Identification and Molecular Characterization of Polyethylene Degrading Bacteria from Garbage Dump Sites in Adama, Ethiopia

Ethiopia Gezahegn Nedi

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirements for the Degree of Master of Science in Applied Biology (Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

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Adama, Ethiopia

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DECLARATION

I hereby declare that this Master's Thesis entitled "**Identification and Molecular Characterization of Polyethylene Degrading Bacteria from Garbage Dump Sites in Adama, Ethiopia**" is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this Thesis have been duly acknowledged through citation.

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RECOMMENDATION OF ADVISORS

We, the advisor(s) of this thesis, hereby certify that we have read the revised version of the thesis entitled "**Identification and Molecular Characterization of Polyethylene Degrading Bacteria from Garbage Dump Sites in Adama, Ethiopia**" prepared under our guidance by Ethiopia Gezahegn submitted in partial fulfillment of the requirements for the degree of Master of Science in Applied Biology (Biotechnology). Therefore, we recommend the submission of revised version of the thesis to the department following the applicable procedures.

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Major-Advisor	Signature	Date
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Co-advisor	Signature	Date

APPROVAL OF ADVISORS

we, the advisors of the thesis entitled "**Identification and Molecular Characterization of Polyethylene Degrading Bacteria from Garbage Dump Sites in Adama, Ethiopia**" and developed by Ethiopia Gezahegn hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Major Advisor	Signature	Date
Co-advisor	Signature	Date

APPROVAL OF BOARD OF REVIEWER

We, the undersigned, members of the Board of Examiners of the thesis by Ethiopia Gezahegn have read and evaluated the thesis entitled "**Identification and Molecular Characterization of Polyethylene Degrading Bacteria from Garbage Dump Sites in Adama, Ethiopia**" and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Applied Biology (Biotechnology).

Chairperson	Signature	Date
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Final approval and acceptance of the thesis is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC).

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ABBREVIATIONS

BLAST: Basic Local Alignment Search Tool

CDW: Cell Dry Weight

HDPE: High Density Polyethylene

LDPE: Low Density Polyethylene

MALDI-TOF MS: Matrix Assisted Laser Desorption Ionization-Time of Flight Mass spectrometry

MSM: Mineral Salt Media

NA: Nutrient Agar

NAHDIC: National Animal Diagnostic and Investigation Center

NB: Nutrient Broth

NCBI: National Center for Biotechnology Information

OD: Optical Density

PCR: Polymerase Chain Reaction

PDI: Polyethylene Degrading Isolate

PE: Polyethylene

PET: Polyethylene Terephthalate

PP: Polypropylene

PS: Polystyrene

PU: Polyurethane

PVC: Polyvinyl Chloride

VP: Voges-proskaure

ABSTRACT

Degradation of polyethylene is a great challenge due to its ever-increasing production and resulting pollution. This study was focused on identifying and characterizing polyethylene degrading bacterial isolates. Microorganisms were isolated from waste disposal site in Adama, Ethiopia. Carbon free basal medium supplemented with polyethylene was used to screen the bacterial isolates for biodegradation potential. The pure bacterial isolates were characterized by Gram's staining, biochemical tests, and MALDI-TOF MS analysis. The detection of alk B gene was carried out using PCR and 16s rRNA sequencing was employed for identification of an isolate that wasn't identified by MALDI-TOF MS. Sequencing revealed the bacterium as Bacillus sp. with a 91% similarity with Rossellomorea oryzaecorticis and a phylogenetic assessment was carried out using MEGA 11. The bacterial isolates were screened for enzymes such as: esterase, protease, and pectinase and the optimum pH, temperature and polyethylene concentration were determined for the isolates. The isolates were then subjected to biodegradation assay with untreated and UV treated polyethylene films in mineral salt medium for 1 month. The highest results based on weight loss % of untreated polyethylene were 4.25% and 1.93% Pseudomonas Sp.; 1.96%, 1.14%, and 1.53% Bacillus sp. with UV treatment the weight loss % was 17.96% and 19.9% Pseudomonas sp., 13.75%, 8.63%, and 4.79% Bacillus sp., and 6.77% Acinetobacter sp. Scanning Electron Microscopy used to observe changes in surface showed cracks on the polyethylene material after incubation with bacterial isolates. Pseudomonas and Bacillus sp. were found to have great capacity in polyethylene degradation and can therefore be used to facilitate an eco-friendly removal of plastic waste from the environment and further studies can be carried out to improve their enzymatic activity and thereby increasing degradative ability of the newly found isolates.

Key words: Biodegradation, Enzymes, MALDI-TOF MS, Polyethylene, 16s rRNA

CHAPTER ONE

1. INTRODUCTION

1.1. Background of the study

Industrialization and urbanization have led to massive accumulation of plastic garbage in the environment. Plastics are involved in many aspects of our lives as they are used in many applications. Plastics are long hydrocarbon chain polymers with high molecular weights which are mainly derived from petrochemicals, which are further synthetically arranged to produce long-chain polymers (Gan and Zhang, 2019). Despite their advantages, these plastics remain non-degradable and their extensive usage results in a tremendous amount of waste that is hard to remove from the environment. A biodegradation approach to this increasing plastic waste in the environment is shaping to be a needed solution as it is eco-friendly. The main methods used to treat plastic waste are recycling, landfill, and incineration which have their own drawbacks such as being expensive, taking a long time to decompose, and a release of toxic gases to the atmosphere respectively (Al-Salem *et al.,* 2009; Gan & Zhang, 2019). Microbial degradation however is a cheap and ecofriendly approach of eliminating plastic wastes.

Plastics can be categorized into two: thermoplastics and thermosets. Thermoplastics have the ability to be melted and reshaped whereas thermosets cannot be reshaped upon heating (Shah *et al.,* 2008). There are several conventional plastic types that are used in our day to day lives for different applications. These are polyethylene, polypropylene, polystyrene, polyvinylchloride, polyethylene terephthalate and polyurethane. Out of this polyethylene has been considered as the major polluting agent with over 25 million tons produced per year. Polyethylene's high demand in the market is due to its desirable characteristics such as extreme flexibility, good impact resistance, light weight, and chemical and corrosion resistance making it widely used in different applications (Munir *et al.,* 2018).

The increase in plastic production and waste accumulation releases a high amount of gaseous substances that are a threat to the environment as well as to human health into the air, including carbon monoxide, dioxins, nitrogen oxides and hydrogen cyanide as a result of the improper

disposal of plastic wastes has created a major problem for the environment (Ali *et al.,* 2021). Furthermore these plastic materials persist in the environment they get incorporated into the food chain and move from lower to higher trophic levels (John *et al.,* 2012).

Several microorganisms have been attributed to the degradation of plastic waste but *Arthrobacter* sp, *Aspergillus* sp, and *Pseudomonas* sp. are considered to be more effective (Munir *et al.,* 2018). The main approach where by microbes degrade plastics is by the use of their enzymes. The enzymes produced attach to the plastic material and depolymerize it into its monomers without producing a toxic substrate that harms the environment making it ecofriendly (Skariyachan *et al.,* 2015). Each of these enzymes capable of degradation are coded for by specific genes. One of the genes responsible for the degradation of polyethylene is the *alk B* gene from *P. aeruginosa* (Smits *et al.,* 2002). Based on this, the present research aimed to isolate bacterial species from waste disposal sites and identify their polyethylene degrading ability. This will offer an eco-friendly way to remove plastic waste especially polyethylene from the environment.

1.2. Statement of the problem

Plastic production is increasing at exponential rate and recent report suggested that the plastic waste management has become a major environmental concern. Waste products containing both liquid and solid plastic are being dumped into the soil and aquatic environments (Skariyachan *et al.,* 2015). These Plastic wastes take many years to decompose and are accumulating in landfills, toxic substances from the plastic is sipping into the soil and water bodies and after a very long time they accumulated in living issues and undergo bio-magnifications (John *et al.,* 2012). Tons of plastic material that is being dumped in the ocean each year is killing marine mammals, birds, sea turtles and fishes. Serious action should be taken to reduce plastic production and increase its removal from the environment. In light to these issues studies aimed at finding a safe, efficient and ecofriendly method for plastic degradation is of the utmost importance.

1.3. Significance of the study

This study identified bacterial isolates that are capable of degrading plastic waste from the environment. The screening of microbial consortia from highly polluted areas has a great application towards the degradation of plastics. Different bacteria were tested for the highest

ability for biodegradation and the one with the highest potential can be applied to cleanup polyethylene plastic wastes. The study also identified the enzyme activity and gene associated with the degrading bacteria. This helps in further understanding the bacteria's role in the activity and substrate specificity which will probably be helpful for engineering novel microbial enzymes capable of degrading various non-degradable compounds.

1.4. Objectives of the study

1.4.1. General objective

- To identify and molecular characterization of polyethylene degrading bacterial isolates from garbage dump sites in Adama, Ethiopia

1.4.2. Specific objectives

- To isolate and screen polyethylene degrading bacteria from dump site in Adama, Ethiopia
- To determine the rate of polyethylene degradation
- To identify polyethylene degrading bacterial isolate using 16SrRNA sequence
- To screen for Esterase, Amylase, Pectinase, and protease enzyme activity responsible for degradation
- To check for the presence of *alk B* gene responsible for the biodegradation of polyethylene

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Plastics

High molecular weight organic polymers created from various hydrocarbon and petroleum derivatives are called plastics. The word ‘‘Plastic” is derived from the Greek word ‘‘Plastikos”, which means a material that can be molded into different shapes (Zeenat *et al.,* 2021). Since its invention in 1907, plastic has been involved in our day-to-day life. Plastics are versatile, lightweight, flexible and durable; these properties make them important for use in packaging, machine parts, inside electronics, for making pipes, tableware and many others applications which causes an exponential increase in plastic waste generation. These plastics are resilient and non-biodegradable and adversely affect the environment through accumulation in landfills, leaching in to the soil and aquatic ecosystems, and being ingested by animal and cause intestinal blockage (Amobonye *et al.,* 2021). Even through these adverse effects, its production is still on the climb. In 2019, global plastics production almost reached 370 million tons with 2.51% increase from the previous year. Plastic production took a sharp drop due to the covid-19 pandemic in 2020 but started to recover during the second half of the year and has continued throughout 2021, However it is estimated that it will not reach its’ pre-pandemic level of production before 2022 (Plastics Europe, 2020).

2.1.1. Classification of Plastics

Thermoplastics: Plastics are usually classified by their thermal properties in two categories: Thermoplastics and Thermoset polymers. Polymers whose structure can be modified both either by heating or cooling repeatedly because the monomers joined end-to-end into a series of long carbon chains are independent of the others. Some of the most used polymers are within this category and they represent 77% of the total plastic produced (Danso *et al.,* 2019). This high C-C backbone structure makes thermoplastics resistant to degradation or hydrolytic cleavage of the chemical bonds. Some examples of thermoplastics are: Polyethylene (PE), Polypropylene (PP) Polyvinyl-chloride (PVC), Polyethylene Terephthalate (PET), Polystyrene (PS) and Polyamides

(PA), Polybutylene succinate (PBS), Polycaprolactone (PCL) and Polyethersulfone (PES) (Jenkins *et al.,* 2019; Shah *et al.,* 2008).

Thermoset polymers: on the other hand, are polymers that undergo a chemical change when heated, once they are shaped, they are unable to be recycled by heat action. Their structure is highly cross-linked and their main chains are made of hetero-atoms (e.g. ester or amide bonds), which make them potentially more susceptible to degradation. These thermosets account for 18% of the total plastic produced market share. Some examples are: Polyurethane (PUR), unsaturated polyesters, Epoxy resins, Melamine resin and Silicone (Shah *et al.,* 2008; Jenkins *et al.,* 2020).

2.1.2. Type of conventional plastics

2.1.2.1. Polyethylene

Polyethylene is the most commonly produced plastic these days. The synthesis of polyethylene was done accidentally by Hans von Pechmann in 1894 with the decomposition of diazomethane yielding what was called polymethylene, a white powdery substance with long sequences of methylenes. In 1933 Eric Fawcett and Reginald Gibson at the Imperial Chemical Industries in England discovered the first polymerization of ethylene while studying ethylene and benzaldehyde mixture at very high temperatures but they were not able to replicate their results because they did not realize that it was the presence of oxygen that made the reaction possible. Finally in 1935 Michael Perrin was successful in producing the practical polyethylene which lead to the production of LDPE at high pressure at the Imperial Chemical Industries in England in 1939 (Demirors, 2011).

Commercially produced polyethylene is a polymer of ethylene and has a chemical formula $(C_2H_4)n$. It is composed of a linear chain of carbons held together by hydrogen bonds and has low strength, hardness and rigidity, but has a high ductility (Mohanan *et al.,* 2020a). Polyethylene is divided into three main classes depending on the mode of polymerization as High Density Polyethylene (HDPE), Low Density Polyethylene (LDPE), and Linear Low Density Polyethylene (LLDPE) (Agboola *et al.,* 2017). HDPE has a linear structure with no branching and a density between 0.941 and 0.967 g/cm^3 and a molecular weight 10,000 to several million and also has good tensile strength and crystallinity. LDPE undergoes a high

pressure polymerization and has a more branched structure resulting in lower density of 0.910 - 0.940 g/cm^3. LDPE also has a good chemical resistance and higher rate of biodegradation because of its more branched structure. LLDPE is linear but has some branching structure. It has greater flexibility and a density of 0.910 - 0.920 g/cm^3 (Kupolati *et al.,* 2017; Dhakal & Ismail, 2021). LDPE is applicable in agricultural mulch, greenhouse films, and packaging. LLDPE can also be used in packaging industries and also in producing LLDPE films. HDPE is mainly used in milk jugs, garbage containers, detergent bottles, plastic bags and water pipes.

2.1.2.2.Polypropylene

Polypropylene (PP) was first produced by the polymerization of propylene monomers $(C_3H_6)n$. The first polymerization of propylene was accomplished by Giulio Natta of the Politecnico di Milano University in 1954 and soon after the industrial production of polypropylene started in 1956 by Montecatini in Ferrara (Cathelin *et al.,* 2018). PP is a very popular high-volume commodity plastic. It has a higher stiffness at lower density, fatigue resistance, good chemical and detergent resistance, resistance to higher temperatures and ease of machining; these key properties make PP advantageous. The main applications of PP are in house hold goods for buckets, bowls, bottle caps, and luggage, in automotive industry for steering wheel covers, bumpers, battery cases, tool boxes and mudguards (Tripathi, 2002).

2.1.2.3. Polyvinylchloride

PVC is the 3rd most widely produced plastic after PE and PP. It is produced by the polymerization of vinyl chloride monomer $[C_2H_3Cl]n$. PVC is a very durable and long lasting material which can be used in a variety of applications, either rigid or flexible. Applications of PVC include: in construction for Cables ducting and conduits; Flooring; Wall coverings; Reservoir linings; Sheets and panels; and Window frames and doors. Use in automotive industry, for making Window encapsulation, Dashboard skins, and coated fabrics. In Clothing for making Raincoats, Life vests, Shoe soles, Rubber boots, and Imitation leather. For making Credit cards, Smart cards, Identity cards, Telephone cards and food packaging (Yu *et al.,* 2016).

2.1.2.4. Polyethylene terephthalate

Polyethylene terephthalate (PET) $(C_{10}H_8O_4)n$ is a polymer commonly referred to as polyester. It is produced by the polymerization of ethylene glycol and terephthalic acid. Ethylene glycol is a colorless liquid obtained from ethylene, and terephthalic acid is a crystalline solid obtained from xylene. The first polyethylene terephthalate was industrially produced by DuPont in 1957. PET has both semi-crystalline and amorphous forms where the semi-crystalline form is stiff, hard, and strong and the amorphous is ductile but has lower heat resistance, stiffness and hardness (Ji, 2013).

The applications of Polyethylene terephthalate (PET) can be divided into three major categories: fiber, bottles, and industrial use. It can be used in synthetic fibers; beverage, food and other liquid containers; thermoforming applications; film, plastic and engineering resins often in combination with glass fiber. The two widely used applications are PET fabrics and PET bottles (Ji, 2013). Properties of PET such as fatigue and crease resistance, transparency, and good barrier properties make PETs suitable for medical applications such as constructing large-diameter vessels (>6 mm inner diameter) and artificial vascular grafts (Zeng *et al.,* 2019). Besides these PET nanofibers prepared by electrospinning which are coated and surface modified are being used for blood vessel engineering (Ma *et al.,* 2005). As the other plastic polymers, PET is non-degradable and persists in the environment; it is mainly removed by either recycling or by the action of microorganisms

2.2. Effect of synthetic plastics on the environment

As the world's capacity to deal with the rapid rising production of disposable plastic goods is overwhelmed, plastic pollution has emerged as one of the most urgent environmental issues. Plastics are used in a wide range of industries, including packaging, construction, electronics, an d many others, making it extremely difficult to recognize their detrimental effects on the environ ment. For instance, singleuse plastics make up 40% of the annual plastic production. Many of the se items, like plastic bags and food wrappers, have short shelf lives but can linger in the environ ment for hundreds of years. In 2020, 195 countries were estimated to produce about 400 Mt. of plastic waste, with about 8.8 Mt. entering the ocean. Plastic wastes can act as a carrier for organic pollutants, chemicals, heavy metals, and pathogens. Furthermore, abiotic degradation of

plastic releases highly toxic compounds, deteriorating the quality of soil and water (Chen *et al.*, 2019). The majority of the plastic trash washes up to the sea, and there sunlight, wind, and wave action break down plastic waste into small particles creating microplastics These microplastics have side effects on human health. Human body is exposed to microplastics through ingestion of food containing microplastics, inhalation of microplastics in the air and by dermal contact of these particles, contained in products (Prata *et al.,* 2020). Ingestion is the major way of exposure which may occur from contaminated food or mucociliary clearance after inhalation and results in inflammatory response, increased permeability, and changes in gut microbe composition and metabolism (Salim *et al.*, 2014). The presence of plastic wastes in water bodies disturbs natural flow, limits the ability of fish to reproduce and destroys vital organisms; also, polymers in the oceans contribute to global warming by creating a shaded canopy hindering plankton growth. Plastic wastes added to soil affect soil organisms, biomass, and biodiversity in total it can affect the ecosystem equilibrium (Ali *et al.*, 2021). This detrimental side effect to the environment has led several countries to take action on plastic products. Several European countries and Japanese government imposed a plastic bag fee to limit production and use. South Africa also has restricted the manufacture and usage of plastic bags. This prohibitions, the identification of novel plastic waste degraders, proper waste disposal, and the use of environmentally friendly plastic alternatives may help in combating the increasing plastic pollution (Jalil *et al.*, 2013)

2.3. Types of degradation

One of the effective ways to lessen the negative consequences brought on by plastic trash is recycling. The recovery rates for PET and HDPE are 19.5% and 10%, respectively. However, depending on their uses, plastics like PVC, LDPE, PP, and PS that have a recovery rate of less than 5% are hardly recyclable. PVC has a recovery rate of zero percent, indicating that it is fully unrecyclable. This is mainly because PVC contains a lot of dangerous chemicals and raw materials that are rich in chlorine. On average, less than 10% of plastic garbage is recycled, allowing more than 80% of it to build up in the environment. Therefore, degradation methods come in handy in removing these plastic polymer wastes (Liang *et al.,* 2021). Heat, mechanical energy, action of microorganisms, and radiation are the main factors causing degradation of polymers. Based on these types of degradation can be classified as thermal, photo, mechanical and bio degradation.

2.3.1. Thermal degradation

Thermal degradation is a process by which an action of increased temperature causes a change in properties of the polymer (Izdebska, 2016). The molecular weight of the plastic typically changes during thermal breakdown. Other changes to a material's attributes include cracking, chalking, color changes, and a general loss of good physical qualities. At a high temperature several polymers such as polyethylene can depolymerize in to their smaller monomeric forms (Ramis *et al.,* 2004). In a study Singh et al. (2019) indicated that plastics such as PS, PE, PET, and PP degrade in a single step and the degradation temperatures varies according to the plastic involved. PS required a higher temperature followed by PET and PP whereas HDPE had the lowest temperature requirement as compared to the rest. Furthermore, virgin polymers show a higher degradation temperature than polymer wastes.

2.3.2. Photo degradation methods

Is a type of degradation in which polymer chains are degraded with UV rays. Physical and chemical changes of the polymer are caused by irradiation with ultraviolet or visible light. This can only happen if the polymer has chromophoric groups with the ability to absorb light(Yousif & Haddad, 2013). UV radiations can break C-C bonds of the polymer and also produce ester, aldehyde, propyl and format groups (Zeenat *et al.,* 2021). Due to its enormous energy, UV radiation is known as the most harmful source for polymers. The physical and chemical structure of polymers will therefore deteriorate as a result of persistent exposure to UV radiation, which is known as photodegradation. Chain scission, altered molecule shape, decreased molecule weight, and deterioration of polymer characteristics are all examples of photodegradation of polymers, which often occurs when UV rays and oxygen are present. Overall, this is a very drawn-out process that frequently takes up to 50 years or longer to complete, with the sun totally degrading the plastic and the bacteria assimilating the polymer molecules. When this operation is carried out underwater, it becomes significantly more difficult and drawn out. The process can take a lot longer due to the poor rate of hydrolysis of most plastics in the ocean as well as the lower temperatures, sunshine exposure, and oxygen availability in seawater (Yousif and Haddad, 2013).

2.3.3. Mechanical degradation

Mechanical degradation occurs when a polymer is broken down due to high mechanical stresses such as compression, shear forces, or tension. Mechanical stresses cause the breakage of the molecule and reduction of the polymers molecular weight which can also be of use in accelerating biodegradation (Al-Shakry *et al.,* 2018). The three basic processes that produce mechanical degradation during processing are agitation, grinding, and extrusion. The fracture of the molecule is the primary phenomenon involved when the polymer is subjected to extremely strong shearing pressures. The average molecular weight of the polymer decreases as a result of mechanical degradation. Despite not being the primary element during biodegradation, mechanical factors can nonetheless activate or speed it up. In real-world settings, mechanical stresses interact synergistically with other environmental factors as humidity, UV rays, and temperature (Niaounakis, 2015).

2.3.4. Biodegradation

Biodegradation is changes in the physical or chemical properties of the polymer due to the action of microorganisms. Polymers are converted to small molecular weight fragments which are broken down to carbon dioxide and water molecules (Glaser, 2019). Microbial organisms such as bacteria, fungi, or actinomycetes are crucial in degrading plastic polymers. Depending on the microorganisms involved, it can be either aerobic or anaerobic biodegradation. In aerobic biodegradation the microorganisms break down the plastic compound in to smaller compounds using oxygen as an electron acceptor and produce water and carbon dioxide whereas anaerobic degradation uses sulphate, iron, manganese, nitrate and CO_2 as electron acceptor and results in the production of methane, carbon dioxide, and water (Müller, 2002). In order for microbes to utilize polymers as carbon source, the production of extracellular enzymes is necessary. These enzymes play part in creating smaller molecules capable of passing the microbe's membrane and being used as a source of energy. In biodegradation the first step is usually the microorganism attaching itself on the polymer surface, after attachment it begins to secret extracellular enzymes to obtain molecules of low molecular weight which are easier for utilization (Danso *et al.,* 2019).

2.4. Properties of plastic materials as an obstacle for their biodegradation

Thermoplastics such as polyethylene and polypropylene are produced by breaking the double bond in the original olefin (C_nH_{2n}) by additional polymerization to form new carbon-carbon bonds. This long carbon chain structure of thermoplastics makes them resistant to degradation or hydrolytic cleavage of chemical bonds and therefore thermoplastics are considered non-biodegradable plastics (Zheng *et al.,* 2005). However, thermosets like polyurethane are produced by condensation between a carboxylic acid and an alcohol or amine to form polyester or polyamide giving thermosets a highly cross-linked structure. Since the main chain of thermoset plastics is made of heteroatoms, there is a possibility of degradation by the hydrolytic cleavage of chemical bonds such as ester bonds or amide bonds (Zheng *et al.,* 2005).

The initial phase of biodegradation in which microorganisms launch a chemical attack on the polymer chain aimed at the breaking of chemical bonds, and a second phase of real biodegradation are affected by several factors. These two phases are strongly controlled by the presence of numerous factors, both endogenous (as molecular weight, crystallinity, flexibility of the molecule, morphology and hydrophobicity) and exogenous (temperature, humidity, pH, availability of oxygen, enzymatic activity) that can, therefore, alter and/or modify the outcome of the biodegradation process itself (Gironi & Piemonte, 2011). High molecular weight and crystalline morphology generally relates to lower degree of biodegradability. Polymers with highly crystalline structures, such as polyethylene and PET, tend to be more resistant than more amorphous polymers such as polycarbonate. PET-based plastics possess a high degree of crystallinity (30–50%), which is one of the principal reasons for their low rate of microbial degradation, it takes more than 50 years for their complete degradation in the natural environment, and hundreds of years if discarded into the oceans, due to their lower temperature and oxygen availability (Mohanan *et al.,* 2020). The amorphous regions of polymers, where the molecules are less densely packed, are preferentially attacked by plastic-degrading enzymes. Thus, the more amorphous a polymer is, the more access the enzymes have and the faster the degradation can proceed. The relatively lower hydrophobicity, denoted by presence of functional groups like the ester linkages in PBS and PCL, facilitates plastic degradation (Jenkins *et al.,* 2019).

2.5. Microbial degradation of synthetic plastic and enzymes involved

Petroleum-derived polymers such as PE, PET, PU, PS, PP, and PVC are extremely recalcitrant to natural biodegradation pathways. But some microorganisms have the ability to degrade petro-polymers under *in vitro* conditions and they have been isolated and characterized in different researchs. *Pseudomonas* spp. was found to be the most efficient followed by *Arthrobacter spp.*in the degradation of PE. In a research Rajandas *et al.,* (2012) *P. aeruginosa* was able to degrade 0.25 g of LDPE by 50.5% in 2 months after pretreatment with nitric acid. Other bacteria capable of degrading PE are Gram positives such as *Bacillus spp., Streptocococcus spp., Staphylococcus spp.,* and gram negatives *Acinetobacter ursingii* and *Pseudomonas fluorescens* (Munir *et al.,* 2018).

Enzymatic degradation is one of the main approaches for plastic waste biodegradation. These microbial enzymes are successful in increasing the rate of biodegradation of plastic polymers effectively without any impact to the ecosystem as it produces nontoxic end products such as CO_2, H_2O and CH_4 (Skariyachan *et al.,* 2015). Plastic-degrading enzymes are classified in to extracellular and intracellular enzymes with the extracellular enzyme being the most studied. These enzymes are involved in the de-polymerization of the long carbon chains of the plastic polymers to a mixture of oligomers, dimers and sometimes, monomers. These groups of enzymes include laccases, peroxidases, lipases, esterases and cutinases (Amobonye *et al.,* 2021). Polyethylene being one of the major sources of plastic waste in the environment has several enzymes involved in its biodegradation. Enzymes hydroxylases, laccases, peroxidases and reductases have been identified as polyethylene degrading enzymes from bacterial and fungal sources. The enzyme alkane hydroxylase was found to be important in the breaking down of PE by *Pseudomonas* spp. E4 (Amobonye *et al.,* 2021).

2.6. Genes related to plastic degrading bacteria

In most analysis of plastic degrading bacteria *Pseudomonas* sp. has the highest degradation potential. This *Pseudomonas* sp. has several genes that aid in the degradation of plastic wastes. Belhaj *et al.,* (2002) and Smits *et al.,*(2002) indicated that *alk B*-related sequences were mostly found in Gram-negative bacteria growing with short chain n-alkanes and are capable of degrading alkanes. Some bacterial species with their genes and enzymes responsible for degrading specific plastic materials are shown in Table 1.

Table 1: Potential bacterial species for biodegradation and their associated genes

NO	Bacterial spp. involved in plastic degradation	Gene associated with conventional plastic degradation	Enzymes involved in plastic degradation	Type of plastic degraded by respective bacterial isolates	References
1	*P. aeruginosa* PAO1	*alk B1* and *alk B2*	Alkane hydroxylases	PE	(Smits *et al.*, 2002)
2	*p. fluorescens* ST	*styAB*	Styrene monooxygenase	Polystyrene	(Leoni *et al.*, 2003)
		styC	Epoxy-styrene isomerase		
		styD	Phenylacetaldehyde dehydrogenase		
3	*P. putida* GPo1	*alk B*	alkane hydroxylases	PE	(Smits *et al.*, 2002)
4	*P. chlororaphis*	*pueA*	Polyurethanase	Polyurethane	(Stern & Howard, 2000)
5	*Ideonellasakaiensis 201-F6*	ISF6_4831	PETase	PET	(Yoshida *et al.*, 2016)
6	*Delftiaacidovorans*	*pudA*	Carboxylic ester hydrolase	Polyurethane	(Nomura *et al.*, 1998)
7	*Paenibacillus sp. DK1*	*alk B*	Alkane monooxygenaseAlkB	PE	(Bardají *et al.*, 2019)

2.7. Plastic waste management and pollution in Ethiopia

Plastic pollution is becoming an increasing concern in the country as the number of plastic items being produced and disposed of has risen sharply over the past few years. The modern waste collection methods of economically developed countries can collect more than 90% of waste and recycle around 20% of what is collected. On the other hand, in Africa, including Ethiopia, the waste collection is less than 40%, with only around 4% recycled. In developing countries, the least preferred waste management techniques like incineration and landfilling are practiced, and they make poor management decisions related to waste control. In comparison to other African countries, Ethiopia has a high level of waste. This is due to the large population (approximately 115 million) and rapid urbanization, which increases the amount of waste generated (Habtom, 2022). According to several research studies conducted in Ethiopia, plastic waste is the second most common type of solid waste generated, after food waste. Plastic waste accounts for 15.5% of the total waste generated in Addis Ababa. PET used for plastic bottles is the most popular plastic in Addis Ababa, followed by HDPE used in shopping bags and LDPE used for garbage bags and agriculture (Natural Resources Stewardship Programme, 2021). According to (Hailemariam & Ajeme, 2014), 20% of the total solid waste generated in Adama City came from households, while 29% came from organizations. This increased plastic waste is also seen in other cities; plastic bottles and bags from both residential and commercial areas accounted for 28% of Jigjiga City's total waste (Birhanu & Berisa, 2015). Another study (Lema *et al.,* 2019) indicated that of all the waste generated in Assela, Ethiopia, 34.8% consisted of plastic. (Hussein *et al.,* 2021) showed the waste generated in Gode town, Eastern Ethiopia, to have plastic bags (46%), water and soft drink bottles (34%), household utensils (16%), and others (4%).

In many Ethiopian cities, the municipal services of waste collection, removal, and administration are lacking. The majority of plastic waste is disposed of in landfills, but the remainder is disposed of in drainage systems, open spaces, street sides, rivers, forests, or is burned informally. Such a lack of waste management and disposal infrastructure further aggravates the already existing plastic waste problem in Ethiopian cities (Hussein *et al.,* 2021). The following ways could be used to reduce plastic pollution in the environment: household level waste sorting, where pre-collection waste is sorted at the household level into different bags; creating awareness of waste management and recycling; designing a modern waste collection system that

suits the city's waste generation and capacity for removal; replacing the non-degradable plastic with biodegradable plastic; reducing plastic usage; and increasing recycling capacity. These strategies would help reduce the amount of plastic waste in Ethiopia and allow for more efficient waste management and disposal (Prata *et al.,* 2020; Habtom, 2022).

2.8. Identification of bacteria isolated from environmental sources

Methods of reliable and accurate microbial identification are valuable for a wide range of applications. Observations of the microorganism's physical characteristics, such as shape, size, and staining are usually the first step in identifying a microbe. The crucial details required for correctly identifying the genera of various bacteria within a sample can be revealed by biochemical reactions. Bacteria naturally produce large amounts of enzymes, and it is thanks to these enzymes that they can be identified using biochemical techniques. Given that bacteria have distinct enzymatic profiles, it is typically possible to classify a bacterium's species based on the type of enzymes it produces (Aslanzadeh, 2006). In addition to biochemical tests, MALDI-TOF MS analysis and 16S rRNA are usually used as identification tools. In recent years, matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has become a go to tool for microbial identification because of its rapid and accurate identification of microorganisms (Feucherolles *et al*., 2019). During the MALDI-TOF process, the sample is coated with the matrix solution, which is an organic compound that absorbs energy. Bacterial identification has relied on matrix compounds such as -cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxy benzoic acid (DHB), and 3,5-dimethoxy-4-hydroxycinnamic acid. After the matrix and the sample crystallize, they are ionized with a laser beam to generate a single protonated ion. The protonated ions are then accelerated at a fixed potential, where they separate based on their mass-to-charge ratio (m/z). The mass-to-charge ratio is then analyzed by time of flight (TOF) analyzers. The TOF analyzer then generates a peptide mass fingerprint; this fingerprint is then compared to others in the database to successfully identify the given microorganism (Singhal *et al.,* 2015). Isolates are separated depending on the output score value, as isolates with a score of $\geq$ 2.0 for a given species were considered high-confidence identifications to the species level, 1.70–1.99 were considered low-confidence identifications, and 0.00–1.69 were characterized as no organism identification possible .

Another tool for the precise identification of microbes is 16S rRNA sequencing. This technique is most commonly used because of its presence in almost all bacteria, the fact that the function of the 16S rRNA gene has not changed over time (it has a slow rate of evolution), and the fact that it's large enough for informatics purposes (Janda & Abbott, 2007). 16S rRNA sequencing allows for the identification of species and subspecies that are difficult to differentiate with peptide mass fingerprinting. Additionally, 16S rRNA sequencing allows for the identification of closely related organisms, as the technique can detect the differences in gene sequence between two organisms. The 16S rRNA sequencing technique involves an initial PCR amplification of a small segment of the 16S rRNA gene, followed by either Sanger or next-generation sequencing to obtain the complete sequence (Janda & Abbott, 2007).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Description of the study area

The study was conducted in Adama city. Adama forms a special zone of Oromia and is surrounded by East Shewa Zone. It is located at 8° 30' 52.1172" N and 39° 16' 9.3252" E at an elevation of 1712 meters, 99 km Southeast of Addis Ababa. The city is located in Ethiopian rift valley and has annual rainfall that ranges from 400-800 mm and annual mean temperature of 22^{o}C. The city is divided into 14 sub cities and has a total population of 324,000 (*Ethiopia Population, 2022*).

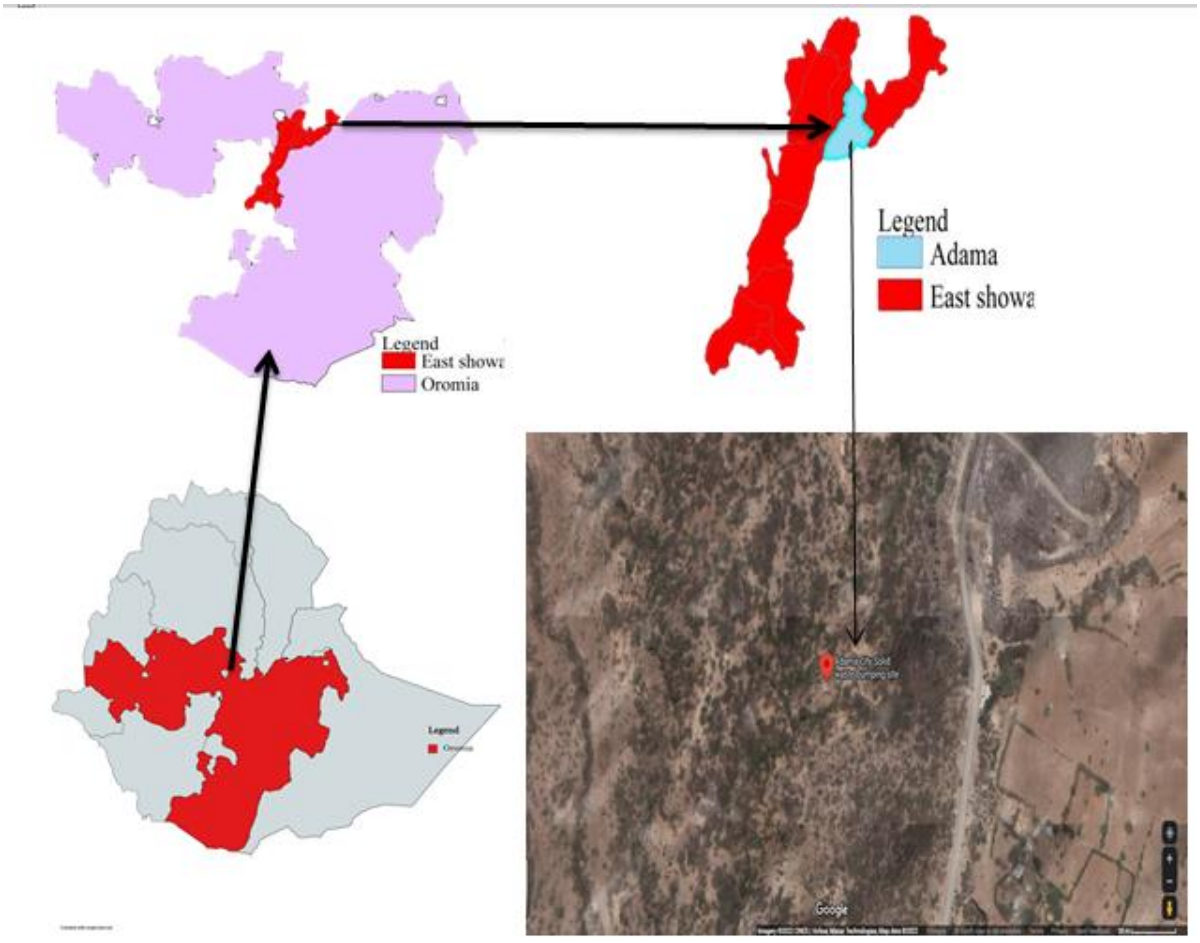

Figure 1: Map of Oromia, Adama and the site of sample collection (Google maps, n.d.)

3.2. Sample collection

Partially degraded polyethylene material along with adhered soil samples (approximately 100g) dug 10-20 cm deep were collected aseptically from the main dump site in Adama city and transported in a sealed Ziploc bag to the biology laboratory at Adama Science and Technology University for analysis. These collected polyethylene samples mainly consisted of HDPE bags and they were processed for the isolation of polyethylene degrading bacteria within 24h. of their arrival at the laboratory.

3.3. Isolation and screening of polyethylene degrading bacteria

The collected partially degraded polyethylene samples (10g) were placed in 90ml of 0.9% (w/v) saline solution and gently shaken on 120rpm shaker incubator at room temperature for 24h to remove adhered soil particles. After 24h, 10ml of the saline solution was aseptically inoculated into 40ml of liquid carbon-free basal medium (g/l) (KH2PO4, 0.7; K_2HPO_4, 0.7; $MgSO_4.7H_2O$, 0.7; NH_4NO_3, 1; NaCl, 0.005; $FeSO_4.7H_2O$, 0.002; $ZnSO_4.7H_2O$, 0.002; and $MnSO_4.H_2O$, 0.001) with 1g of polyethylene plastic pieces as the sole source of carbon. The cultures were incubated at 37^{o}C for 14 days (Sivan *et al.,* 2006).

Microorganisms from the previous culture were grown on a salt medium (%) (K_2HPO_4, 0.016; $CaCl_2.2H_2O$, 0.02; $(NH_4)_2SO_4$, 0.01; $MgSO_4.7H_2O$, 0.02; $ZnSO_4.7H_2O$, 0.005; $MnSO_4.H_2O$, 0.005; $FeSO_4.7H_2O$, 0.001; and agar, 1.5. polyethylene pieces of 0.1% (w/v) was added as a carbon source and the culture was incubated for 3-5 days at 37^{o}C. After completion of the incubation period, the bacterial isolates were purified by streak plate technique on nutrient agar and pure cultures were maintained in nutrient broth (Himedia) at 4^{o}C for further study (Nadeem *et al.,* 2021).

3.4. Identification of newly isolated Polyethylene degrading bacterial isolates

3.4.1. Gram Staining

Gram staining technique was used to categorize the bacteria into Gram positive and Gram negative. The Gram staining was carried out according to (Beveridge, 2001). Pure bacterial colones of 24h were taken and smeared on glass slide. The smear was then heat fixed. A few

drops of crystal violet (0.0124 g/mL) (w/v) were added to the smear test. The smear was then allowed to stand for 0.5 min then rinsed with water. Then, a few drops of Gram's iodine (a mixture of 0.33 g iodine and 0.67 g potassium iodide in 100 ml water) was directly added the smear test with crystal violet for 0.5 min. The crystal violet-Gram's iodine mixture was washed off from the slide and the slide was washed rapidly with tap water. Then, the slide was washed with ethanol (95% v/v ethanol in water) for 20 sec. Then, the slide was rapidly washed using tap water and followed by, safranin as a counter stain for 1 min. The slide was rinsed with tap water. Finally, the slides were air dried and viewed under a microscope (100X).

3.4.2. Biochemical characterization

Biochemical tests such as Catalase, citrate utilization, Indole, hydrogen sulfide, Methyl red, and urease were used to identify bacterial isolates from dump sites these tests are considered as traditional methods of identifying bacteria.

3.4.2.1. Catalase test

This test was applied to identify the presence of catalase which converts hydrogen peroxide in to water and oxygen as follows. Four drops of 3% (v/v) H_2O_2 was added to in a test tube and a small amount of isolated bacterial colony was added to the test tube and the formation of bubbles was examined and the result was recorded (Bullock & Aslanzadeh, 2013).

3.4.2.2. Citrate utilization test

Citrate test was carried out to test the ability of the bacteria to use citrate as a carbon source. Fresh 18-24 h colonies were streaked on Simmons citrate agar slants (g/l) (Bromothymol blue 0.08; $MgSO_4$ 0.2; $(NH_4)_2HPO_4$ 1 and Agar 15) and incubated at 37°C 2- 4 days. Citrate metabolization is indicated by a color change from green to blue (MacWilliams, 2009).

3.4.2.3. Sulfur Indole Motility test

The SIM medium tests for hydrogen sulfide production, indole production, and motility. A sterilized tube containing SIM medium was inoculated with 18-24h old culture and incubated at 37°C for 24 - 48h. Hydrogen sulfide production was indicated by presence of black precipitate and motility was indicated by growth that spreads out from the initial stab line. Indole production

was indicated by the presence or absence of a red ring after 0.5ml of kovac's reagent was added into test tube (Darkoh *et al.*, 2015).

3.4.2.4. Methyl red test

This assay determines if an organism metabolizing glucose utilizes mixed acid fermentation pathway and produces strong acid end products (lactic, acetic, or formic) that are detected by the indicator methyl red. 5 ml MR-VP broth tube was inoculated with the organism and was incubated at 35 °C for 48–72 h. A 2.5 ml of the broth culture was transferred to a fresh tube and inoculated with five drops of methyl red indicator then the presence or absence of color change from yellow to red was recorded (Bullock & Aslanzadeh, 2013).

3.4.2.5. Urease test

Urease test was used to determine the bacteria's ability to produce urease and convert urea into CO_2, H_2O, and NH_4. 18-24 h pure colony was streaked on urea agar slants prepared using urea agar base (g/l) (Dextrose 1; Disodium phosphate 1.2; Monopotassium phosphate 0.8; NaCl 5; Peptone 1; Phenol red 0.012 and Agar 15) with addition of 40% (w/v) urea which was added to the agar base after sterilization and cooling down. The culture was incubated 35-37^{o}C for up to 5 days. The presence of urease is indicated by color change from yellow to pink (Bullock & Aslanzadeh, 2013).

3.4.2.6. Voges-proskaure test

The VP test detects the production of acetoin by organisms that utilize the butylene glycol fermentation pathway. In the presence of air and potassium hydroxide, acetoin is oxidized to diacetyl, which produces a red-colored complex. A 5 ml MR-VP broth tubes were inoculated with pure bacterial culture and incubated at 35 °C for 18–24 h. A 2.5 ml of the broth cultures were transferred to a fresh tube and inoculated with six drops of α-naphthol followed by three drops of KOH. Then, The tubes were shaken and left for 10 min and a pink-red color change was considered positive (Bullock & Aslanzadeh, 2013).

3.4.3. Identification using MALDI-TOF MS

Pure isolates of plastic degrading bacterial sp. were identified following the methods of (Toubal *et al.,* 2018). Matrix-assisted laser desorption ionization time flight mass-spectroscopy (MALDI-TOF MS) identification was carried out at NAHDIC. Briefly, all isolates were initially purified and screened based on their ability to degrade plastics prior to identification using MALDI-TOF MS. These isolates classifications were carried out using the direct transfer method (MALDI Biotyper 3.1. User Manual, Bruker Daltonics Inc.). The representative single colonies of the possible polyethylene degrading isolates (PDI) were smeared as a thin film directly into a spot on MALDI targeted plate using tooth applicator. The MALDI-TOF MS target plate was overlaid with 1µl of 70% (v/v) of formic acid and allowed to dry at a room temperature. Immediately the spot was overlaid with 1µl of matrix solution α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile (CHCA) solution and allowed to dry at room temperature. The resulting spectra were compared with reference spectra by using the Biotyper 3.1 software (Bruker MALDI Biotyper, UK). The identification score cutoff values were applied to each measurement according to the manufacturer's instructions. Isolates with a score of ≥2.0 for a given species were considered High confidence identification to the species level, 1.70-1.99 were considered Low confidence identification, and 0.00-1.69 were characterized as No organism identification possible as prescribed by Tomazi et al. (2015). *E. coli* ATCC 25922^{T} was used as a standard for calibration and quality control.

3.5. Molecular characterization for identification of newly obtained Polyethylene degrading bacterial isolates

3.5.1. Bacterial DNA extraction

All molecular characterization in this study was carried out at NAHDIC. Bacterial DNA was extracted using QiagenDNeasy DNA extraction protocol adapted from QiagenDNeasy handbook, July, 2006. A 200µl of bacterial cultures were added to a labeled 2 ml tube then 180 µl of enzymatic lysis buffer was added and the tubes were vortexed for 10-20 s. The tubes were incubated for 30min in a water bath at 37^{o}C. After incubation 25 µl of proteinase K and 200 µl of

buffer AL were added to the tubes and vortexed for 1min. The mixtures were then incubated at 60^{o}C for 30min. Then 200 µl of 100% ethanol was added to the tube and vortexed briefly.

The entire content of the tube (600 µl) was transferred to a mini spin column placed in a collection tube and centrifuged at 10000 x g for 1 min. The column was removed and placed in a new collection tube and 500 µl of buffer AW1 was added to the column and centrifuged at 10,000 x g for 1 min. Then again, the column was removed and placed in a new collection tube and 500 µl of buffer AW2 was added to the column and centrifuged at 20,000 x g for 3 min. The column was transferred to a 1.5 ml tube and 200 µl of buffer AE was added to the column. After 1 minute room temperature the column was centrifuged at 10,000 x g for 1 min. And finally, the column was discarded and the DNA was appropriately stored (4° C for short term, -20°C for long term).

3.5.2. DNA amplification and agarose gel electrophoresis

PCR amplification was performed according to the methods of (Kohno *et al.*, 2002; Kloos *et al.*, 2006). Partial *alkB* genes were amplified using the forward primer ALK-3F 5'-TCGAGCACATCCGCGGCCACCA-3' and reverse ALK-3R 5'-CCGTAGTGCTCGACGTAGTT-3'. The PCR master mix included 10.95µl ultra-pure water, 5µl buffer, 0.8 µl dNTPs, 1 µl enzyme, 0.5 µl of each forward and reverse primer, 1.25 µl $MgCl_2$, and 5 µl of DNA. PCR cycling conditions were, an initial enzyme activation step at 95°C for 15 min, followed by an additional 39 cycles of denaturation 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1min.

The amplified DNA samples were analyzed by gel electrophoresis in 1.5% (w/v) agarose gel in 1X TBE buffer stained with 20µl ethidium bromide. 6µl of amplified DNA was mixed with 2 µl of loading dye and the mixture was loaded onto the gel. In addition, DNA ladder, water as a negative control, and a known Pseudomonas and Bacillus isolates as a positive control were loaded onto the gel. After an hour of electrophoresis at 100v, the gel was transferred to Gel doc^{TM}XR+ for visualization.

3.6. Identification of isolated bacteria by 16S rRNA sequencing

DNA isolation was carried out using Quick-DNA™ Fungal/BacterialMiniprep Kit Catalog No. D6005 from Zymo Research. The amplified DNA was separated by electrophoresis in 0.8% agarose gel run in 1× TAE buffer at 50V for 30 to 45 minutes till DNA fragments are migrated well. The gel was photographed on gel documentation system. The concentration of DNA sample was then measured by Nanodrop (Biotech instruments, USA). DNA was stored at−80◦C for further use. The ratio of absorbance at 260 nm and 280nm is used to assess the purity of DNA. A ratio of ~1.8 to 2.0 is generally accepted as "pure" for DNA. After this PCR amplification was performed in a total volume of 25 µl containing 10 pmol each of forward and reverse primers (8F- AGAGTTTGATCCTGGCTCAG and 1492R- TACGGTTACCTTGTTACGACTT), 2.5 mM of MgCl2, 200 µM each of the four deoxyribonucleotide triphosphates (dNTPs), 0.5 U of Taq DNA polymerase, 1x concentration of PCR buffer (Invitrogen, Life Technologies, Brazil) and 50 to 100 ng of isolated bacterial genomic DNA. The template was denatured by heating at pre-denaturation of 95°C for 5 min. This was followed by 39 cycles of denaturation 30 sec at 95 °C, 45 sec annealing and 1 min elongation at 72°C, with a final extension of 7 min at 72°C. The amplicons were resolved in 1.5% agarose gel using 0.5x trisacetate-EDTA (TAE) buffer. And finally, the PCR amplicon was subjected to Sanger Sequencing and 16SrRNA sequences fragments were sequenced with an automated ABI 3730XL DNA sequencer. The 16S rRNA gene sequences were analysed with Basic Local Alignment Search Tool (BLAST) and strains were aligned by multiple sequence alignment program (Clustal W) and finally a phylogenetic tree was constructed.

3.7. Detection of enzymes responsible for biodegrading isolates

3.7.1. Detection of Amylase

The ability of amylase production was identified on NA medium (g/L) (Glucose 10; NaCl 5; Peptone 10; Yeast extract 10 and Agar 18 at pH 7.0-7.2) which supplemented with 1%(w/v) of starch. The sterilized medium was supplemented with separately sterilized starch and the medium was then incubated at 37^{o}C for 48h. After completing incubation they were flooded with Gram's Iodine (Nxumalo *et al.*, 2020).

3.7.2. Detection of Protease

Ability of protease production was identified with NA (g/L) (Glucose, 10; NaCl, 5; Peptone, 10; Yeast extract, 10; Agar, 18 and pH 7.0-7.2) supplemented with 1% skim milk (w/v) with the addition of 0.0015% (w/v) Bromocresol green (BCG) dye. After sterilization at 121˚C for 15 min, the medium was inoculated with the isolate and incubated at 37^{o}C for 48h. Zone of proteolysis was checked after completion of incubation period (Vijayaraghavan & Vincent, 2013).

3.7.3. Detection of Esterase

Esterase activity was determined using a salt medium (g/l) (NaCl 5.0, $CaCl_2.2H_2O$ 0.1, agar 18.0, pH 7.0). The sterilized medium was supplemented with separately sterilized Tween 80 (1%) (v/v). The medium was then incubated at 28°C for 5 days. The appearance of a halo zone was indicative of esterase activity (Nxumalo *et al.,* 2020).

3.7.4. Detection of Pectinase

Pectinase activity was detected using a pectin agar medium containing ($FeSO_4.7H_2O$, 0.01 g/L; K_2HPO_4, 1.8 g/L; $MgSO_4.7H_2O$, 0.2 g/L; NaCl, 0.1 g/L; NH_4Cl, 4.0 g/L; Pectin 10 g/L and Agar, 15 g/l) agar plate. After sterilization, the medium was inoculated with a fresh culture and incubated at 30^{o}C for 4 days and after completion of incubation the medium was flooded with iodine solution (Beg *et al.,* 2000).

3.8. Optimization for Growth Parameters

To identify which are the optimum temperature, pH, and concentration of polyethylene requirement of the bacterial isolates, the isolates were cultured under a set of conditions with varying pH, temperature, and concentration.

3.8.1. Optimization for growth temperature

The effect of temperature on the ability of the selected isolates to degrade polyethylene was determined by using Mineral Salt Medium (MSM) in g/l (NH_4NO_3, 1.0; K_2HPO_4, 1.0; $MgSO_4$.7H2O, 0.2; KCL, 0.15; $CaCl_2$.2H2O, 0.1; $FeSO_4.6H_2O$, 0.001; $ZnSO_4.7H_2O$, 0.001; $MnSO_4$, 0.001) supplemented with 0.1% (w/v) polyethylene as a sole carbon source. The pH of

the medium was adjusted to 7.2 and fresh 18 -24 h old culture was inoculated and incubated on a shaker incubator (150rpm) at various temperatures (25, 30, and 37^{o}C) for 10 days. The incubation period is extended to 10 days because the bacterial isolates take longer time to grow in a medium with polyethylene as a carbon source. After completion of the incubation period, OD_{600nm} and Biomass (g/l) were recorded in triplicates (Sivan *et al.,* 2006).

3.8.2. Optimization for pH

The effects of pH value on the growth of Polyethylene Degrading Isolate (PDI) were evaluated using MSM that supplemented with polyethylene (0.1%, w/v) as a main carbon source at various pH values (5.5, 7.2, and 8.5). The cultures were incubated at 30^{o}C on a shaker incubator (150rpm) for 10 days. After completion of the incubation period, OD_{600nm} and Biomass (g/l) were measured in triplicates (Al-Jailawi *et al.,* 2015).

3.8.3. Optimization of substrate concentration

In order to determine the optimum concentration of polyethylene films, each isolate capable of degrading polyethylene was grown in MSM containing different polyethylene concentrations (0.05, 0.1, and 0.5%). PH of the medium was adjusted to 7.1 and was incubated at 30^{o}C on a shaker incubator (150rpm) for 10 days. After completion of the incubation period, OD_{600nm} and Biomass (g/l) were measured in triplicates (Al-Jailawi *et al.,* 2015).

3.9. Biodegradation Assay

The biodegradation assay was performed with samples of untreated polyethylene films (2×2 cm) that were weighed, disinfected (30 min in 70% ethanol) (v/v), and dried over night at 60^{o}C for degradation assay without any pretreatment. For pretreated assay, the polyethylene films were exposed to UV radiation for two weeks under a UV lamp (245nm) in a hood followed by disinfection with 70% (v/v) ethanol and overnight drying. These polyethylene samples were then added to flasks each containing 50ml of MSM. Each flask was inoculated with 2ml of freshly grown culture and was incubated at 30° c for 1 month. After incubation period was completed, the polyethylene samples were removed from the medium and washed off with 2% (v/v) of aqueous sodium dodecyl sulfate (SDS) solution for 4h at 60^{o}C to remove the microbial

biofilm colonizing the polyethylene surface this was further washed with distilled water and then dried overnight at 60^{o}C. The final weight loss was calculated using the formula:

Percentage of weight loss = [(FW - IW)/IW] × 100 where FW and IW are Final Weight and Initial Weight respectively (Sivan *et al.,* 2006; Sangeetha *et al.,* 2015).

3.10. Scanning electron microscopy (SEM) of polyethylene

The polyethylene films removed from the culture medium were observed for surface erosion. Polyethylene samples were washed with a 2% SDS followed by warm distilled water to completely remove surface-adhered cells. Then the samples were fixed in 2% glutaraldehyde for 2 h, washed twice (30 min each) in 50% ethanol, incubated overnight in 70% (w/v) ethanol, and finally washed again (30 min 3) in 100% ethanol. After fixation, the samples were dried in a vacuum, coated with gold and scanned in a LEO1430 VP SEM (Harshvardhan & Jha, 2013).

3.11. Statistical analysis

All the experiments were performed in triplicates. Statistical analysis was performed using Origin Pro 8.0 software and one-way analysis of ANOVA (SPSS version 20.0). The data obtained was expressed as mean ± standard deviation (SD) and the differences was considered to be significant at $p<0.05$.

CHAPTER FOUR

4. RESULTS

4.1. Isolation and screening of polyethylene degrading bacteria

In the screening for bacteria capable of growing on a carbon-free basal Medium supplemented with polyethylene, 52 bacterial colonies were shown to utilize polyethylene as a sole carbon source, as was evident from their growth on the medium with no additional carbon supply (Figure 2). From these pure colonies were isolates and labeled as Polyethylene Degrading Isolate (PDI).

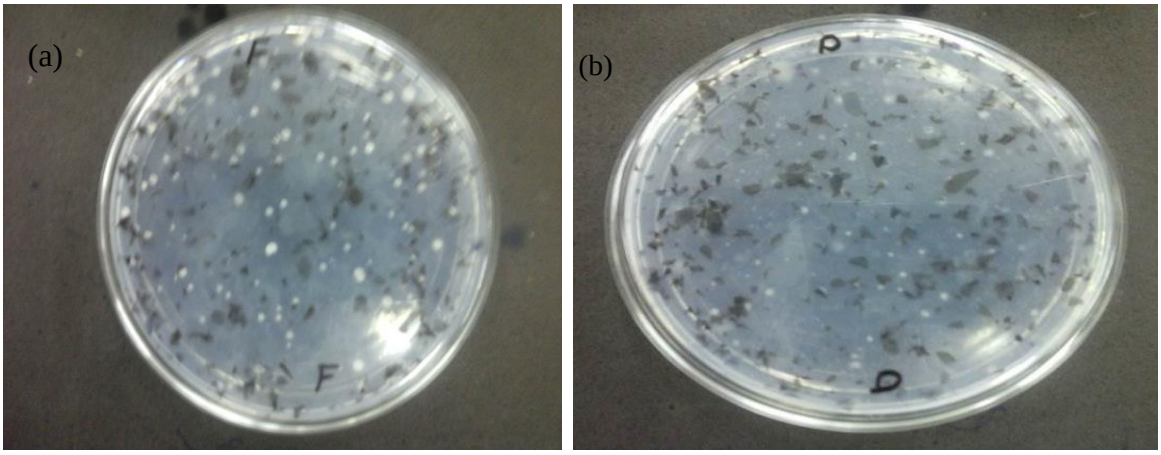

Figure 2: Screening for bacterial isolates which have polyethylene degrading ability. Both (a) and (b) show polyethylene degrading isolates grown on carbon free basal medium supplemented with polyethylene.

4.2. Identification and Characterization of polyethylene degrading bacterial isolates

In the present study, out of 52 bacterial isolates, 30 (57.69%) of them were found to be Gram positive and 22 (42.30%) were Gram negative. In this study, Gram negative (Figure 3a) and Gram positive (Figure 3b) PDIs were obtained from soil samples containing polyethylene collected from dumpsite.

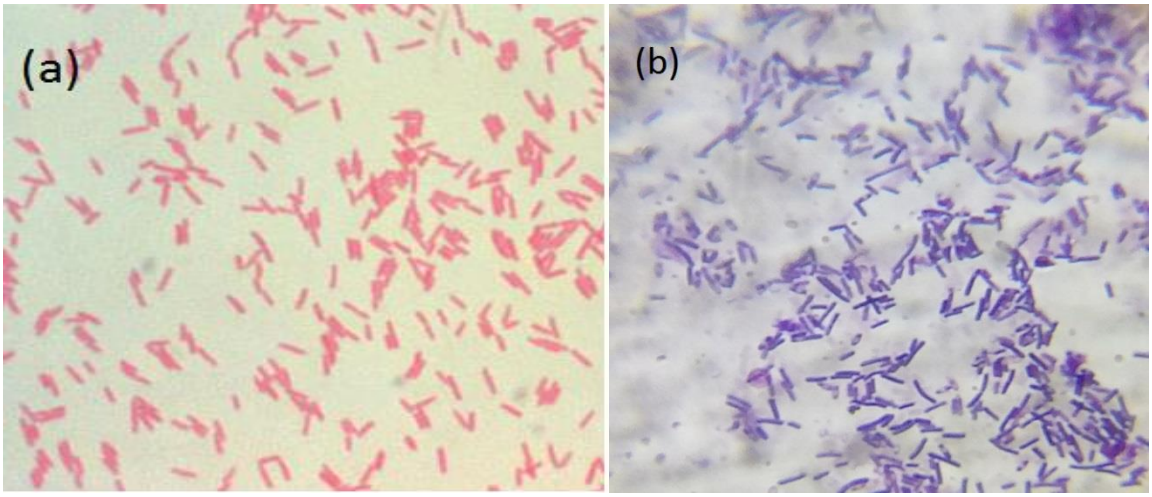

Figure 3: Gram's staining of isolated polyethylene degrading bacteria viewed under 100 x magnifications. (a) A Gram-negative rod shaped PDI and (b) a Gram-positive rod shaped PDI isolated from waste disposal site

Out of the 52 isolates, 25 bacterial isolates were identified by MALDI-TOF MS. The majority of the isolates (72%) were found to be of *Bacillus* spp. In addition to *Bacillus* spp. the other isolates were identified as *Pseudomonas* sp. and *Acinetobacter* sp. (Table 2). From the identified 25 bacterial species, *Bacillus cereus* takes the top spot with 15 of the isolates, 3 isolates were *Bacillus subtilis*, 2 isolates were *Pseudomonas aeruginosa*, 2 isolates were *Pseudomonas balearica*, and finally 1 species of *Acinetobacter kookii* were obtained from MALDI-TOF analysis. Two bacterial isolates however were not able to be identified by MALDI-TOF analysis.

Table 2: MALDI-TOF MS results for Polyethylene degrading isolates

S. N	Name of PDIs	Species	Score value	S. N	Name of PDIs	Species	Score value
1	PDI-1	*P. aeruginosa*	2.34	14	PDI 27	*B. subtilis*	2.18
2	PDI-2	*P. aeruginosa*	2.39	15	PDI 29	*B. cereus*	2.32
3	PDI-3	*B. cereus*	2.32	16	PDI-30	*B. cereus*	2.26
4	PDI-6	*B. cereus*	2.10	17	PDI-31	*B. cereus*	2.08
5	PDI-8	*B. cereus*	2.08	18	PDI-33	*B. subtilis*	2.23
6	PDI-13	*B. cereus*	1.91	19	PDI-34	*B. cereus*	2.29
7	PDI-14	*B. cereus*	2.17	20	PDI-36	*B. cereus*	2.33
8	PDI-17	*P. balearica*	2.20	21	PDI-39	*P. balearica*	1.77
9	PDI-20	Not identified	1.41	22	PDI-42	*Acinetobacter kookii*	1.98
10	PDI-21	Not identified	1.60	23	PDI-46	*B. cereus*	2.29
11	PDI-24	*B. cereus*	2.16	24	PDI-48	*B. cereus*	2.11

12	PDI-25	*B. subtilis*	2.13	25	PDI-50	*B. cereus*	1.95
13	PDI-26	*B. cereus*	2.28				

Key: score of ≥2.0 indicates High confidence identification to the species level,

1.70-1.99 - Low confidence identification, and

0.00-1.69 - No organism identification possible

Biochemical tests were carried out on the PDI that wasn't identified by MALDI-TOF MS, tests such as catalase test, indole test, methyl red test, and voges-proskaure tests were employed and the results were analyzed to give expected bacterial genus (Table 3).

Table 3: Characteristics of MALDI-TOF unidentified PDI

NO	Characteristics	Results
1	Gram's staining	+
2	Catalase test	+
3	Citrate utilization test	+
4	Indole test	-
5	Urease test	+
6	Methyl red test	-
7	Voges-proskaure test	+
8	Motility	-
9	H_2S utilization	-
Expected isolate species		*Bacillus sp.*

Key: + indicates positive result for a given biochemical test whereas – indicates negative result

4.3. Molecular characterization for identification of newly obtained Polyethylene degrading bacterial isolates

The presence of catabolic genes encoding alkane hydroxylase *alk B* genes in the isolated strains was determined by PCR using specific primers for this functional gene. The results of the PCR (Figure 4) revealed that strains *P. aeruginosa* PDI-1, *P. aeruginosa* PDI-2, *P. balearica* PDI-17, PDI-11, and *P. balearica* PDI-39 are positive for *alk B* gene amplification with ~ 330 bp fragment. In this study certain PDIs were shown negative for *alk B* genes amplifications. These

isolates were including such as *B. cereus* PDI-8, *B. subtilis* PDI-27, and *Acinetobacter kookii* PDI-42.

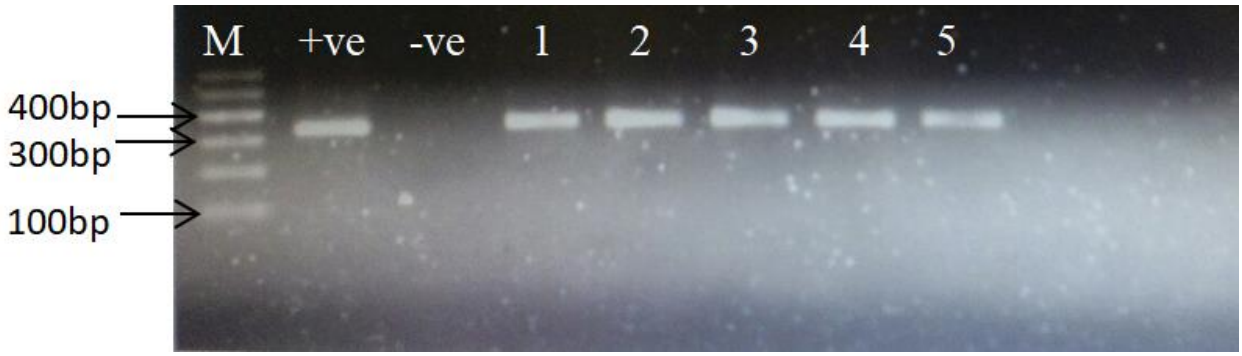

Figure 4: PCR Amplification of *alk B* alkane hydroxylase genes. In this Figure M indicates DNA Marker with 100bp size, +ve indicates a known pseudomonas sample as positive control, -ve indicates water as negative Control, Lane 1, 2, and 3 indicates *alk B* alkane hydroxylase genes amplicons *P. aeruginosa* PDI-1, *P. aeruginosa* PDI-2, and PDI 11 respectively. However, lane 4 and 5 indicates *alk B* alkane hydroxylase genes amplicons *P. balearica* PDI-17, and *P. balearica* PDI-39, respectively.

4.4. 16S rRNA sequencing and phylogenetic tree construction for Polyethylene degrading isolate

In the present study, 16SrRNA sequencing was performed for one of the bacterial species that was not identified by MALDI-TOF MS and found to be *Bacillus* sp. PDI-21 (Appendix 12). This isolate was shown closest sequence similar with the Bacillus *Rossellomorea oryzaecorticis* R1^T KF548480, and *Bacillus manusensis* Ma50-6^T MF582328 in which it is 91.88% similarity with 53.20 GC% and 82.03% similarity with 53.74 GC% respectively. The recent PE degrading isolate also close ally with *B. paramycoides* NH24A2^T MAOI01000012, *B. toyonensis* BCT-7112^T (CP006863), and *B. mobilis* 0711P9-1^T (MACF01000036) with 80.67% sequence similarity (Figure 5, Table 4). As indicated in the Table 4, the same genus that shared the lowest sequence similarity (80.49%) was predicted to be *Neobacillus drentensis* LMG 21831^T AJ542506. *Bacillus* sp. PDI-21 also shown sequence similarity with certain none *Bacillus* strains such as *Metabacillus sediminilitoris* DSL-17^T MN067806, *Cytobacillus oceanisediminis* H2^T (GQ292772), *Peribacillus huizhouensis* GSS03^T (KJ464756) and others as illustrated in Figure5.

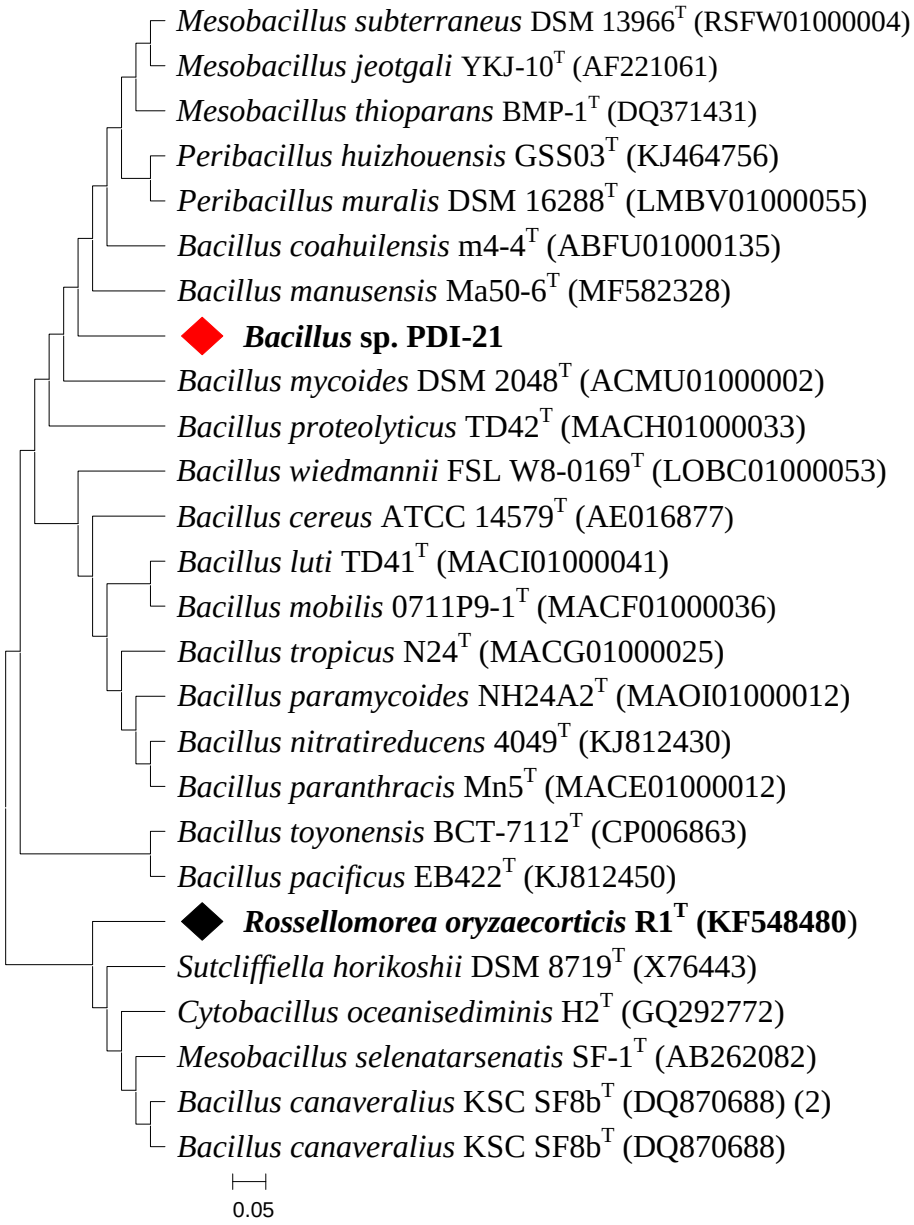

Figure 5: Evolutionary analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale bar (0.05 substitutions per nucleotide position), with branch lengths measured in the number of substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. Evolutionary analyses were conducted in MEGA 11 (Tamura *et al.*, 2021).

Table 4: Sequence similarity between our isolate and other strains with their respective GC% and accession numbers

S.N.	Sequence	Strain	Accession number	Total length (bp)	GC%	Sequence similarity with newly isolates strain (%)
1	*Bacillus sp.*	PDI-21	Not given	1498	53.20	Current isolate
2	*Rossellomorea oryzaecorticis*	R1 ^{T}	KF548480	1109	53.74	91.88
3	*Bacillus manusensis*	Ma50-6 ^{T}	MF582328	1426	56.31	82.03
4	*B. mycoides*	DSM 2048 ^{T}	ACMU01000002	1622	52.10	81.60
5	*Peribacillus huizhouensis*	GSS03 ^{T}	KJ464756	1460	54.73	81.52
6	*Mesobacillus selenatarsenatis*	SF-1	AB262082	1413	55.77	81.37
7	*B. canaveralius*	KSC SF8b ^{T}	DQ870688	1323	55.33	80.97
8	*Mesobacillus boroniphilus*	JCM 21738 ^{T}	BAUW01000204	1572	55.53	80.94
9	*Cytobacillus oceanisediminis*	H2 ^{T}	GQ292772	1393	54.99	80.87
10	*B. pacificus*	EB422 ^{T}	KJ812450	1509	53.48	80.75
11	*Sutcliffiella horikoshii*	DSM 8719 ^{T}	X76443	1529	54.91	80.72
12	*Cytobacillus purgationiresistens*	DS22 ^{T}	FR666703	1450	53.86	80.71
13	*B. paramycoides*	NH24A2 ^{T}	MAOI01000012	1710	51.29	80.67
14	*B. paranthracis*	Mn5 ^{T}	MACE01000012	1588	52.52	80.67
15	*B. toyonensis*	BCT-7112 ^{T}	CP006863			80.67
16	*B. mobilis*	0711P9-1 ^{T}	MACF01000036	1680	51.85	80.67
17	*Bacillus nitratireducens*	4049 ^{T}	KJ812430	1509	53.41	80.67
18	*B. cereus*	ATCC 14579 ^{T}	AE016877	1474	53.54	80.58
19	*B. wiedmannii*	FSL W8-0169	LOBC01000053	1474	55.53	80.50
20	*B. pseudomycoides*	DSM 12442 ^{T}	ACMX01000133	1657	52.32	80.50
21	*Bacillus luti*	TD41 ^{T}	MACI01000041	1664	51.86	80.50
22	*B. albus*	N35-10-2 ^{T}	MAOE01000087	1630	52.33	80.50
23	*Neobacillus drentensis*	LMG 21831 ^{T}	AJ542506	1438	55.42	80.49

4.5. Detection of enzymes responsible for biodegradation

Production of enzymes such as amylase, esterase, protease, and pectinase were detected and might be indirectly contributed for polyethylene biodegradation. The secretion and release of

these and other extracellular enzymes from the newly isolated bacterial cells are crucial in their ability to act on polyethylene materials.

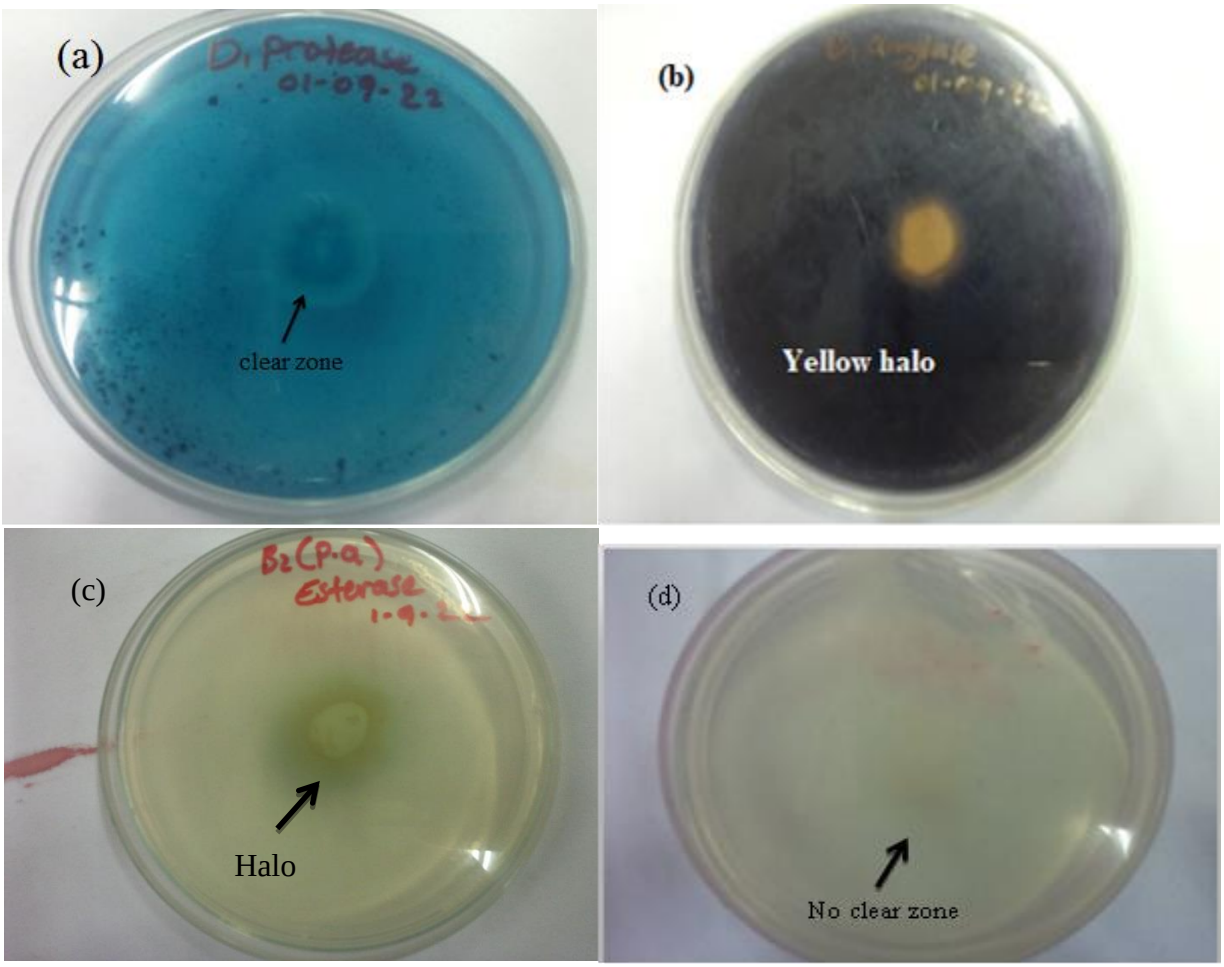

Figure 6: Enzyme activity of isolated bacterial species obtained from waste dump site. (a) Protease producing PDI-21. (b) Amylase producing isolates *Bacillus* sp. PDI-21. (c) Esterase producing ability of isolate PDI-1. (d) Negative pectinase production of isolate *P. aeruginosa* PDI-1.

Skimmed milk agar plate assays allow for qualitative determinations of protease activity. With the aid of the added Bromocresol green, clear zone formed by the bacterial species were easily visualized (Figure 6a). All isolates *P. aeruginosa* PDI-1, *P. balearica* PDI-17, and *Bacillus* sp. PDI-21 were able to produce protease.

Amylase activity was determined on starch agar. *P. aeruginosa* PDI-1 and *P. balearica* PDI-17 gave a negative result whereas *Bacillus* sp. PDI-21 was positive for amylase production (Figure

6b). Esterase activity of the isolates was tested on a medium containing Tween 80 supplements. *P. aeruginosa* PDI-1 showed a halo around the colony indicative of esterase activity (Figure 6c). But on the rest of the isolates, *P. balearica* PDI-17 and *Bacillus* sp. PDI-21, no halo was visible indicating no esterase activity. For pectinase test formation of a clear zone around the colonies indicates presence of pectinase however in this study none of the isolates showed a positive result (Figure 6d).

4.6. Optimization for growth parameters

Three of the newly isolated polyethylene degrading bacteria (i.e. *P. aeroginosa* PDI-1; *P. balearica* PDI-17, and *Bacillus sp.* PDI-21) were used in growth optimization parameters. These bacterial isolates were chosen because of their higher degradation capacity relative to the other isolates. The effect of Temperature, pH, and polyethylene concentration were tested and the result was presented as Mean ± SD of Biomass or Cell Dry Weight (CDW) (g/l) and Optical Density (OD_{600nm}) measurements.

4.6.1. Optimization for pH

In this study, various CDW and OD_{600nm} were obtained from newly isolated PDIs at various pH (5.5, 7.2, and 8.5). As illustrated in the Figure 7a, the minimum and maximum CDW (g/L) were recorded for *P. aeruginosa* PDI-1 (0.00005±0.00002g/L) and *P. balearica* PDI-17 (0.000353±0.000057g/L) at pH 5.5 & pH 7.2, respectively. *P. aeruginosa* PDI-1 also gave higher CDW (g/L) and OD_{600nm} at pH 8.5 with 0.0002±0.000064g/L and 0.077±0.002, respectively (Figure 7a). Considerable amount of CDW (0.000197±0.000057g/L) and OD_{600nm} (0.071±0.001) (Figure 7a) were recorded for *Bacillus sp.* PDI-21 at pH7.2. However, the minimum CDW (0.00010±0.000015g/L) and OD_{600nm} (0.060±0.00115) were recorded for the same isolate at pH 8.5 (Figure 7a, Supplementary file: Appendix 4). As it was demonstrated in the Figure 7a, OneWay Anova test at LSD test shown that significant variations were observed between *Bacillus sp.* PDI-21 & *P. aeruginosa* PDI-1 (*P=0.018*) at pH 5.5, among *Bacillus sp.* PDI-21, *P. aeruginosa* PDI-1, and *P. balearica* PDI-17 (*P=0.000*) at pH 7.2, between *Bacillus sp.* PDI-21 & *P. aeruginosa* PDI-1 (*P=0.017*) at pH8.5, and between *Bacillus sp.* PDI-21 & *P. balearica* PDI-17 (*P=0.036*) against CDW (g/L). However, no variations (*P>0.05*) were observed among

Bacillus sp. PDI-21, *P. aeruginosa* PDI-1 and *P. balearica* PDI-17 interms of CDW (g/L) as illustrated in Multiple Comparisons of Post Hoc Tests at LSD (Supplementary file: Appendix 4)

4.6.2. Optimization for Temperature

The temperature of MSM was adjusted to 25, 30, and 37^{o}C for temperature optimization. The PDI *P. aeroginosa* PDI-1; *P. balearica* PDI-17, and *Bacillus sp.* PDI-21 showed various CDW and OD_{600nm} under a given different temperatures. The minimum CDW (g/L) was recorded for PDI-21 isolates (0.000093±0.0000115) with OD_{600nm} (0.0623±0.00153) at 25^{o}C (Figure 7). However, the maximum CDW (0.00015±0.0000252g/L) was recorded for PDI-17 with considerable amount of OD_{600nm} (0.0883±0.00416) at 37^{o}C (Figure 7b, supplementary file: Appendix 3). Variations were observed ($p \leq 0.05$) among PDI 1, PDI 17, and PDI 21 were indicated by different letters as in figure 7b and no variation ($p > 0.05$) at 95% confidence interval was indicated by the same letters.

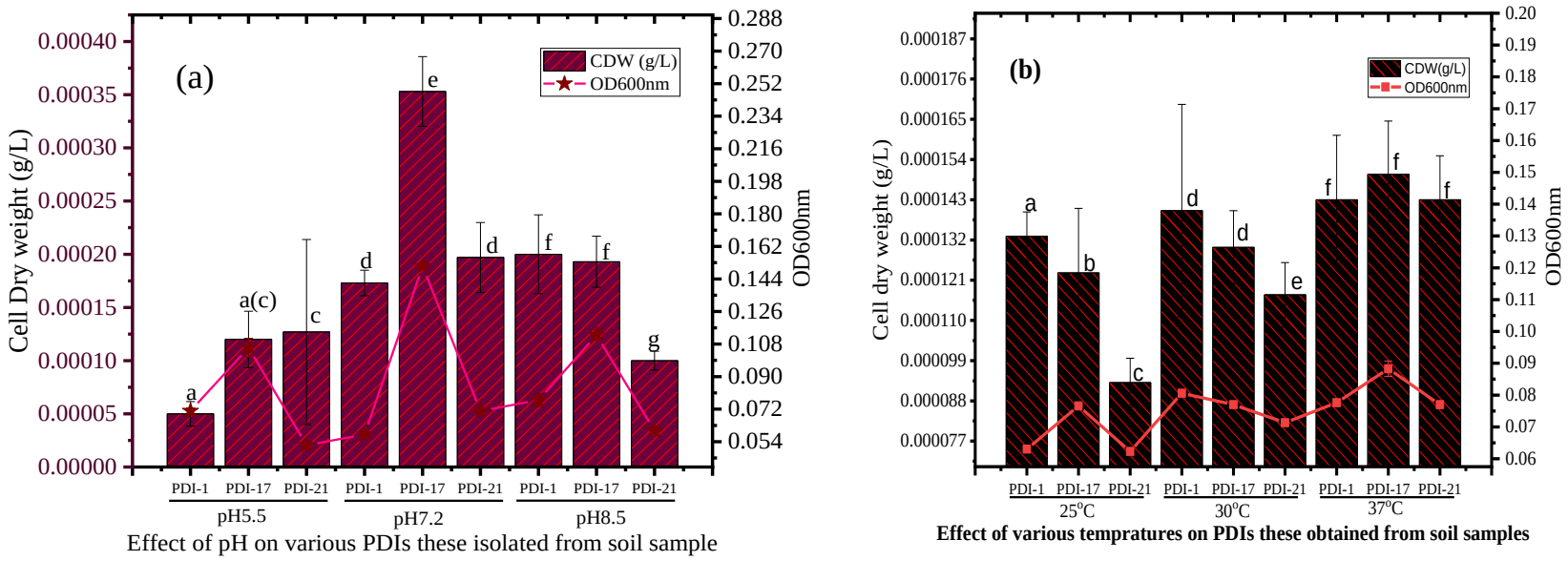

Figure 7: Effect of pH and Temperatures on PDIs. (a) CDW (g/L) and OD_{600nm} obtained from certain isolated PDIs at various pH values. (b) Effect of temperatures against certain PDIs these obtained from area where conventional plastic (polyethylene) available. Data represented mean ± SEM of three replicate (n = 3) of CDW (g/L) against pH and temperature. According to one way ANOVA, the CDW (g/L) has no significant variation at 95% confidence interval by using LSD test (P> 0.05) for these isolates during growth on Mineral Salt Medium (MSM) with the same letter on their respective bars among the same pH and temperature value.

4.6.3. Optimization for Concentration of polyethylene

Certain polyethylene concentrations (0.5, 0.1, and 0.05%) were evaluated and found to be employed as a main carbon source for microbial growth. The minimum CDW (0.00009±0.00002g/L) was obtained from *Bacillus sp.* PDI-21 when polyethylene 0.1% (w/v) (Figure 8) was provided in the salt medium. However, the highest CDW (g/L) (0.001±0.00008g/L) was recorded for *P. aeruginosa* PDI-1 when the salt medium was supplied with 0.5% (w/v) of polyethylene concentrations (Figure 8) with the highest OD_{600nm} (0.995± 0.005). Higher amount of CDW (0.00039±0.000083g/L) was also obtained from *P. balearica* PDI-17 at 0.5% (w/v) polyethylene as a main carbon source with higher (0.208± 0.00057) OD_{600nm} as indicated in Figure 8. It was also noted that as the concentration of polyethylene increased, the CDW (g/L) and OD_{600nm} increased with longer incubation. As shows in the Figure 8, the highest significant variation (*P=0.000*) was observed between *Pseudomonas aeruginosa* PDI-1 and other isolates at 0.5% (w/v) polyethylene concentration. However, no significant variations were observed among certain PDIs with the same letter at LSD test using One Way Anova at post hoc test with multiple comparisons (Figure 8, supplementary file: Appendix 5).

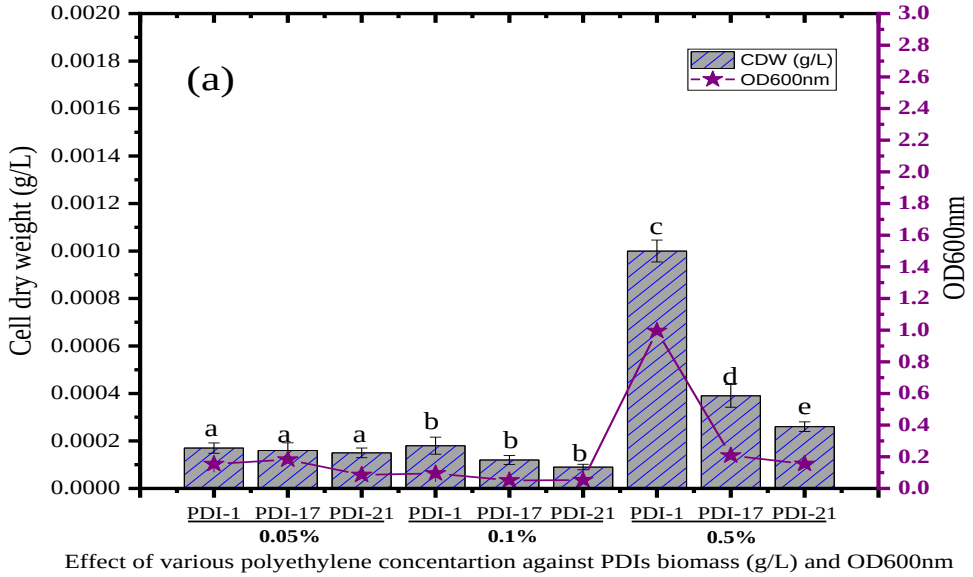

Figure 8: Effect of various Polyethylene concentrations against certain PDIs biomasses (g/L) and their OD_{600nm}. Data represented as mean ± SEM of three replicate (n = 3) of

CDW (g/L) against various concentration of polyethylene. According to one way ANOVA, the CDW (g/L) has no significant variation at 95% confidence interval by using LSD test (P> 0.05) for these isolates during growth on MSM supplemented with various concentration of polyethylene with the same letter on their respective bars among the same polyethylene concentration.

4.7. Biodegradation assay

All the bacterial isolates were tested for polyethylene degradation ability. After the completion of the 1-month incubation period with and without any pretreatment, weight losses of polyethylene were calculated and presented as weight loss ± SD. Among the 52 PDIs, 5 bacterial isolates displayed higher weight loss of the polyethylene films without any pretreatment and there was no weight loss observed in the control group. The highest percentage of weight loss was observed in *Pseudomonas balearica* PDI-17 (4.25±0.000053%), followed by *Bacillus* sp. PDI-21 (1.96 ± 0.0000346%) and *Pseudomonas aeruginosa* PDI-1 (1.93 ± 0.000035%). Also, other bacterial isolates such as *Bacillus subtilis* PDI-33 and *Bacillus* sp. PDI-27 showed polyethylene degradation although lower than the previous once with 1.53 ± 0.000051% and 1.14 ± 0.000056% respectively as shown in Figure 9 (Supplementary file: Appendix 1).

In this study the highest weight reduction without any pretreatment was 4.25±0.000053% but with the action of UV radiation the rate of degradation was significantly increased. After 2 weeks UV radiation combined with 1 month incubation period, the highest percentage of weight loss was *Pseudomonas balearica* PDI-17 (19.9 ± 0.00039%) followed by (*Pseudomonas aeruginosa* PDI-1 (17.96 ± 0.00050%), *Bacillus subtilis* PDI-33- (13.75 ± 0.00025%), and *Bacillus* sp. PDI 21 (8.63 ± 0.00037%). PDI 42 (*Acinetobacter kookii)* was able to give a weight loss of 6.77 ± 0.00046%. Also, the *Bacillus* sp. PDI-27 degraded 4.78 ± 0.00007% of the polyethylene sheet (Figure 9) (Supplementary file: Appendix 2).

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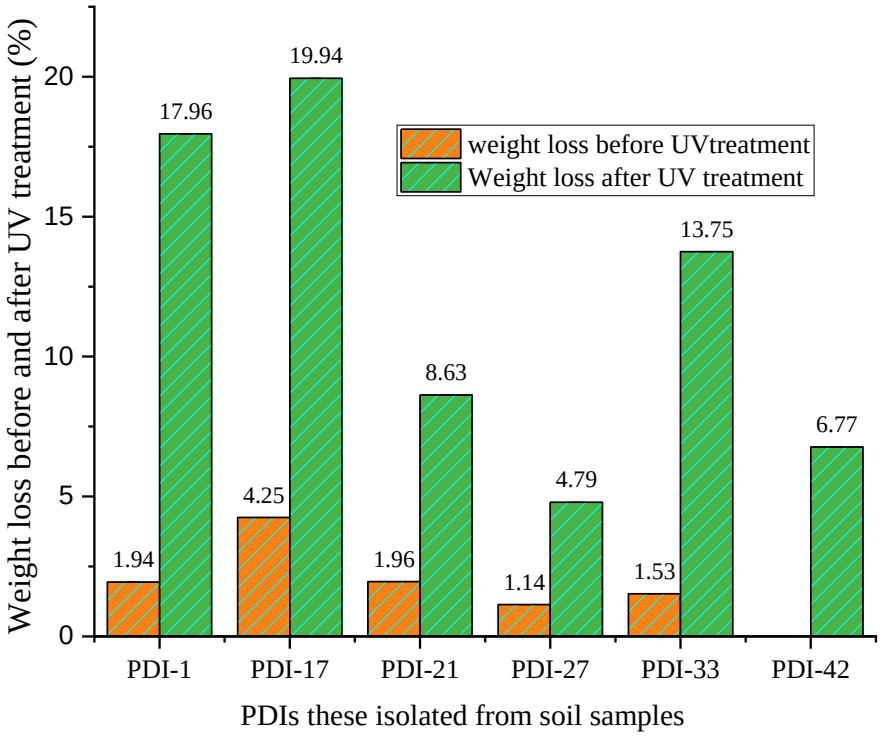

Figure 9: Polyethylene weight loss due to certain PDIs before and after polyethylene was treated with UV radiation.

4.8. Scanning electron microscopy of polyethylene

SEM analysis examines changes in surface morphology due to biodegradation. After 30 days of incubation without pretreatment the surface of polyethylene colonized by *Pseudomonas* sp. (Figure 10c) and *Bacillus* Species (Figure 10b) has cracks and distortions whereas the polyethylene film that hasn't been incubated and the control group (Figure 10d) show no signs of distortions on the polyethylene film surface.

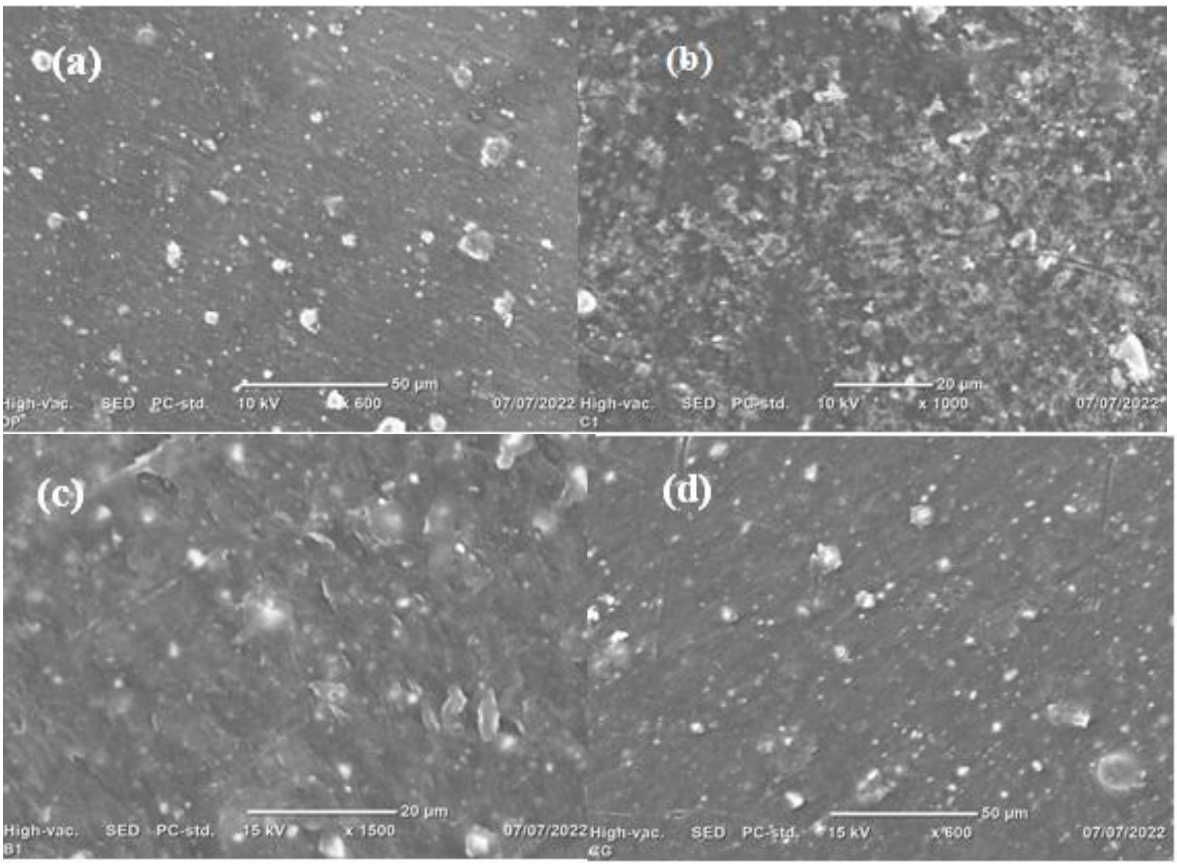

Figure 10: Scanning electron microscopy of polyethylene films. With (a) indicating Polyethylene film without incubation, (b) Polyethylene film after 1 month incubation with *Bacillus sp.,* (c) Polyethylene film after 1 month incubation with *Pseudomonas sp*., and (d) polyethylene film after 1 month incubation without any bacterial isolates.

CHAPTER FIVE

5. DISCUSSION

This study assessed the ability of bacterial isolates from dump site in the degradation of polyethylene. Results of screening shows that dump sites are good source of polyethylene degrading organism. Based on the morphological, biochemical, and MALDI-TOF characterization carried out, these results reveal that most bacterial isolates capable of degrading polyethylene belong to the genus *Bacillus* and *Pseudomonas sp.* This indicates that *Bacillus* and *Pseudomonas* are rich in dumpsites and have the capacity for polyethylene degradation. However, a couple of isolates were not successfully identified by MALDI-TOF technique. Until recent times 16S rRNA and 18S rRNA gene sequencing were the best ways to identify a certain microorganism but now MALDI-TOF MS is becoming the go to tool for microbial identification (Singhal *et al.,* 2015). These isolates could not be identified either because of impurity problems or one of the limitations of this technology which is isolates can't be identified unless specific peptide mass fingerprints of species or strain is found in the data base. *Bacillus* and *Pseudomonas sp.* have the ability to provide the necessary requirements for breaking down and utilizing carbon and energy in the polyethylene. Similarly (Sangeetha *et al.,* 2015) also states that *Bacillus* and *Pseudomonas* species are highly efficient in degrading polyethylene. (Balasubramanian *et al.,* 2010) demonstrate that *Arthrobacter sp*. and *Pseudomonas sp*. isolated from plastic waste dumpsites were capable of utilizing HDPE as a sole carbon source.

The *alk B* genes that code for production of hydrolase enzymes are responsible for alkane degradation. In the present study the gene was amplified in 4 bacterial samples. *alk B* and *alkB* related genes code for an alkane-degrading enzyme, alkane hydroxylase. The analysis of the bacterial samples revealed presence of *alk B* 1 gene in 4 of the bacterial samples. Bacterial isolates that were positive for *alkB* 1 gene were *Pseudomonas aeruginosa* and *Pseudomonas balearica* species. The rest of the PDIs were found to be negative. Non-*alk B* possessing strains can either use different enzymatic systems for alkane degradation or require a more specific gene but either way this study has shown that they do have degradative capacities. Multiple genes of *alkB* with significant sequence divergence have been described for strains of *Acinetobacter* and

Pseudomonas. (Kloos *et al.,* 2006) states that the encoded enzymes can vary in their specificity and are differentially regulated by substrate availability and suggests the use of combined PCR/hybridization method for the detection of *alkB* without an obvious specificity for any bacterial group. Alkane biodegradation is initiated through terminal oxidation to the corresponding primary alcohol, which is further oxidized by dehydrogenases to fatty acids which can enter the TCA cycle. This is an indication of the genetic ability of the microorganisms to degrade long-chain alkanes through production of this enzyme (Van Beilen *et al.,* 2003). In a similar study by (Muhonja *et al.,* 2018) *Brevibacillus borstelensis, Pseudomonas putida*, and *Bacillus cereus* were found to be positive for the presence of the *alk B* gene.

One of the PDI that was capable of polyethylene degradation but was not identified by MALDI TOF MS was subjected to 16S ribosomal RNA (rRNA) gene sequencing and was predicted to be *Bacillus* sp. PDI-21. This strain has been shown sequence similarity with *Bacillus* and other bacterial strains (91.88 to 80.49%) (Figure 5, Table 4). Similarly, it was demonstrated that (Skariyachan *et al.,* 2018) strains such as IS1, IS3, ISA and ISC which contributed for plastic degradation shown identity with *Aneurinibacillus aneurinilyticus* (78%), *Brevibacillus* sp. DB-3 (87%), *Brevibacillus* sp. HL-2 (91%), and *Brevibacillus brevis* NBRC 100599(94%), respectively. The present result for 16SrRNA gene sequence reveals that the highest sequence similar has been observed between *Rossellomorea oryzaecorticis* $R1^T$ KF548480 and *Bacillus* sp. PDI-21 (91.88%) which is less than 98.00% sequence similarity. This indicated that the present our isolate could be new species and contributed for microbial diversity with biodegradation of polyethylene features. It has been documented that (Reller *et al.*, 2007) 16S rRNA gene sequence data on an individual strain with a nearest neighbor showing a similarity score of <97% represents a new species which is a confirmation to the recent estimation of our *Bacillus* sp. PDI-21.

The optimization of temperature, pH, and concentration of polyethylene for PDIs involved in the study is crucial in attaining the maximum possible degradation capacity. The temperature of a medium is one important factor for the enzyme activity of the microorganisms present, regardless of whether the enzymes are intracellular or extracellular. Enzymes are susceptible to heat change and the level of enzyme activity often multiplies as environmental temperatures rise. Higher temperatures, after an optimum temperature has been reached, cause the enzyme to

degrade quickly and irreversibly lose activity. The result of this study indicated that a temperature of 37^{o}C was the optimum temperature required by the PDIs. Temperatures between 30 and 44 degrees Celsius are generally ideal for *P. aeruginosa* (Zhang *et al.*, 2018). (Mouafo *et al.*, 2021) stated that the degradation rates of polyethylene fragments increased with increasing incubation temperature. In his study, the highest rate value of degradation was 2.10^{-4} g/10 days, and it was recorded under 44 °C. The lowest was 8.10^{-5} g/10 days and it was recorded under 7°C and 23 °C. However a study done by (Pischedda *et al.*, 2019) indicates temperature of 23 °C seems the most favorable for the cells and that plastic biodegradation tests in the soil gave an increased result in the incubation temperature (from 15 to 28 °C).

Another major factor is the pH of the medium in which the PDIs grow in. the results indicated that a pH of 7.2 was most desirable. Similarly (Veethahavya *et al.*, 2016) indicated that many bacteria species such as *Pseudomonas fluorescens* and *P. aeruginosa* show high ability to degrade polyethylene in aquatic environment under pH 7 and that an acidic pH has a higher negative impact than an alkaline one. Bacterial consortium (*Anabena* species, *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas stutzeri, Flavobacterium* species and *Vibrio alginolytious*) also degrade plastic under slightly alkaline condition (pH 7.5). The pH7.0 was also found to be the optimum pH for maximum degradation of LDPE in a study by (Rani *et al.*, 2021). Results suggest that a polyethylene concentration of 0.5% was the optimum concentration at which the maximum CDW (g/l) and OD_{600nm} were recorded. Similar to this (Al-Jailawi *et al.*, 2015) indicated with their study on *P. putida* S3A degradation ability that 0.5% was the optimum concentration for bacterial growth and also that gradual increasing of Polyethylene concentration increases growth up until the optimum stage then higher than 0.5% showed decrease in bacterial growth.

Production of extracellular enzymes plays an important role in biodegradation. These enzymes break down the plastic polymer into smaller components that can be utilized as a source of carbon by the bacterial species (Muhonja *et al.*, 2018). *Bacillus sp*. was positive for protease and amylase activities whereas *Pseudomonas sp.* was positive for esterase and protease activity. Esterases able to catalyze hydrolysis of specific molecules containing ester group, have been frequently identified in many hydrocarbon-degrading bacteria (Martínez *et al.*, 2017). (Francis *et*

al., 2009) used amylase producing *Vibrios* to degrade LDPE-dextrin blends. Because of their enzymes, these PDIs could also degrade other plastic polymers in addition to polyethylene.

Biodegradation assay in this study was measured by weight loss of the polyethylene films. The weight loss in polyethylene film was the action of microorganisms on the surface of the polyethylene film. When the organisms utilize the polyethylene as a carbon source by breaking down carbon backbone due to extracellular enzymes, reduction in its weight caused. This reduction in weight of the polyethylene piece indicates that it has been utilized by the organism. The weight loss without pretreatment reported in this study was higher than reported by (Albertsson *et al.,* 1995) which documented the degradation of thermooxidized LDPE only after 3.5 years of incubation with *Arthrobacter paraffineus* but showed a lower degradation result than (Sangeetha *et al.,* 2015) which reported a maximum of 40.39 ± 1.94%. (Kyaw *et al.,* 2012) conducted a 120 days degradation assay and the percentage of weight reduction was 20% in *Pseudomonas aeruginosa* (PAO1), 11% in *Pseudomonas aeruginosa* (ATCC) strain, 9% in *Pseudomonas putida,* and 11.3% in *Pseudomonas syringae* strain. Another LDPE degradation study was conducted by (Biki *et al.,* 2021) and the results demonstrated that *Ralstonia* sp. strain SKM2 and Bacillus sp. strain were capable of 39.2% and 18.9% degradation respectively in 180 days.

The biodegradation assay with UV pretreatment gave a 19.9% maximum weight loss; this suggests that UV pretreatment enhances degradation capability. Results in this study were higher than once reported by (Mukherjee *et al.,* 2018) with a Weight-loss of 7.13 ± 0.05% which was observed in polyethylene samples treated with SDS in the first month, then bacterially treated with *P. fluorescens* in the second month and finally treated with bio-surfactant in the third month. Similarly (Hadad *et al.,* 2005) reported 11% loss after Incubation of UV treated polyethylene with *B. borstelensis* and the polyethylene weight loss achieved in this study was much higher. The difference in the percentage of polyethylene degradation can be attributed to the different strains of isolated bacteria and also to the expression of different polyethylene degrading genes. The results of non-pretreatment and 2 weeks UV radiation pretreatment have shown a clear distinction on the biodegradation capacity of the PDIs. UV radiation alters the physical characteristics of PE by breaking carbon and hydrogen bonds, releasing free radicals. This action aids plastic degradation and improve the biodegradability of PE by increasing the

hydrophilicity of the polymer which facilitates bacterial binding and degradation (Arkatkar *et al.*, 2010; Minh *et al.*, 2018)

SEM investigated the changes in the surface of the Polyethylene films. The control polyethylene film revealed smooth and homogeneous morphology whereas the polyethylene with PDIs revealed uneven surface. As evident in the SEM micrographs the extracellular enzymes secreted by these PDI on the surface of the polyethylene result the physical breakdown and loss of functional group of the polyethylene. These findings are consistent with (Bardají *et al.*, 2019) where surface deformation was observed on LDPE film incubated with the *Paenibacillus* sp. DK1 for 90 days. (Montazer *et al.*, 2020) observed similar alteration in surface topology for LDPE films that were treated with bacterium *Sphingobacterium moltivorum, Delftia tsuruhatensis, and Pseudomonas putida LS46.* The treated HDPE film showed rough surface with a number of cracks and grooves.

6. CONCLUSION AND RECOMMENDATION

6.1. Conclusion

Biodegradation of polyethylene is a promising eco-friendly method whereby plastic material waste is managed with minimum adverse effect to the environment. In this study 52 isolates collected from waste disposal sites were tested for polyethylene degrading ability. These isolates were identified by biochemical and MALDI-TOF MS analysis. A potentially new bacterium species was characterized using 16s rRNA and belongs to *Bacillus sp*. And it showed highest similarity with *Rossellomorea oryzaecorticis.* The biodegradation assay conducted demonstrated that *Pseudomonas, Bacillus,* and *Acinetobacter* sp. have a great capacity to remove polyethylene from the environment and their degradation ability aided by pretreatments may improve plastic pollution removal. Pretreatments such as UV radiation can potentially increase plastic degradation efficiency of isolates and the effect of this degradation process was evident in the cracks present on the surface of polyethylene films. A pH of 7.2 and temperature of 37^{o}C with 0.5% polyethylene concentration was found as optimal condition for isolates in degrading polyethylene. Overall this study showed that waste dumping sites can be a great source of polyethylene degrading bacteria.

6.2. Recommendation

- In this study degradation capacity of a microbial consortia was not studied so further investigation of the combined abilities of each individual polyethylene degrading bacteria may result in better environmental cleanup strategy.
- Further studies on the bacterial enzymes that play an important role in biodegradation and if possible, to increase production as to give microbes a better degradative ability.
- To visit a genetic engineering approach where by a novel genetically engineered microorganism with very high plastic degrading ability and very little adverse effect can be generated.

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APPENDICES

Appendix 1: Polyethylene weight loss without pre treatment

Isolates	Replication	Initial weight	Final weight	Weight loss (g)	Mean	SD	SEM	Weight loss percentage
PDI-1	R1	0.01506	0.01480	0.00026	0.0002567	3.5E-5	2.03E-5	1.93%
	R2	0.01129	0.01100	0.00029				
	R3	0.01347	0.01325	0.00022				
PDI-17	R1	0.00465	0.00452	0.00013	0.000143	1.53E-5	8.82E-6	4.25%
	R2	0.00254	0.00238	0.00016				
	R3	0.00292	0.00278	0.00014				
PDI-21	R1	0.01173	0.01155	0.00018	0.0002	3.46E-5	2.0E-5	1.96%
	R2	0.0097	0.00952	0.00018				
	R3	0.00915	0.00891	0.00024				
PDI-27	R1	0.01725	0.01700	0.00025	0.00019	5.57E-5	3.21E-5	1.14%
	R2	0.01658	0.01644	0.00014				
	R3	0.01592	0.01574	0.00018				
PDI-33	R1	0.01225	0.01199	0.00026	0.00020	5.13E-5	2.96E-5	1.53%
	R2	0.01473	0.01457	0.00016				
	R3	0.01279	0.01260	0.00019				

Appendix 2: Polyethylene weight loss with UV pretreatment

Isolates	Replications	Initial weight	Final weight	Weight loss (g)	Mean	SD	SEM	Weight loss Percentage
PDI-1	R1	0.02300	0.01850	0.0045	0.004	0.00050	0.00029	17.96%
	R2	0.01975	0.0158	0.0039				
	R3	0.0238	0.0203	0.0035				
PDI-17	R1	0.02150	0.01712	0.00438	0.0044	0.00039	0.00022	19.9%
	R2	0.02477	0.01998	0.00479				
	R3	0.01984	0.01583	0.00401				
PDI-21	R1	0.02143	0.01926	0.00217	0.0019	0.00037	0.00021	8.63%
	R2	0.02027	0.01821	0.00206				
	R3	0.02448	0.02300	0.00148				
PDI-27	R1	0.02085	0.01972	0.00113	0.00106	0.00007	4.055E-5	4.788%
	R2	0.02335	0.02230	0.00105				
	R3	0.0221	0.02111	0.00099				
PDI-33	R1	0.02460	0.02114	0.00346	0.0032	0.00025	0.00014	13.75%
	R2	0.02515	0.02219	0.00296				
	R3	0.01992	0.01676	0.00316				
PDI-42	R1	0.02183	0.01993	0.0019	0.0014	0.00046	0.00026	6.77%
	R2	0.02030	0.01930	0.001				
	R3	0.01988	0.01858	0.0013				

Appendix 3: Effect of temperature on growth of polyethylene degrading bacteria

Temp.	**Isolates**	**CDW (g/l)**						**OD_{600nm}**					
		R1	R2	R3	Mean	SD	SEM	R1	R2	R3	Mean	SD	SEM
25	PDI-1	0.00012	0.00014	0.00014	0.000133	0.0000115	6.67E-6	0.064	0.063	0.063	0.063	0.000577	0.00033
25	PDI-17	0.00009	0.00013	0.00015	0.000123	0.0000305	1.76E-5	0.077	0.077	0.076	0.0766	0.000577	0.00033
25	PDI-21	0.00008	0.00010	0.00010	0.000093	0.0000115	6.67E-6	0.064	0.061	0.062	0.0623	0.00153	0.00088
30	PDI-1	0.00015	0.00009	0.00019	0.00014	0.0000503	2.91E-5	0.080	0.081	0.081	0.0806	0.000577	0.00033
30	PDI-17	0.00011	0.00014	0.00014	0.00013	0.0000173	1.0E-5	0.077	0.076	0.078	0.077	0.001	0.00058
30	PDI-21	0.00012	0.0001	0.00013	0.000117	0.0000153	8.82E-6	0.071	0.072	0.071	0.07133	0.000577	0.00033
37	PDI-1	0.00015	0.00017	0.00011	0.000143	0.0000305	1.76E-5	0.077	0.079	0.077	0.0776	0.00115	0.00067
37	PDI-17	0.00018	0.00013	0.00015	0.00015	0.0000252	1.45E-5	0.085	0.093	0.087	0.0883	0.00416	0.0024
37	PDI-21	0.00015	0.00016	0.00012	0.000143	0.0000208	1.20E-5	0.076	0.077	0.078	0.077	0.001	0.00058

Appendix 4: Effect of pH on growth of polyethylene degrading bacteria

pH	Isolates	CDW (g/l)						OD_{600nm}					
		R1	R2	R3	Mean	SD	SEM	R1	R2	R3	Mean	SD	SEM
5.5	PDI-1	0.00005	0.00007	0.00003	0.00005	0.00002	1.15E-5	0.072	0.071	0.070	0.071	0.001	0.00058
5.5	PDI-17	0.00008	0.00017	0.00011	0.00012	0.000046	2.65E-5	0.103	0.106	0.109	0.106	0.003	0.0017
5.5	PDI-21	0.00003	0.00005	0.0003	0.000127	0.00015	8.68E-5	0.052	0.051	0.052	0.052	0.00058	0.00033
7.2	PDI-1	0.00015	0.00019	0.00018	0.000173	0.000021	1.20E-5	0.058	0.058	0.057	0.058	0.00058	0.00033
7.2	PDI-17	0.00029	0.00037	0.00040	0.000353	0.000057	3.28E-5	0.152	0.150	0.151	0.151	0.001	0.00058
7.2	PDI-21	0.00026	0.00018	0.00015	0.000197	0.000057	3.28E-5	0.072	0.070	0.071	0.071	0.001	0.00058
8.5	PDI-1	0.00015	0.00027	0.00017	0.0002	0.000064	3.71E-5	0.079	0.075	0.076	0.077	0.002	0.0012
8.5	PDI-17	0.00018	0.00024	0.00016	0.000193	0.000042	2.40E-5	0.112	0.115	0.112	0.113	0.0017	0.001
8.5	PDI-21	0.00010	0.00012	0.00009	0.00010	0.000015	8.82E-6	0.061	0.061	0.059	0.060	0.00115	0.00067

Appendix 5: Effect of concentration of polyethylene on growth of polyethylene degrading bacteria

Conc. (%)	**Isolates**	**CDW (g/l)**						**OD_{600nm}**					
		R1	R2	R3	Mean	SD	SEM	R1	R2	R3	Mean	SD	SEM
0.05	PDI 1	0.00013	0.0002	0.00019	0.00017	0.000038	2.18E-5	0.155	0.153	0.153	0.154	0.00115	0.00067
0.05	PDI 17	0.00021	0.00017	0.00010	0.00016	0.000056	3.21E-5	0.183	0.180	0.183	0.182	0.0017	0.001
0.05	PDI 21	0.00015	0.00018	0.00011	0.00015	0.000035	2.03E-5	0.085	0.086	0.088	0.086	0.0015	0.00088
0.1	PDI 1	0.00011	0.00023	0.00020	0.00018	0.000062	3.60E-5	0.096	0.095	0.095	0.095	0.00058	0.00033
0.1	PDI 17	0.00016	0.00010	0.00011	0.00012	0.000032	1.85E-5	0.053	0.050	0.051	0.051	0.0015	0.00088
0.1	PDI 21	0.00011	0.00007	0.00009	0.00009	0.00002	1.15E-5	0.055	0.053	0.051	0.053	0.002	0.0011
0.5	PDI 1	0.00113	0.00106	0.00097	0.0010	0.000080	4.63E-5	1.00	0.996	0.990	0.995	0.005	0.0029
0.5	PDI 17	0.00036	0.00032	0.00048	0.00039	0.000083	4.80E-5	0.207	0.208	0.208	0.208	0.00057	0.00033
0.5	PDI 21	0.00029	0.00026	0.00022	0.00026	0.000035	2.03E-5	0.154	0.157	0.155	0.155	0.0015	0.00088

Appendix 6: Descriptive against pH for CDW (g/L)

Descriptive									
		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
CDW	CDW at pH5.5 for PDI-2	3	.0000500	.00002000	.00001155	3E-7	.0000997	.00003	.00007
	CDW at pH5.5 for PDI-3	3	.0001333	.00003215	.00001856	.0000535	.0002132	.00011	.00017
	CDW at pH5.5 for PDI-1	3	.0001590	.00012804	.00007392	-.0001591	.0004771	.00005	.00030
	CDW at pH7.2 for PDI-2	3	.0001810	.00000854	.00000493	.0001598	.0002022	.00017	.00019
	CDW at pH7.2 for PDI-3	3	.0003743	.00002380	.00001374	.0003152	.0004335	.00035	.00040
	CDW at pH7.2 for PDI-1	3	.0001757	.00002380	.00001374	.0001165	.0002348	.00015	.00020
	CDW at pH8.5 for PDI-2	3	.0002133	.00005132	.00002963	.0000859	.0003408	.00017	.00027
	CDW at pH8.5 for PDI-3	3	.0001977	.00004020	.00002321	.0000978	.0002975	.00016	.00024
	CDW at pH8.5 for PDI-1	3	.0001033	.00001528	.00000882	.0000654	.0001413	.00009	.00012
	Total	27	.0001764	.00009618	.00001851	.0001384	.0002145	.00003	.00040
pH	CDW at pH5.5 for PDI-2	3	5.5000000	0E-8	0E-8	5.5000000	5.5000000	5.50000	5.50000
	CDW at pH5.5 for PDI-3	3	5.5000000	0E-8	0E-8	5.5000000	5.5000000	5.50000	5.50000
	CDW at pH5.5 for PDI-1	3	5.5000000	0E-8	0E-8	5.5000000	5.5000000	5.50000	5.50000
	CDW at pH7.2 for PDI-2	3	7.2000000	0E-8	0E-8	7.2000000	7.2000000	7.20000	7.20000
	CDW at pH7.2 for PDI-3	3	7.2000000	0E-8	0E-8	7.2000000	7.2000000	7.20000	7.20000
	CDW at pH7.2 for PDI-1	3	7.2000000	0E-8	0E-8	7.2000000	7.2000000	7.20000	7.20000
	CDW at pH8.5 for PDI-2	3	8.5000000	0E-8	0E-8	8.5000000	8.5000000	8.50000	8.50000
	CDW at pH8.5 for PDI-3	3	8.5000000	0E-8	0E-8	8.5000000	8.5000000	8.50000	8.50000
	CDW at pH8.5 for PDI-1	3	8.5000000	0E-8	0E-8	8.5000000	8.5000000	8.50000	8.50000
	Total	27	7.0666667	1.25176798	.24090286	6.5714837	7.5618496	5.50000	8.50000

Appendix 7: One-way Anova test against pH for CDW (g/L) at 0.05 significant variations

Multiple Comparisons							
LSD							
Dependent Variable	(I) CODING	(J) CODING	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
CDW	CDW at pH5.5 for PDI-2	CDW at pH5.5 for PDI-3	-.00008333	.00004174	.061	-.0001710	.0000043
		CDW at pH5.5 for PDI-1	-.00010900*	.00004174	.018	-.0001967	-.0000213
		CDW at pH7.2 for PDI-2	-.00013100*	.00004174	.006	-.0002187	-.0000433
		CDW at pH7.2 for PDI-3	-.00032433*	.00004174	.000	-.0004120	-.0002367
		CDW at pH7.2 for PDI-1	-.00012567*	.00004174	.008	-.0002133	-.0000380
		CDW at pH8.5 for PDI-2	-.00016333*	.00004174	.001	-.0002510	-.0000757
		CDW at pH8.5 for PDI-3	-.00014767*	.00004174	.002	-.0002353	-.0000600
		CDW at pH8.5 for PDI-1	-.00005333	.00004174	.218	-.0001410	.0000343
	CDW at pH5.5 for PDI-3	CDW at pH5.5 for PDI-2	.00008333	.00004174	.061	-.0000043	.0001710
		CDW at pH5.5 for PDI-1	-.00002567	.00004174	.546	-.0001133	.0000620
		CDW at pH7.2 for PDI-2	-.00004767	.00004174	.268	-.0001353	.0000400
		CDW at pH7.2 for PDI-3	-.00024100*	.00004174	.000	-.0003287	-.0001533
		CDW at pH7.2 for PDI-1	-.00004233	.00004174	.324	-.0001300	.0000453
		CDW at pH8.5 for PDI-2	-.00008000	.00004174	.071	-.0001677	.0000077
		CDW at pH8.5 for PDI-3	-.00006433	.00004174	.141	-.0001520	.0000233
		CDW at pH8.5 for PDI-1	.00003000	.00004174	.481	-.0000577	.0001177
	CDW at pH5.5 for PDI-1	CDW at pH5.5 for PDI-2	.00010900*	.00004174	.018	.0000213	.0001967
		CDW at pH5.5 for PDI-3	.00002567	.00004174	.546	-.0000620	.0001133
		CDW at pH7.2 for PDI-2	-.00002200	.00004174	.605	-.0001097	.0000657

		CDW at pH7.2 for PDI-3	-.00021533*	.00004174	.000	-.0003030	-.0001277
		CDW at pH7.2 for PDI-1	-.00001667	.00004174	.694	-.0001043	.0000710
		CDW at pH8.5 for PDI-2	-.00005433	.00004174	.209	-.0001420	.0000333
		CDW at pH8.5 for PDI-3	-.00003867	.00004174	.366	-.0001263	.0000490
		CDW at pH8.5 for PDI-1	.00005567	.00004174	.199	-.0000320	.0001433
	CDW at pH7.2 for PDI-2	CDW at pH5.5 for PDI-2	.00013100*	.00004174	.006	.0000433	.0002187
		CDW at pH5.5 for PDI-3	.00004767	.00004174	.268	-.0000400	.0001353
		CDW at pH5.5 for PDI-1	.00002200	.00004174	.605	-.0000657	.0001097
		CDW at pH7.2 for PDI-3	-.00019333*	.00004174	.000	-.0002810	-.0001057
		CDW at pH7.2 for PDI-1	.00000533	.00004174	.900	-.0000823	.0000930
		CDW at pH8.5 for PDI-2	-.00003233	.00004174	.449	-.0001200	.0000553
		CDW at pH8.5 for PDI-3	-.00001667	.00004174	.694	-.0001043	.0000710
		CDW at pH8.5 for PDI-1	.00007767	.00004174	.079	-.0000100	.0001653
	CDW at pH7.2 for PDI-3	CDW at pH5.5 for PDI-2	.00032433*	.00004174	.000	.0002367	.0004120
		CDW at pH5.5 for PDI-3	.00024100*	.00004174	.000	.0001533	.0003287
		CDW at pH5.5 for PDI-1	.00021533*	.00004174	.000	.0001277	.0003030
		CDW at pH7.2 for PDI-2	.00019333*	.00004174	.000	.0001057	.0002810
		CDW at pH7.2 for PDI-1	.00019867*	.00004174	.000	.0001110	.0002863
		CDW at pH8.5 for PDI-2	.00016100*	.00004174	.001	.0000733	.0002487
		CDW at pH8.5 for PDI-3	.00017667*	.00004174	.001	.0000890	.0002643
		CDW at pH8.5 for PDI-1	.00027100*	.00004174	.000	.0001833	.0003587
	CDW at pH7.2 for PDI-1	CDW at pH5.5 for PDI-2	.00012567*	.00004174	.008	.0000380	.0002133
		CDW at pH5.5 for PDI-3	.00004233	.00004174	.324	-.0000453	.0001300
		CDW at pH5.5 for PDI-1	.00001667	.00004174	.694	-.0000710	.0001043
		CDW at pH7.2 for PDI-2	-.00000533	.00004174	.900	-.0000930	.0000823
		CDW at pH7.2 for PDI-3	-.00019867*	.00004174	.000	-.0002863	-.0001110
		CDW at pH8.5 for PDI-2	-.00003767	.00004174	.379	-.0001253	.0000500
		CDW at pH8.5 for PDI-3	-.00002200	.00004174	.605	-.0001097	.0000657
		CDW at pH8.5 for PDI-1	.00007233	.00004174	.100	-.0000153	.0001600
	CDW at pH8.5 for PDI-2	CDW at pH5.5 for PDI-2	.00016333*	.00004174	.001	.0000757	.0002510
		CDW at pH5.5 for PDI-3	.00008000	.00004174	.071	-.0000077	.0001677
		CDW at pH5.5 for PDI-1	.00005433	.00004174	.209	-.0000333	.0001420

		CDW at pH7.2 for PDI-2	.00003233	.00004174	.449	-.0000553	.0001200
		CDW at pH7.2 for PDI-3	-.00016100*	.00004174	.001	-.0002487	-.0000733
		CDW at pH7.2 for PDI-1	.00003767	.00004174	.379	-.0000500	.0001253
		CDW at pH8.5 for PDI-3	.00001567	.00004174	.712	-.0000720	.0001033
		CDW at pH8.5 for PDI-1	.00011000*	.00004174	.017	.0000223	.0001977
	CDW at pH8.5 for PDI-3	CDW at pH5.5 for PDI-2	.00014767*	.00004174	.002	.0000600	.0002353
		CDW at pH5.5 for PDI-3	.00006433	.00004174	.141	-.0000233	.0001520
		CDW at pH5.5 for PDI-1	.00003867	.00004174	.366	-.0000490	.0001263
		CDW at pH7.2 for PDI-2	.00001667	.00004174	.694	-.0000710	.0001043
		CDW at pH7.2 for PDI-3	-.00017667*	.00004174	.001	-.0002643	-.0000890
		CDW at pH7.2 for PDI-1	.00002200	.00004174	.605	-.0000657	.0001097
		CDW at pH8.5 for PDI-2	-.00001567	.00004174	.712	-.0001033	.0000720
		CDW at pH8.5 for PDI-1	.00009433*	.00004174	.036	.0000067	.0001820
	CDW at pH8.5 for PDI-1	CDW at pH5.5 for PDI-2	.00005333	.00004174	.218	-.0000343	.0001410
		CDW at pH5.5 for PDI-3	-.00003000	.00004174	.481	-.0001177	.0000577
		CDW at pH5.5 for PDI-1	-.00005567	.00004174	.199	-.0001433	.0000320
		CDW at pH7.2 for PDI-2	-.00007767	.00004174	.079	-.0001653	.0000100
		CDW at pH7.2 for PDI-3	-.00027100*	.00004174	.000	-.0003587	-.0001833
		CDW at pH7.2 for PDI-1	-.00007233	.00004174	.100	-.0001600	.0000153
		CDW at pH8.5 for PDI-2	-.00011000*	.00004174	.017	-.0001977	-.0000223
		CDW at pH8.5 for PDI-3	-.00009433*	.00004174	.036	-.0001820	-.0000067

*. The mean difference is significant at the 0.05 level.

Appendix 8: Descriptive test against various Temperatures against CDW (g/L)

Descriptives									
		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
CDW g/l	CDW at 25oC for PDI-1	3	.000133	.0000115	.0000067	.000105	.000162	.0001	.0001
	CDW at 25oC for PDI-17	3	.000123	.0000306	.0000176	.000047	.000199	.0001	.0002
	CDW at 25oC for PDI-21	3	.000093	.0000115	.0000067	.000065	.000122	.0001	.0001
	CDW at 30oC for PDI-1	3	.000143	.0000503	.0000291	.000018	.000268	.0001	.0002
	CDW at 30oC for PDI-17	3	.000130	.0000173	.0000100	.000087	.000173	.0001	.0001
	CDW at 30oC for PDI-21	3	.000117	.0000153	.0000088	.000079	.000155	.0001	.0001
	CDW at 37oC for PDI-1	3	.000143	.0000306	.0000176	.000067	.000219	.0001	.0002
	CDW at 37oC for PDI-17	3	.000153	.0000252	.0000145	.000091	.000216	.0001	.0002
	CDW at 37oC for PDI-21	3	.000143	.0000208	.0000120	.000092	.000195	.0001	.0002
	Total	27	.000131	.0000281	.0000054	.000120	.000142	.0001	.0002
Temp	CDW at 25oC for PDI-1	3	25.0000000	0E-8	0E-8	25.0000000	25.0000000	25.00000	25.00000
	CDW at 25oC for PDI-17	3	25.0000000	0E-8	0E-8	25.0000000	25.0000000	25.00000	25.00000
	CDW at 25oC for PDI-21	3	25.0000000	0E-8	0E-8	25.0000000	25.0000000	25.00000	25.00000
	CDW at 30oC for PDI-1	3	30.0000000	0E-8	0E-8	30.0000000	30.0000000	30.00000	30.00000
	CDW at 30oC for PDI-17	3	30.0000000	0E-8	0E-8	30.0000000	30.0000000	30.00000	30.00000
	CDW at 30oC for PDI-21	3	30.0000000	0E-8	0E-8	30.0000000	30.0000000	30.00000	30.00000
	CDW at 37oC for PDI-1	3	37.0000000	0E-8	0E-8	37.0000000	37.0000000	37.00000	37.00000
	CDW at 37oC for PDI-17	3	37.0000000	0E-8	0E-8	37.0000000	37.0000000	37.00000	37.00000
	CDW at 37oC for PDI-21	3	37.0000000	0E-8	0E-8	37.0000000	37.0000000	37.00000	37.00000
	Total	27	30.6666667	5.01536102	.96520668	28.6826559	32.6506774	25.00000	37.00000

Appendix 9: One-way Anova test against various Temperatures against CDW (g/L) at 0.05 significant variations

Multiple Comparisons							
LSD							
Dependent Variable	(I) cod	(J) cod	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
CDW g/l	CDW at 25oC for PDI-1	CDW at 25oC for PDI-17	.0000100	.0000215	.648	-.000035	.000055
		CDW at 25oC for PDI-21	.0000400	.0000215	.080	-.000005	.000085
		CDW at 30oC for PDI-1	-.0000100	.0000215	.648	-.000055	.000035
		CDW at 30oC for PDI-17	.0000033	.0000215	.879	-.000042	.000049
		CDW at 30oC for PDI-21	.0000167	.0000215	.449	-.000029	.000062
		CDW at 37oC for PDI-1	-.0000100	.0000215	.648	-.000055	.000035
		CDW at 37oC for PDI-17	-.0000200	.0000215	.366	-.000065	.000025
		CDW at 37oC for PDI-21	-.0000100	.0000215	.648	-.000055	.000035
	CDW at 25oC for PDI-17	CDW at 25oC for PDI-1	-.0000100	.0000215	.648	-.000055	.000035
		CDW at 25oC for PDI-21	.0000300	.0000215	.181	-.000015	.000075
		CDW at 30oC for PDI-1	-.0000200	.0000215	.366	-.000065	.000025
		CDW at 30oC for PDI-17	-.0000067	.0000215	.761	-.000052	.000039
		CDW at 30oC for PDI-21	.0000067	.0000215	.761	-.000039	.000052
		CDW at 37oC for PDI-1	-.0000200	.0000215	.366	-.000065	.000025
		CDW at 37oC for PDI-17	-.0000300	.0000215	.181	-.000075	.000015
		CDW at 37oC for PDI-21	-.0000200	.0000215	.366	-.000065	.000025
	CDW at 25oC for PDI-21	CDW at 25oC for PDI-1	-.0000400	.0000215	.080	-.000085	.000005
		CDW at 25oC for PDI-17	-.0000300	.0000215	.181	-.000075	.000015
		CDW at 30oC for PDI-1	-.0000500*	.0000215	.032	-.000095	-.000005
		CDW at 30oC for PDI-17	-.0000367	.0000215	.106	-.000082	.000009

		CDW at 30oC for PDI-21	-.0000233	.0000215	.293	-.000069	.000022
		CDW at 37oC for PDI-1	-.0000500*	.0000215	.032	-.000095	-.000005
		CDW at 37oC for PDI-17	-.0000600*	.0000215	.012	-.000105	-.000015
		CDW at 37oC for PDI-21	-.0000500*	.0000215	.032	-.000095	-.000005
	CDW at 30oC for PDI-1	CDW at 25oC for PDI-1	.0000100	.0000215	.648	-.000035	.000055
		CDW at 25oC for PDI-17	.0000200	.0000215	.366	-.000025	.000065
		CDW at 25oC for PDI-21	.0000500*	.0000215	.032	.000005	.000095
		CDW at 30oC for PDI-17	.0000133	.0000215	.544	-.000032	.000059
		CDW at 30oC for PDI-21	.0000267	.0000215	.232	-.000019	.000072
		CDW at 37oC for PDI-1	0E-7	.0000215	1.000	-.000045	.000045
		CDW at 37oC for PDI-17	-.0000100	.0000215	.648	-.000055	.000035
		CDW at 37oC for PDI-21	0E-7	.0000215	1.000	-.000045	.000045
	CDW at 30oC for PDI-17	CDW at 25oC for PDI-1	-.0000033	.0000215	.879	-.000049	.000042
		CDW at 25oC for PDI-17	.0000067	.0000215	.761	-.000039	.000052
		CDW at 25oC for PDI-21	.0000367	.0000215	.106	-.000009	.000082
		CDW at 30oC for PDI-1	-.0000133	.0000215	.544	-.000059	.000032
		CDW at 30oC for PDI-21	.0000133	.0000215	.544	-.000032	.000059
		CDW at 37oC for PDI-1	-.0000133	.0000215	.544	-.000059	.000032
		CDW at 37oC for PDI-17	-.0000233	.0000215	.293	-.000069	.000022
		CDW at 37oC for PDI-21	-.0000133	.0000215	.544	-.000059	.000032
	CDW at 30oC for PDI-21	CDW at 25oC for PDI-1	-.0000167	.0000215	.449	-.000062	.000029
		CDW at 25oC for PDI-17	-.0000067	.0000215	.761	-.000052	.000039
		CDW at 25oC for PDI-21	.0000233	.0000215	.293	-.000022	.000069
		CDW at 30oC for PDI-1	-.0000267	.0000215	.232	-.000072	.000019
		CDW at 30oC for PDI-17	-.0000133	.0000215	.544	-.000059	.000032
		CDW at 37oC for PDI-1	-.0000267	.0000215	.232	-.000072	.000019
		CDW at 37oC for PDI-17	-.0000367	.0000215	.106	-.000082	.000009
		CDW at 37oC for PDI-21	-.0000267	.0000215	.232	-.000072	.000019

	CDW at 37oC for PDI-1	CDW at 25oC for PDI-1	.0000100	.0000215	.648	-.000035	.000055
		CDW at 25oC for PDI-17	.0000200	.0000215	.366	-.000025	.000065
		CDW at 25oC for PDI-21	.0000500*	.0000215	.032	.000005	.000095
		CDW at 30oC for PDI-1	0E-7	.0000215	1.000	-.000045	.000045
		CDW at 30oC for PDI-17	.0000133	.0000215	.544	-.000032	.000059
		CDW at 30oC for PDI-21	.0000267	.0000215	.232	-.000019	.000072
		CDW at 37oC for PDI-17	-.0000100	.0000215	.648	-.000055	.000035
		CDW at 37oC for PDI-21	0E-7	.0000215	1.000	-.000045	.000045
	CDW at 37oC for PDI-17	CDW at 25oC for PDI-1	.0000200	.0000215	.366	-.000025	.000065
		CDW at 25oC for PDI-17	.0000300	.0000215	.181	-.000015	.000075
		CDW at 25oC for PDI-21	.0000600*	.0000215	.012	.000015	.000105
		CDW at 30oC for PDI-1	.0000100	.0000215	.648	-.000035	.000055
		CDW at 30oC for PDI-17	.0000233	.0000215	.293	-.000022	.000069
		CDW at 30oC for PDI-21	.0000367	.0000215	.106	-.000009	.000082
		CDW at 37oC for PDI-1	.0000100	.0000215	.648	-.000035	.000055
		CDW at 37oC for PDI-21	.0000100	.0000215	.648	-.000035	.000055
	CDW at 37oC for PDI-21	CDW at 25oC for PDI-1	.0000100	.0000215	.648	-.000035	.000055
		CDW at 25oC for PDI-17	.0000200	.0000215	.366	-.000025	.000065
		CDW at 25oC for PDI-21	.0000500*	.0000215	.032	.000005	.000095
		CDW at 30oC for PDI-1	0E-7	.0000215	1.000	-.000045	.000045
		CDW at 30oC for PDI-17	.0000133	.0000215	.544	-.000032	.000059
		CDW at 30oC for PDI-21	.0000267	.0000215	.232	-.000019	.000072
		CDW at 37oC for PDI-1	0E-7	.0000215	1.000	-.000045	.000045
		CDW at 37oC for PDI-17	-.0000100	.0000215	.648	-.000055	.000035

*. The mean difference is significant at the 0.05 level.

Appendix 10: Descriptive test against various concentration of polyethylene

Descriptive								
CDW								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
CDW at 0.05 g/L concentration of Polyethylene for PDI-2	3	.000173	.0000379	.0000219	.000079	.000267	.0001	.0002
CDW at 0.05 g/L concentration of polyethylene for PDI-3	3	.000160	.0000557	.0000321	.000022	.000298	.0001	.0002
CDW at 0.05 g/L concentration of polyethylene for PDI-1	3	.000147	.0000351	.0000203	.000059	.000234	.0001	.0002
CDW at 0.1 g/L concentration of polyethylene for PDI-2	3	.000180	.0000624	.0000361	.000025	.000335	.0001	.0002
CDW at 0.1 g/L concentration of polyethylene for PDI-3	3	.000123	.0000321	.0000186	.000043	.000203	.0001	.0002
CDW at 0.1 g/L concentration of polyethylene for PDI-1	3	.000090	.0000200	.0000115	.000040	.000140	.0001	.0001
CDW at 0.5 g/L concentration of polyethylene for PDI-2	3	.001053	.0000802	.0000463	.000854	.001253	.0010	.0011
CDW at 0.5 g/L concentration of polyethylene for PDI-3	3	.000387	.0000833	.0000481	.000180	.000594	.0003	.0005
CDW at 0.5 g/L concentration of polyethylene for PDI-1	3	.000257	.0000351	.0000203	.000169	.000344	.0002	.0003
Total	27	.000286	.0002924	.0000563	.000170	.000401	.0001	.0011

Appendix 11: One way Anova test against various concentration of polyethylene at 0.05 confidence interval with Post Hoc Tests

Multiple Comparisons						
Dependent Variable: CDW LSD						
(I) Coding	(J) Coding	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CDW at 0.05 g/L concentration of Polyethylene for PDI-2	CDW at 0.05 g/L concentration of Polyethylene for PDI-3	.0000133	.0000436	.763	-.000078	.000105
	CDW at 0.05 g/L concentration of Polyethylene for PDI-1	.0000267	.0000436	.549	-.000065	.000118
	CDW at 0.1 g/L concentration of Polyethylene for PDI-2	-.0000067	.0000436	.880	-.000098	.000085
	CDW at 0.1 g/L concentration of Polyethylene for PDI-3	.0000500	.0000436	.267	-.000042	.000142
	CDW at 0.1 g/L concentration of Polyethylene for PDI-1	.0000833	.0000436	.072	-.000008	.000175
	CDW at 0.5 g/L concentration of Polyethylene for PDI-2	-.0008800*	.0000436	.000	-.000972	-.000788
	CDW at 0.5 g/L concentration of Polyethylene for PDI-3	-.0002133*	.0000436	.000	-.000305	-.000122
	CDW at 0.5 g/L concentration of Polyethylene for PDI-1	-.0000833	.0000436	.072	-.000175	.000008
CDW at 0.05 g/L concentration of Polyethylene for PDI-3	CDW at 0.05 g/L concentration of Polyethylene for PDI-2	-.0000133	.0000436	.763	-.000105	.000078
	CDW at 0.05 g/L concentration of Polyethylene for PDI-1	.0000133	.0000436	.763	-.000078	.000105

	CDW at 0.1 g/L concentration of Polyethylene for PDI-2	-.0000200	.0000436	.652	-.000112	.000072
	CDW at 0.1 g/L concentration of Polyethylene for PDI-3	.0000367	.0000436	.412	-.000055	.000128
	CDW at 0.1 g/L concentration of Polyethylene for PDI-1	.0000700	.0000436	.126	-.000022	.000162
	CDW at 0.5 g/L concentration of Polyethylene for PDI-2	-.0008933*	.0000436	.000	-.000985	-.000802
	CDW at 0.5 g/L concentration of Polyethylene for PDI-3	-.0002267*	.0000436	.000	-.000318	-.000135
	CDW at 0.5 g/L concentration of polyethylene for PDI-1	-.0000967*	.0000436	.040	-.000188	-.000005
CDW at 0.05 g/L concentration of Polyethylene for PDI-1	CDW at 0.05 g/L concentration of polyethylene for PDI-2	-.0000267	.0000436	.549	-.000118	.000065
	CDW at 0.05 g/L concentration of polyethylene for PDI-3	-.0000133	.0000436	.763	-.000105	.000078
	CDW at 0.1 g/L concentration of polyethylene for PDI-2	-.0000333	.0000436	.455	-.000125	.000058
	CDW at 0.1 g/L concentration of polyethylene for PDI-3	.0000233	.0000436	.599	-.000068	.000115
	CDW at 0.1 g/L concentration of polyethylene for PDI-1	.0000567	.0000436	.210	-.000035	.000148
	CDW at 0.5 g/L concentration of polyethylene for PDI-2	-.0009067*	.0000436	.000	-.000998	-.000815
	CDW at 0.5 g/L concentration of polyethylene for PDI-3	-.0002400*	.0000436	.000	-.000332	-.000148
	CDW at 0.5 g/L concentration of polyethylene for PDI-1	-.0001100*	.0000436	.021	-.000202	-.000018

CDW at 0.1 g/L concentration of Polyethylene for PDI-2	CDW at 0.05 g/L concentration of polyethylene for PDI-2	.0000067	.0000436	.880	-.000085	.000098
	CDW at 0.05 g/L concentration of polyethylene for PDI-3	.0000200	.0000436	.652	-.000072	.000112
	CDW at 0.05 g/L concentration of polyethylene for PDI-1	.0000333	.0000436	.455	-.000058	.000125
	CDW at 0.1 g/L concentration of Polyethylene for PDI-3	.0000567	.0000436	.210	-.000035	.000148
	CDW at 0.1 g/L concentration of Polyethylene for PDI-1	.0000900	.0000436	.054	-.000002	.000182
	CDW at 0.5 g/L concentration of Polyethylene for PDI-2	-.0008733*	.0000436	.000	-.000965	-.000782
	CDW at 0.5 g/L concentration of Polyethylene for PDI-3	-.0002067*	.0000436	.000	-.000298	-.000115
	CDW at 0.5 g/L concentration of Polyethylene for PDI-1	-.0000767	.0000436	.096	-.000168	.000015
CDW at 0.1 g/L concentration of Polyethylene for PDI-3	CDW at 0.05 g/L concentration of Polyethylene for PDI-2	-.0000500	.0000436	.267	-.000142	.000042
	CDW at 0.05 g/L concentration of Polyethylene for PDI-3	-.0000367	.0000436	.412	-.000128	.000055
	CDW at 0.05 g/L concentration of Polyethylene for PDI-1	-.0000233	.0000436	.599	-.000115	.000068
	CDW at 0.1 g/L concentration of Polyethylene for PDI-2	-.0000567	.0000436	.210	-.000148	.000035
	CDW at 0.1 g/L concentration of Polyethylene for PDI-1	.0000333	.0000436	.455	-.000058	.000125
	CDW at 0.5 g/L concentration of Polyethylene for PDI-2	-.0009300*	.0000436	.000	-.001022	-.000838

	CDW at 0.5 g/L concentration of Polyethylene for PDI-3	-.0002633*	.0000436	.000	-.000355	-.000172
	CDW at 0.5 g/L concentration of Polyethylene for PDI-1	-.0001333*	.0000436	.007	-.000225	-.000042
CDW at 0.1 g/L concentration of Polyethylene for PDI-1	CDW at 0.05 g/L concentration of Polyethylene for PDI-2	-.0000833	.0000436	.072	-.000175	.000008
	CDW at 0.05 g/L concentration of Polyethylene for PDI-3	-.0000700	.0000436	.126	-.000162	.000022
	CDW at 0.05 g/L concentration of Polyethylene for PDI-1	-.0000567	.0000436	.210	-.000148	.000035
	CDW at 0.1 g/L concentration of Polyethylene for PDI-2	-.0000900	.0000436	.054	-.000182	.000002
	CDW at 0.1 g/L concentration of Polyethylene for PDI-3	-.0000333	.0000436	.455	-.000125	.000058
	CDW at 0.5 g/L concentration of polyethylene for PDI-2	-.0009633*	.0000436	.000	-.001055	-.000872
	CDW at 0.5 g/L concentration of polyethylene for PDI-3	-.0002967*	.0000436	.000	-.000388	-.000205
	CDW at 0.5 g/L concentration of polyethylene for PDI-1	-.0001667*	.0000436	.001	-.000258	-.000075
CDW at 0.5 g/L concentration of Polyethylene for PDI-2	CDW at 0.05 g/L concentration of polyethylene for PDI-2	.0008800*	.0000436	.000	.000788	.000972
	CDW at 0.05 g/L concentration of polyethylene for PDI-3	.0008933*	.0000436	.000	.000802	.000985
	CDW at 0.05 g/L concentration of polyethylene for PDI-1	.0009067*	.0000436	.000	.000815	.000998
	CDW at 0.1 g/L concentration of polyethylene for PDI-2	.0008733*	.0000436	.000	.000782	.000965

	CDW at 0.1 g/L concentration of polyethylene for PDI-3	.0009300*	.0000436	.000	.000838	.001022
	CDW at 0.1 g/L concentration of polyethylene for PDI-1	.0009633*	.0000436	.000	.000872	.001055
	CDW at 0.5 g/L concentration of polyethylene for PDI-3	.0006667*	.0000436	.000	.000575	.000758
	CDW at 0.5 g/L concentration of polyethylene for PDI-1	.0007967*	.0000436	.000	.000705	.000888
CDW at 0.5 g/L concentration of Polyethylene for PDI-3	CDW at 0.05 g/L concentration of polyethylene for PDI-2	.0002133*	.0000436	.000	.000122	.000305
	CDW at 0.05 g/L concentration of polyethylene for PDI-3	.0002267*	.0000436	.000	.000135	.000318
	CDW at 0.05 g/L concentration of polyethylene for PDI-1	.0002400*	.0000436	.000	.000148	.000332
	CDW at 0.1 g/L concentration of polyethylene for PDI-2	.0002067*	.0000436	.000	.000115	.000298
	CDW at 0.1 g/L concentration of polyethylene for PDI-3	.0002633*	.0000436	.000	.000172	.000355
	CDW at 0.1 g/L concentration of polyethylene for PDI-1	.0002967*	.0000436	.000	.000205	.000388
	CDW at 0.5 g/L concentration of polyethylene for PDI-2	-.0006667*	.0000436	.000	-.000758	-.000575
	CDW at 0.5 g/L concentration of polyethylene for PDI-1	.0001300*	.0000436	.008	.000038	.000222
CDW at 0.5 g/L concentration of Polyethylene for PDI-1	CDW at 0.05 g/L concentration of polyethylene for PDI-2	.0000833	.0000436	.072	-.000008	.000175
	CDW at 0.05 g/L concentration of polyethylene for PDI-3	.0000967*	.0000436	.040	.000005	.000188

	CDW at 0.05 g/L concentration of polyethylene for PDI-1	.0001100*	.0000436	.021	.000018	.000202
	CDW at 0.1 g/L concentration of polyethylene for PDI-2	.0000767	.0000436	.096	-.000015	.000168
	CDW at 0.1 g/L concentration of polyethylene for PDI-3	.0001333*	.0000436	.007	.000042	.000225
	CDW at 0.1 g/L concentration of polyethylene for PDI-1	.0001667*	.0000436	.001	.000075	.000258
	CDW at 0.5 g/L concentration of polyethylene for PDI-2	-.0007967*	.0000436	.000	-.000888	-.000705
	CDW at 0.5 g/L concentration of polyethylene for PDI-3	-.0001300*	.0000436	.008	-.000222	-.000038
*. The mean difference is significant at the 0.05 level.						

Appendix 12: Newly isolated *Bacillus* sp. PDI 21 sequence

>*Bacillus* sp. PDI-21
TTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGC
AAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCC
ACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCC
ATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTA
ACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
ACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGC
CTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA
TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGAT
AGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCCTGTCGTGAGATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGT
GACTGCCGGTGACAACCGGAGGAAGGGGGGGATGACGTCAATCATCATGCCCCTTATGACCTGGGCT
ACACTTGACGGTACCTAACCAGAAAGCCACGGGTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
TGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAA
GCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAA
TTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTC
TGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACT
CCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAG
AGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAG
ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTA
AGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCT
GGGCTACAC