break down LDPE, contributing to the growing understanding of microbial plastic degradation mechanisms. A notable discovery is the bacterium *Nocardia* species, specifically *Nocardia asteroids* (Reddy et al., 2021). This gram-positive *actinomycete* was found to possess the enzymatic machinery capable of initiating LDPE degradation. The study identified extracellular enzymes, including lipases and esterases, as key players in breaking down the polymer chains of LDPE. Such gram-positive bacteria demonstrate the diverse enzymatic tool kit that can be harnessed for plastic degradation. Several *Bacillus* strains, known for their robust enzymatic activities, have demonstrated the ability to degrade LDPE. For example, Moogoi et al. (2018) identified *Bacillus sp*. as a potential LDPE-degrading bacterium, highlighting the significance of Gram-positive bacteria in plastic degradation.

In a study by Sivan (2011), *Pseudomonas sp.* was found to play a crucial role in polyethylene degradation. The research highlighted the importance of extracellular enzymes secreted by *Pseudomonas* in initiating the breakdown of LDPE, emphasizing the potential of gram-negative bacteria in plastic waste management. In a study by Abrusci et al. (2020), *Pseudomonas citronellolis* was found to produce extracellular enzymes capable of degrading LDPE efficiently.

Moreover, the comprehensive work of Hadad et al. (2005) explored the polyethylene-degrading potential of gram-positive bacteria, focusing on *Brevibacillus borstelensis*. This bacterium demonstrated efficient LDPE degradation, providing insights into the role of temperature in enhancing plastic degradation processes. The study identified the production of depolymerase enzymes by *B. borstelensis* as a crucial factor in facilitating LDPE breakdown.

In this study, biochemical tests were conducted to characterize the potential isolates. Biochemical test results are fundamental in bacterial taxonomy and research. The information gathered aids in the classification of bacteria into taxonomic groups, contributing to our understanding of microbial diversity and evolution (Tindall, 1990). The explanation of biochemical rest is mentioned in Table 17.

Table 17: Explanation of biochemical test result

Test	The result of this study	Explanation	
		Positive	Negative
Catalase test	Catalase test was found to be positive for isolate 1,4,5,6, and 8, rest of them showed negative result.	Bubble production means that the bacteria produce catalase and can break down hydrogen peroxide into water and oxygen (Holt et al., 1994)	If no bubbles or only very slow bubbles are produced, the test is negative. This indicates that the bacteria lack catalase activity and cannot break down hydrogen peroxide (Holt et al., 1994).
Oxidase test	Oxidase test was found to be positive for isolate 2,5,6 and	Positive result indicates the presence of cytochrome c oxidase, an enzyme involved in the electron transport chain of aerobic organisms. The test	No color change means that cytochrome c oxidase enzyme is absent (Tille, 2017)

	7, rest of them showed negative result.	is based on the ability of cytochrome c oxidase to oxidize a substrate, typically tetramethyl-phenylenediamine dihydrochloride (TMPD), in the presence of oxygen, producing a characteristic color change (Tille, 2017).	
Methyl Red test	MR test was found to be positive for isolate 1,2,5,6, and 8, rest of them showed negative result.	The Methyl Red test is for determining whether a bacterium can perform mixed acid fermentation. The Methyl Red test specifically checks the ability of an organism to produce stable acids (such as lactic acid and acetic acid) from glucose fermentation. The bacteria which can produce acid shows positive result (Johnson et al., 2013).	No acid production means negative result.
Voges-Proskauer (VP) Test	VP test was found to be positive for isolate 5,7, and 8, rest of them showed negative result.	A positive VP test indicates the presence of acetoin. This means that the bacteria in the culture can ferment glucose and convert the end product, 2,3-butanediol, into acetoin. The development of a red color in the medium after the addition of reagents indicates a positive VP test result (Voges et al., 1898).	A negative VP test means that the bacteria in the culture do not produce acetoin. Instead, they may convert 2,3-butanediol into other products. The absence of a color change (remains yellow) after the addition of reagents indicates a negative VP test result (Voges et al., 1898).
Indole production test	Indole production test was found to be positive for	A positive indole test result indicates that the organism can produce indole from tryptophan. Common indole-	A negative indole test result indicates that the organism cannot produce indole from tryptophan.

	isolate 2,5,7, and 8, rest of them showed negative result.	positive bacteria include Escherichia coli (MacFaddin, 2000).	Common indole-negative bacteria include Shigella species (MacFaddin, 2000).
Citrate utilization test	Citrate utilization test was found to be positive for isolate 2,5,7 and 8, rest of them showed negative result.	The citrate utilization test is a biochemical test often used in microbiology to differentiate bacterial species based on their ability to utilize citrate as the sole carbon source. If the bacteria utilize citrate, the medium's pH increases, leading to a color change from green to blue due to the production of alkaline byproducts (mainly sodium carbonate) (MacFaddin, 2000).	If the bacteria do not utilize citrate, the medium remains green, indicating no change in pH. (MacFaddin, 2000)

In my study, OD (Optical Density) and dry cell weight were used to quantify the growth of microorganisms in a culture. These measurements provide insights into the density and biomass of microbial populations. The OD and dry cell weight of isolate 2 and 7 was found to be the highest for isolate no 2 and 7. Higher OD values generally correspond to a denser microbial population. Higher dry cell weight values indicate a higher concentration of microbial biomass in the culture.

In a study by (Khandakar et al., 2019), the biomass of the isolated bacteria in the LDPE containing MSM broth was measured after 15 days. The OD for isolate 8 was found to be 0.5, which was more than any other isolate. The biomass increased for all the isolates up to 10 th day and drastically reduced at 15 th day. In another study conducted by (Bhatia et al., 2014), the isolates grown in nutrient broth were transferred to enrichment medium for screening LDPE degrading strains. The cultures obtained in the broth were then grown on nutrient broth. Four isolates were screened with the ability to use LDPE as nutrient medium. A growth profile study of individual strains and culture consortium containing all four isolates was performed by taking OD at regular intervals of 6 hours in presence of glucose and LDPE separately as

substrates. The growth curve formed by consortium was quite supportive of the fact that microbial association is good enough to use the LDPE for cell growth and multiplication. A1 and A2 were having better LDPE assimilation rate than B and C. A1 and A2 were found to give better and efficient digestion of LDPE in comparison to B and C. The similar growth profile studies have earlier been reported by Satlewal et al.; Sah et al.; Negi et al.; Soni et al. to increase microbial biomass by supplementation of different polymers like LDPE, HDPE and epoxy blends (Negi et al. 2009; Negi et al. 2011; Sah et al. 2010a; Satlewal et al. 2008; Soni et al. 2009; Soni et al. 2008). The enzyme secretion by bacterium leads to degradation of substrates like LDPE, HDPE, etc.

In this study, the wight loss of LDPE sheet by potential isolate 3 and isolate 2 was found to be 41% and 33%, respectively after 30 days of incubation. These bacterial strains utilized the LDPE sheets as sole carbon source of the MSM broth.

According to Nadeem et al., 2021, four bacterial strains were isolated from the solid-waste dumpsites of Faisalabad, Pakistan, using enrichment culture technique. The isolated bacterial strains could grow on media having polystyrene as the sole carbon source. Based on 16S rRNA gene sequencing and phylogenetic analysis of the isolated strains Serratia sp., Stenotrophomonas sp. and Pseudomonas sp. were identified as the potential strains for the biodegradation of LDPE. Serratia sp. resulted in 40% weight loss of the LDPE plastic pieces after 150 days of treatment. Stenotrophomonas sp. and Pseudomonas species resulted in 32 and 21% weight loss of the treated piece of plastics (LDPE), respectively.

A notable study by Moogoi et al. (2018) conducted a weight loss experiment using *Bacillus sp.* and Pseudomonas sp. isolated from the mud of Sungai Kerian, Penang, Malaysia. The researchers exposed LDPE sheets to these bacteria and observed a decrease in weight, suggesting microbial activity in breaking down the plastic.

Jayanthi et al., 2022 conducted research on determining the weight loss of LDPE sheet, The biodegradation of LDPE was determined by evaluating weight loss and morphological changes of the LDPE samples. The isolated strains: *Aspergillus nomius* had the capacity to degrade 4.9% and *Streptomyces sp*. showed 5.2% of weight loss of LDPE films respectively. Weight loss of LDPE film after inoculation of isolates in degradation medium indicated that it could use polyethylene as carbon and energy source.

Another study (Laleena et al., 2022) employed microbes to develop an environmentally acceptable technique for degrading Low-Density Polyethylene (LDPE). Six of the 36 bacterial

strains obtained from garbage disposal locations shown potential biodegradation activity. *Bacillus siamensis* and *Bacillus wiedmannii* were reported as new strains for LDPE degradation in this investigation. After 90 days of incubation, the percent weight loss of LDPE films for isolates was *B. siamensis* (8.46 0.3%), *B. cereus* (6.33 0.2%), *B. wiedmannii* (5.39 0.3%), *B. subtilis* (3.75 0.1%), *P. aeruginosa* (1.15 0.1%), and *A. iwoffii* (0.76 0.1%).

Pseudomonas sp., Bacillus sp., Staphylococcus sp., Aspergillus nidulans, Aspergillus flavus, and *Streptomyces sp.* were discovered as promising LDPE degrading isolates by Usha et al., 2020. The effectiveness of microorganisms in the breakdown of polythene and plastics was studied using a liquid (shaker) culture method. *Pseudomonas sp* degraded 37.09% of polythene and 28.42% of plastics in a 6-month timeframe. Fungal species contained 20.96% polythene and 16.84% plastics, while *Streptomyces* species contained 46.16% polythene and 35.78% plastics. This study found that *Streptomyces sps* has a stronger ability to digest polythene and plastics than other bacteria and fungi.

Chapter Ⅵ

Conclusion

Bioremediation by bacteria appears to be a potential and environmentally benign solution to the global plastic pollution challenge, particularly for polyethylene. In this study, chosen bacterial strains demonstrated amazing capability in digesting polyethylene, providing a long-term solution to environmental harm. This study can help decreasing the environmental impact of polyethylene accumulation, protecting ecosystems, and paving the way for a cleaner, greener future by developing interdisciplinary cooperation and investing in breakthrough biotechnological solutions.

The sole objective was to isolate the potential LDPE degrading bacteria.The dry cell weight (g/100 L) was determined at 0.1% LDPE powder concentrations, and the biomass was raised for all bacterial isolates. The cell weight values for isolates 2 and 7 were 0.9 g and 0.8 g, respectively. Isolate strains 3 and 2 lost 41% and 33% of their weight in LDPE sheets, respectively. As a result, the potentially isolated bacteria could be used as LDPE degrading microorganisms. By culturing these bacteria, the polyethene waste can be eliminated from the environment without impacting any biotic or abiotic factor.

The isolation and identification of enzymes capable of oxidizing and breaking polyethylene chains, as well as the size of polyethylene chains that they may utilize as substrate, is a fundamental goal in understanding the mechanics of polyethylene degradation. Another major area of research is determining the destiny of polyethylene inside microorganisms. It has been suggested that it is metabolized via the tricarboxylic acid cycle (TCA), although isotopic tagging has not been utilized to establish this.

The bioremediation of LDPE by bacteria holds profound environmental implications. By harnessing nature's own mechanisms, it offers a sustainable solution to plastic pollution, mitigating the adverse impact of non-biodegradable waste on ecosystems.

Successful biodegradation of LDPE reduces landfill usage, lowers carbon emissions from plastic production, and curtails marine and terrestrial contamination. Moreover, it promotes circular economy models, emphasizing recycling and waste reduction. Embracing this eco-friendly approach not only preserves biodiversity but also addresses public health concerns related to plastic pollutants. Continuous research and implementation of bioremediation technologies are essential in fostering a cleaner environment and advancing a greener, more sustainable future.

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