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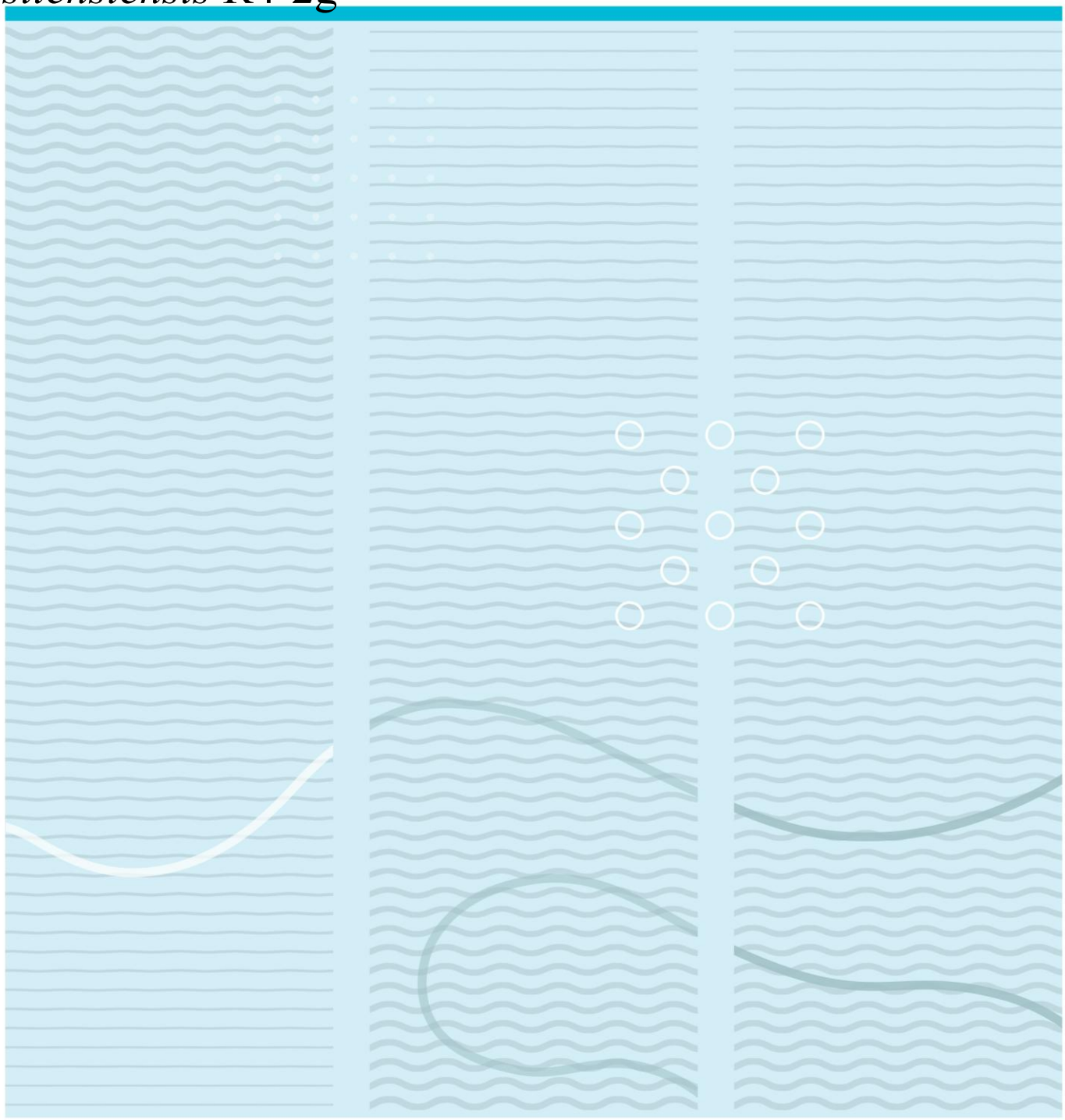
Low-density Polyethylene degradation by strains of *Rhodococcus degradans* R1-1a and *Pseudomonas silensiensis* R4-2g



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This thesis is worth 60 study points

Summary

For all sin betydning i vår daglige bruk, har plastpolymer utgjort en stor fare for miljøet. På grunn av deres stabilitet, høye molekylvekt, hydrofobisitet og lave nedbrytningshastighet, vedvarer de derfor i miljøet og utgjør alvorlig fare for levende organismer blant andre miljøhensyn. Seks mikrobielle stammer ble brukt i denne studien, men to stammer Rhodococcus degradans R1-1a og Pseudomonas silensiensis R4-2g viste tegn på LDPE-nedbrytning. Mikrobiell nedbrytning av LDPE ble analysert ved reduksjon av tørr vekt av LDPE-polymeren, utvikling av CO_2 og platetellingmetoden. Disse bakteriene viste utholdenheten til å leve på LDPE som eneste karbonkilde, med vekttap på 6,5% og 3,5% av LDPE etter 60 dagers inkubasjon for henholdsvis Rhodococcus degradans R1-1a og Pseudomonas silensiensis R4-2g som enkeltkulturer og 21,5% vekttap som en blandet kultur, og CO2-utslipp på henholdsvis 150 ppm og 400 ppm av R1-1a og R4-2g som antyder LDPE-nedbrytning.

Nøkkelord: LDPE, nedbrytning, Rhodococcus degradans, Pseudomonas silensiensis.

Abstract

For all its importance in our daily use, plastic polymer has posed a great danger to the environment. Due to their stability, high molecule weight, hydrophobicity, and low degradability rate, hence, persist in the environment posing grave danger to living organisms amongst other environmental concerns. Six microbial strains were used in this study but two strains *Rhodococcus degradans* R1-1a *and Pseudomonas silensiensis* R4-2g showed evidence of LDPE degradation. Microbial degradation of LDPE was analyzed by dry weight reduction of the LDPE polymer, the evolution of CO_2, and the plate count method. These bacteria showed the tenacity to live on LDPE as the sole carbon source, with weight loss of 6.5%, and 3.5% of LDPE after 60 days of incubation for the *Rhodococcus degradans* R1-1a *and Pseudomonas silensiensis* R4-2g respectively as single cultures and 21.5% weight loss as a mixed culture, and CO_2 emission of 150 ppm and 400 ppm of R1-1a and R4-2g respectively which suggests LDPE breakdown.

Keywords: LDPE, degradation, *Rhodococcus degradans*, *Pseudomonas silensiensis*.

Contents

Summary **3**

Abstract **4**

List of Tables **7**

List of Figures **8**

List of Annexes **9**

List of Abbreviations **10**

Acknowledgment **11**

1. Introduction **12**

1.1 Dangers of Plastics 13

1.2 Plastic Degradation 15

1.3 Biodegradation 16

1.4 Factor affecting plastic biodegradation 18

1.5 Microorganism Implicated in Synthetic Plastic degradation 18

1.5.1 Polyethylene 18

1.5.2 Polyethylene terephthalate 21

1.5.3 Polyurethane 22

1.5.4 Polystyrene 22

1.5.5 Polyvinyl Chloride and Polypropylene 24

1.5.6 Polyamides 25

1.6 Environmental Factors Aiding Bioremediation of Plastic Litters 25

1.7 Aim 25

2. Methods **27**

2.1 Preparation of Erlenmeyer flask 27

2.2 Growth of pure bacterial strain on solid media 27

2.3 Preparation of liquid culture for plastic degradation 28

2.5 Assessing the growth of microbes on LDPE Polymer 29

2.5.1 Total ATP test for bacterial growth 29

2.5.2 Enumeration of number of cells using the pour plate method 29

2.5.3 Enumeration bacterial densities 30

2.6 Measuring Biodegradation 30

2.6.1 Weight loss experiment 30

2.6.2 CO_2 Measurement experiment 31
2.7 Statistical analysis 31
3. Results 32
3.1 Growth of bacteria strains on Tryptic Soy Broth (TSB) 32
3.2 Assessing Bacteria Growth on LDPE. 32
3.2.1 ATP measurements 32
3.2.2 Viable Plate Count 37
3.4 Statistical Analysis on plate count between the stimulated cultures of R1-1a and R4-2g 42
4. Discussion 43
4.1 Assessing growth on LDPE 43
4.2 Biodegradability test 44
4.2.1 Visual observation 44
4.2.2 Weight loss experiment 46
4.2.3 Carbondioxide (CO_2) evolution test 47
5. Recommendation 49
6. Conclusion 50
References/bibliography 51
Annexes 57

List of Tables

Table 1: List of bacterial strain used in the experiment 27
Table 2: Total ATP Values of the bacterial strains after 38 days inoculation with LDPE.. 33
Table 3: Viable Colony count for unstimulated cultures 37
Table 4: Viable colony count for stimulated cultures 38
Table 5:Two-Sample t-Test assuming unequal variance 40
Table 6:Two-Sample t-Test assuming unequal variance 41
Table 7:Two-Sample t-Test assuming unequal variance 41
Table 8: Two-Sample t-Test assuming unequal variance of plate count between stimulated and unstimulated cultures of Rhodococcus degradans..... 42
Table 9: Two-Sample t-Test assuming unequal variance of plate count between stimulated and unstimulated cultures of Pseudomonas silensiensis R4-2g..... 42
Table 10:Percentage change in weight of 2.0g of LDPE upon 60 days of incubation..... 46
Table 11:CO_2 Measurement in Parts per Millions (ppm)..... 48

List of Figures

Figure 1: Structures of major commercial synthetic thermoplastic polymers..... 13
Figure 2: Reaction pathways during degradation of polymers..... 17
Figure 3: Mechanism of biodegradation of polyethylene..... 20
Figure 4: Microbial degradation of Polyethylene Terephthalate (PET)..... 21
Figure 5: Chemical formula of polystyrene..... 23
Figure 6: Pathway for the aerobic degradation routes of styrene by microorganisms... 24
Figure 7: Bacterial strain in TSB..... 32
Figure 8: Total ATP values of Rhodococcus degradans (R1-1a)..... 33
Figure 9: Total ATP value of Pseudomonas silensiensis (R4-2g)..... 34
Figure 10: Total ATP values for Pseudoarthrobacter phenanthrenivorans (R4-3d)..... 34
Figure 11: Total ATP values for Microbacterium hydrocarbonoxydans (R4-2h)..... 35
Figure 12: Total ATP values for Paraburkholderia xenovorans (R4-2c)..... 35
Figure 13: Total ATP values for Pseudoarthrobacter sulfonivorans (R1-4b)..... 36
Figure 14: Total ATP values for Negative control..... 36
Figure 15: Viable colony count curve for unstimulated cultures of Rhadococcus degradans R1-1a..... 38
Figure 16: Viable colony count curve for unstimulated cultures of Pseudomonas silensisnsis R4-2g..... 39
Figure 17: Viable colony count curve for unstimulated cultures of Rhadococcus degradans R1-1a and Pseudomonas silensisnsis R4-2g..... 39
Figure 18 : Viable colony count curve for stimulated cultures of Rhadococcus degradans R1-1a and Pseudomonas silensisnsis R4-2g..... 40
Figure 19: (a) LDPE culture without microorganism (negative control), (b) LDPE cultured with R4-2(g), (c), Visual nature of LDPE after 148days of degradation. Flask 1 (left), contains LDPE inoculated with R4-2(h), plastic is still whitish, viscous, and hydrophobic, Flask 2 (right), inoculated with R1-1a, LDPE is transparent and more water-soluble. 46

List of Annexes

Annex 1: Procedure and Preparation of Stock Solution 57
Annex 2: Bacterial cultures in aluminum foil wrapped Erlenmeyer flasks on rotor. 58
Annex 3: The Accuro setup for CO2 Measurement 58
Annex 4: descriptive statistics of R1-1a; R4-2g; and, R1-1a/R4-2g 59

List of Abbreviations

PE	Polyethylene
PP	Polypropylene
PVC	Polyvinylchloride
PS	Polystyrene
PET	Polyethylene terephthalate
PUR	Polyurethane
PAH	Polyaromatic hydrocarbon
PLA	Polylactic acid
PCL	Polycaprolactone
PES	Polyethylene succinate
MHET	Mono (2-hydroxyethyl) terephthalic acid
LDPE	Low density polyethylene
HDPE	High density polyethylene
VLDPE	Very low density polyethylene
MDPE	Medium density polyethylene
ATP	Adenosine triphosphate
TSA	Tryptic soy agar
TSB	Tryptic soy broth
OD	Optical density
UV	Ultraviolet light
RLU	Relative light unit
FTIR	Fourier transformation infrared
SEM	Scanning electron microscopy

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1. Introduction

Plastics are long-chain polymers produced from petrochemical sources or natural materials like starch, lignin, chitin, or cellulose (bio-polymers).

For all their good qualities in our daily use, plastics litters have become a serious concern to the environment. They are highly stable and not easily degradable or depolymerized and thus persist in the environment resulting in various health and ecological issues (Khan & Majeed, 2019).

Production of plastic has increased by an estimated amount of 8.7% annually since the 1960s evolving into a multi-billion dollar industry globally (Smith et al., 2018). There has been a dramatic rise in plastic production most especially in the last 15 years. Globally, in 2016 an estimated 335 million tons of plastic was produced, and by 2050 a statistically estimated 26 billion tons of plastic wastes will be produced and more than half of these will end up in landfills and in the ecospheres, such as oceans and lakes, leading to serious environmental pollution (Danso et al., 2019; Rodrigues et al., 2019; Ru et al., 2020).

Commonly used plastics are produced from petrochemical materials for example Polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), Polystyrene (PS), polyethylene terephthalate (PET), polyurethane (PUR), (Danso et al., 2019; Muhonja et al., 2018; Urbanek et al., 2018)

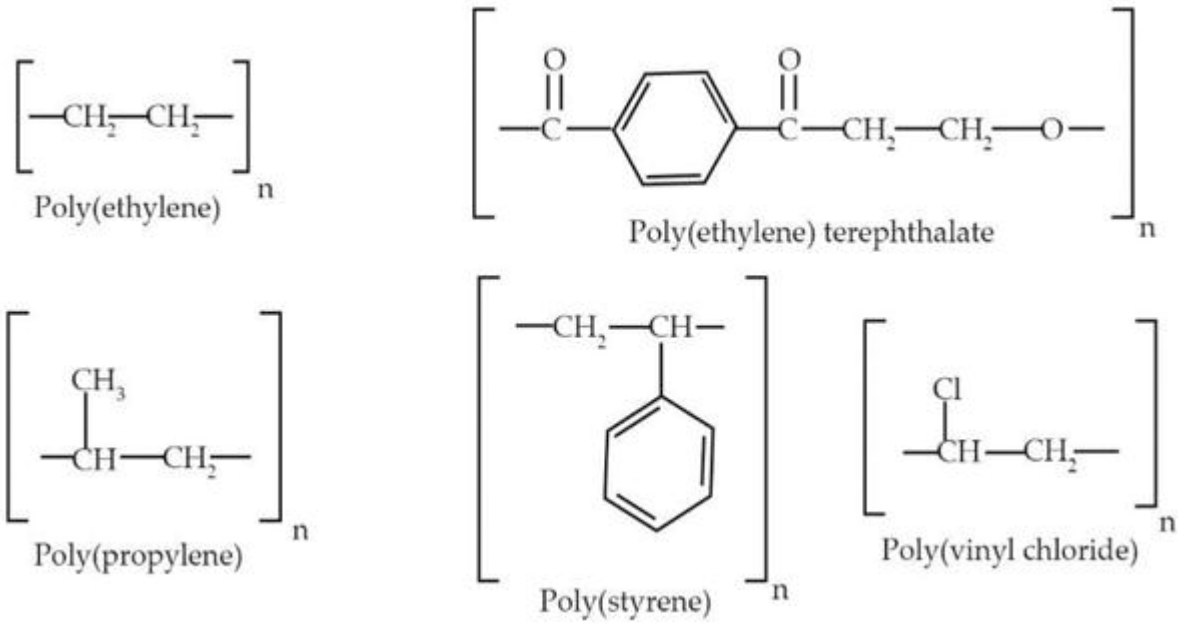


Figure 1: Structures of major commercial synthetic thermoplastic polymers (adapted from Glaser, 2019).

The enormity of the plastic problem came to the fore in 1997 when Charles Moore a US oceanographer discovered huge floating trash dubbed the "Great Pacific Garbage Patch". Such patches which are 70% plastics have been found in high amounts in the Indian Ocean, the North and South Pacific, North and South Atlantic was believed to be created by surface currents or gyres (Christina Reed, 2015).

Most plastic litter eventually ends up in the ocean either as macroplastic, microplastics, or nanoplastics (Urbanek et al., 2018). As a result of degradation by environmental factors, abrasion, shearing, and other mechanical disruption, larger plastics material generate microplastic particles which can spread over long distances by wind-driven circulation and wave actions even affecting places far away from the source of pollution (Urbanek et al., 2018; Wilkes & Aristilde, 2017).

1.1 Dangers of Plastics

In 2010, an estimated 5 – 13 million tonnes of plastic entered the ocean thus contributing to trillions of floating plastic pieces circulating in the marine environment (Amaral-Zettler et al., 2020). Plastics not only litter the environment; it causes health-related issues on wildlife. One problem is the ingestion of plastics by marine life or birds

which mistake it for food. According to a report, over 260 species including turtles, invertebrates, fish, mammals, and seabirds ingest plastic litters which they mistake for food, consequently leading to starvation, impaired feeding, ulcer-causing, or have become entangled in plastic, leading to impaired movement, body harm and distortion and even death (Rodrigues et al., 2019; Zettler et al., 2013). Blended into the plastic during the production process are chemical additives such as BISPHENOL A, PHTHALATES, POLYBROMINATED DIPHENYLETHERS, and ANTIOXIDANTS. These additives are weakly bound to these plastics polymers hence escape during plastics fragmentation by environmental forces or upon ingestion mostly by aquatic organisms. These additives are toxic, carcinogenic and an endocrine disruptor in animals and humans (Rodrigues et al., 2019). During production or burning, PVC plastics may release dioxins which are known to be cancer-causing, immune, and reproductive system disruptors (Chandra, 2015).

Due to their hydrophobicity, plastics can adsorb and concentrate pollutants such as metal ions, polyaromatic hydrocarbon (PAH), polychlorinated biphenyls, organochlorine pesticides, pharmaceutical products, which subsequently are released in animals that feed directly or indirectly on these plastic products (Rodrigues et al., 2019).

The high amount of plastic in the terrestrial environment finds its way into drainage systems and over time due to its accumulation can block drainages thus causing serious flooding (Lebreton & Andrady, 2019).

Christened the “Plastisphere” by Amaral-Zettler, the plastic surface has provided a niche for microbes. Microorganisms can colonize plastic surface and form biofilms which support the growth of other organisms like microalgae, microscopic fungi, plants, and animals. This accumulation of biomass leads to plastic biofouling that has increased the density of some microplastics causing their sinking to the pelagic or benthic zones, changed the ecosystem of the open oceans, and also promotes the transport of invasive organism from one location to another (Caruso, 2020; Pinto et al., 2019). Vibrio a bacterial genus has attracted quite a lot of attention recently due to its high isolation from marine microplastic from which many fish have been infected (Parthasarathy et al., 2019; Reed, 2015). Microplastic has also found its way into the human diet, according to Lisbeth Van Cauwenberghe microplastic can find its way into our body from the consumption of shellfish and particularly mussels (Reed, 2015).

Plastic pollution has become an enormous problem in our environment hence it is vital to solving this problem. Conventional approaches for disposal like incineration, dumping in landfills, dumping into oceans, recycling are effective but only help with about 10% of the plastic waste generated and are still in need of improvement. Hence, it is important nature's decomposers evolve rapidly to produce enzymes in the degradation of these xenobiotic compounds. Microorganisms degrade plastics through enzymatic actions into metabolic products that are of less or no concern to the environment (Alshehrei, 2017; Fesseha & Abebe, 2019; Khan & Majeed, 2019).

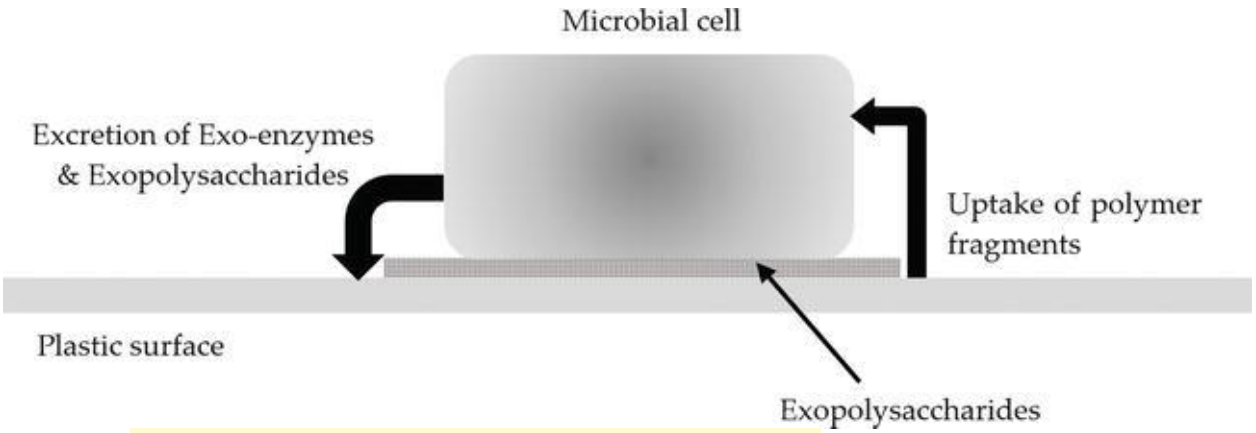


Figure 2*: Microbial attachment processes to a polymer surface (adapted from Glaser, 2019).*

1.2 Plastic Degradation

In the environment, plastic can be degraded by either biotic or abiotic processes. All-be-it a slow process, abiotic processes which mediate plastic degradation include temperature, light (UV-light), pH, moisture, and wave action. While the biotic processes are mediated chiefly by bacteria and fungi that act to decompose these complex xenobiotic plastic compounds. The environmental degradation means include photo-oxidative degradation, thermal degradation, mechanical/physical degradation, and biodegradation.

Many of these degradation methods probably help weakens the bond of the polymer, reduce its size and molecular weight, thus fragmenting the polymer. It is necessary to degrade plastic completely to its inorganic constituent hence biodegradation of plastic is been given much attention lately to achieve this.

1.3 Biodegradation

Biodegradation is a process by which microorganisms (mainly bacteria and fungi) transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment (Urbanek et al., 2018). Microbes attack polymers first by attaching and colonizing the surface of the polymer with biofilm formation, then secretion of extracellular enzyme and depolymerization of the plastic polymer to low molecular weight compounds, microbial uptake, and utilization of the low molecular compounds for growth (increasing biomass and cell number) and production of inorganic constituents (mineralization) (Alshehrei, 2017; Fesseha & Abebe, 2019; Zumstein et al., 2018).

As stated earlier, plastic that enters into the marine environment is first fragmented to form microplastic and nanoplastics via a variety of biotic and abiotic processes. The loss of physical integrity i.e. increases the surface area of the plastics, opens more surface for microbial colonization and biofilms formation on microplastics (Urbanek et al., 2018). It has been reported of biofilms formation on plastic bags after 15days of exposure to the marine environment. The number of biofilms increased significantly within 33 days on polyethylene and biodegradable plastic bags distributed to a shallow benthic and pelagic habitat (Urbanek et al., 2018).

Through enzymatic hydrolysis, microbes can degrade plastics with hydrolyzable bonds through the process of de-polymerization. The plastic polymer's chains bonds are cleaved by enzymes to their oligomers and monomeric units and then to water-soluble components (Montazer et al., 2020). The mechanism of biodegradation of plastics involves bio-deterioration, biofragmentation, assimilation, and mineralization, though this process may mainly apply to polyesters, polyurethanes, and polyamides but not to polyolefins such as PE, PP, and PS.

The degradative pathway and product formed of these plastic polymers largely depend on the pre-existing environmental conditions, under aerobic conditions, aerobic microorganisms are mostly responsible for the biodegradation with microbial biomass, water, and carbon dioxide formation. In anoxic/hypoxic environmental conditions, anaerobic microorganisms are largely involved in plastic degradation with microbial biomass, carbon dioxide, methane, and water formation (Shah et al., 2008).

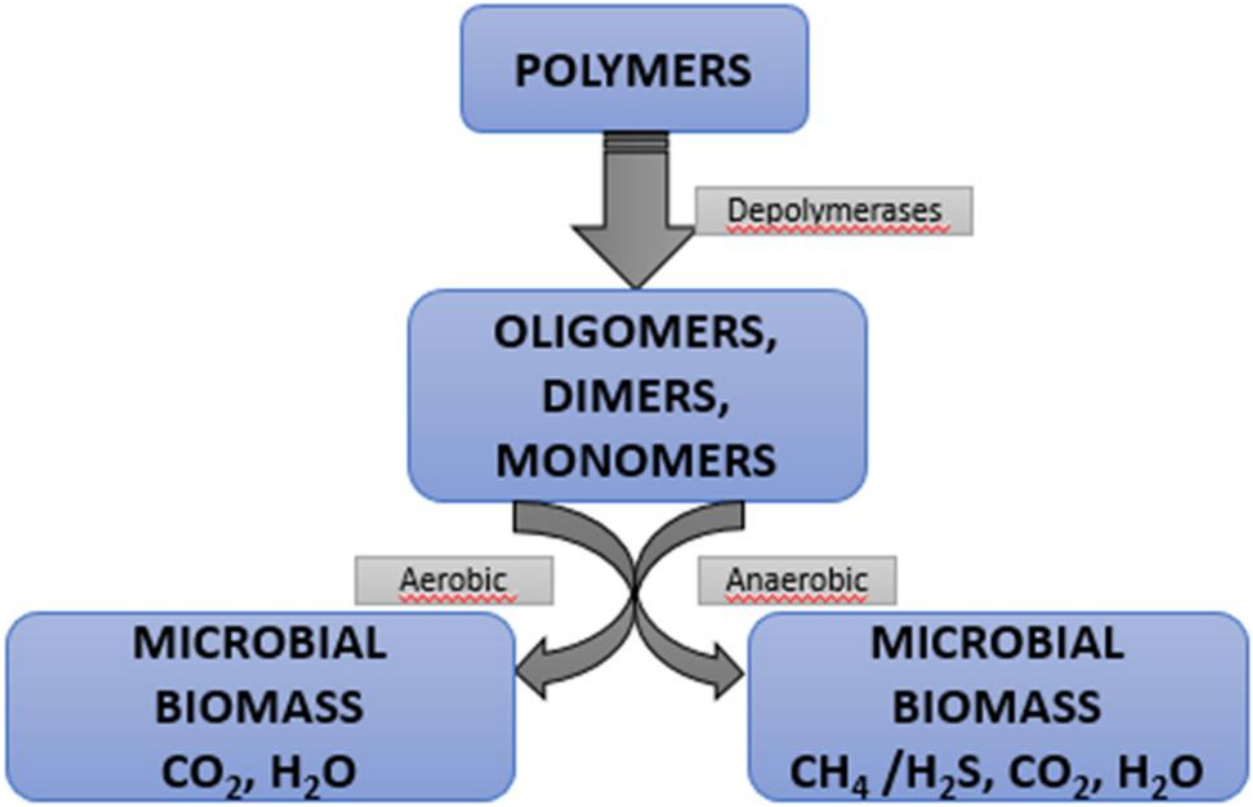


Figure 2: Reaction pathways during degradation of polymers (adapted from Mierzwa-Hersztek et al., 2019)

Plastic can be grouped into biodegradable and non-biodegradable, based on the material the polymer is made from. Some plastic biopolymers are either made from starch such as polylactic acid (PLA) or substances produced by microorganisms such as Polyhydroxyalkanoates (PHA). Some other biodegradable polymers are polymer blends, for example, starch and polyvinyl alcohol blends, starch and polyester blends. Other biodegradable polymers are made from non-renewable sources, for example, polycaprolactone (PCL) and polyethylene succinate (PES) (Khan & Majeed, 2019; Rana, 2019; Shimao, 2001). Reports are replete with microbial degradation of biodegradable plastic. But there are still ongoing researches on plastic that mostly are classified as non-biodegradable. These plastics are mostly produced from non-renewable energy sources, they include; polyethylene, polypropylene, polyvinyl chloride, polystyrene, polyterephthalate, polyurethane, polyamides (Danso et al., 2019; Urbanek et al., 2018). Hence we are going to focus on microbial degradation of these non-biodegradable plastics.

1.4 Factor affecting plastic biodegradation

Polymer degradation is affected by different factors; these factors include the polymer characteristics, prevailing environmental conditions on exposure, and type of microorganisms (Chandra, 2015)

The degree biodegradability of a plastic polymer has been shown to depend essentially on the following polymer characteristics; viz-a-viz degree of its hydrophobicity or hydrophilicity, the molecular weight and density of the polymer, the morphology and structural complexity, type of bond and molecular composition, hardness and physical forms of the plastics, type of additives/plasticizers, functional group present (Alshehrei, 2017; Yuan et al., 2020).

Some important environmental factor that affects polymer degradation includes ultra-violet light, humidity, and heat, chemical and wave action. These factors either affect the growth and metabolic activities of resident microorganisms which in turn alter the degradative rate of the microorganisms or they cause considerable damage, aging of the plastics which weaken its bonds thus accelerating the degradation and bioavailability of the plastics for degradative microorganisms (Yuan et al., 2020).

1.5 Microorganism Implicated in Synthetic Plastic degradation

1.5.1 Polyethylene

Polyethylene $(CH_2{=}CH_2)_n$, is a synthetic thermoplastic polymer made from the polymerization of monomeric units of ethene ($CH_2{=}CH_2$). This polymer marks one of the most commonly produced and used plastic in the world (Grover et al., 2015).

Some bacterial and fungal strains and or genera have been implicated in different plastic degradation for example in the degradation of PE, bacteria belonging to the genera *Pseudomonas, Ralstonia, Stenotrophomonas*, *Staphylococcus, Bacillus,* and species such as *Serratia marcescens, Phormidium lucidium, Oscillatoria subbrevis Brevibacillus borstelensis, Rhadococcus ruber* has been shown to utilize PE as their sole carbon source.

While fungi genera like *Aspergillus, Fusarium, Cladosporium*, *Penicillium* also degrade PE Plastic, with lactase, peroxidase (manganese and soybean peroxidase), oxidase, and proteases as the main enzymes used by the microbial degraders.

Wax moth (*Galleria mellonella*), Indian mealworm (*Plodia interpunctella*) has shown evidence of PE degradation. Mutualistic bacteria identified as *Bacillus sp* strain YP1 as the PE degrading bacterium responsible for the degradation in Indian mealworm.

This discovery shows a more promising source for identifying and screening bacterial for PE degradation (Danso et al., 2019; Khan & Majeed, 2019; Urbanek et al., 2018).

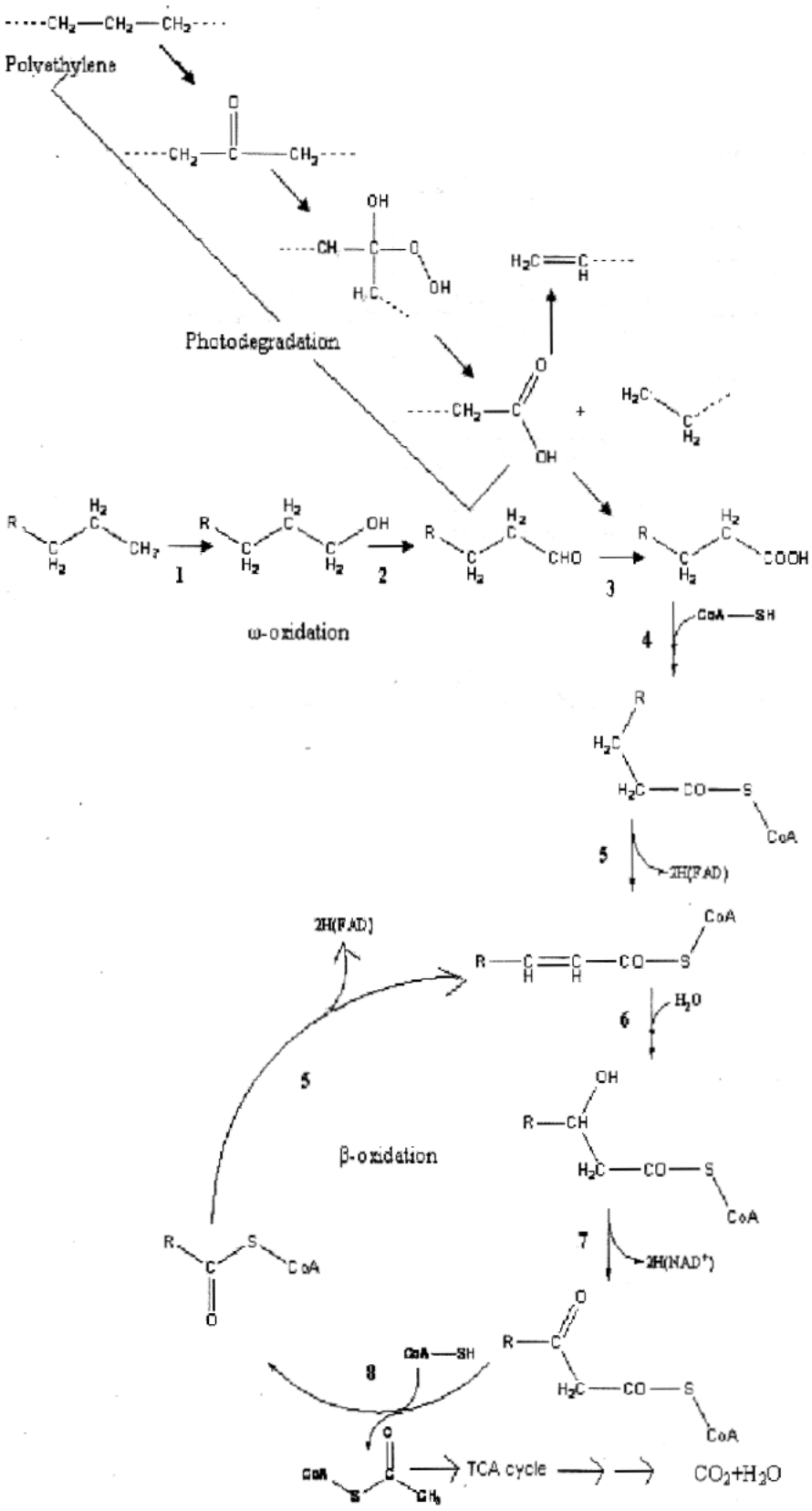


Figure 3: Mechanism of biodegradation of polyethylene (adapted from Arutchelvi et al., 2008)

1.5.2 Polyethylene terephthalate

PET is a long chain polyester formed from the polymerization of monomeric units of ethylene glycol $(CH_2OH)_2$ and terephthalic acid $C_6H_4(CO_2H)_2$ and forms about 50% synthetic fiber manufactured (Moses et al., 2015).

Some bacteria and fungi isolates implicated in PET degradation include include *Pseudomonas mendocina*, *Humicola insolens, Thermobifida fusca, Fusarium solani, Saccharomonospora viridis* AHK190. These microorganisms produce cutinase that can degrade low crystalline PET. A bacterium *Ideonella sakaiensis* was shown to produce two types of enzymes identified as PETase and MHETase that work together simultaneously to hydrolyze PET. Firstly, it was shown that PETase breaks down PET into Mono(2-hydroxyethyl) terephthalic acid (MHET), while MHETase hydrolyzes MHET into terephthalic acid and ethylene glycol (Danso et al., 2019; Khan & Majeed, 2019).

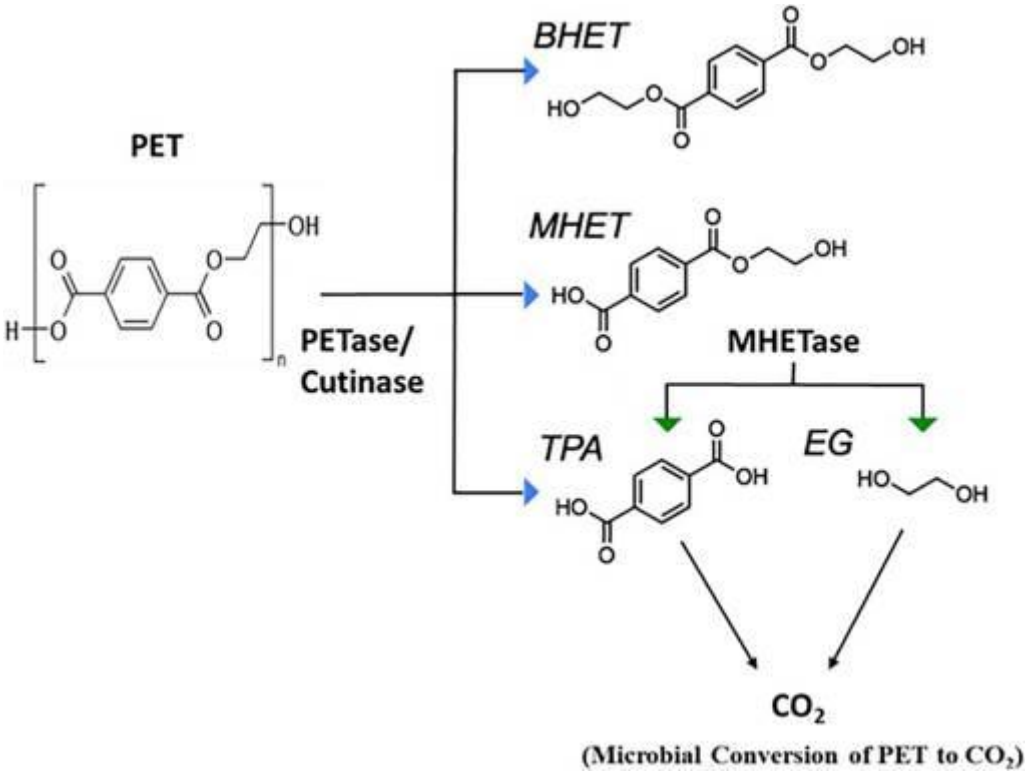


Figure 4**:** Microbial degradation of Polyethylene Terephthalate (PET) (adapted from Levin, 2020)

1.5.3 Polyurethane

Polyurethane (PU) is a polymer formed from the reaction of polyol and an isocyanate and has found application in foam production (Akindoyo et al., 2016).

Some bacteria and fungi have been linked to PUR activities. *Pseudomonas chlororaphis*, *Pseudomonas protegeus* strain pf-5, *Comamonas acidovorans* TB-35 which produces esterases and lipase (PueA and PueB) enzyme as the hydrolyzing enzymes. Reported fungi with PUR degrading capabilities include *Fusarium solani*, *Candida ethanolica, Candida rugosa, Cladosporium spp, penicillium chrysogenum*, *Aspergillus flavus*. Just like the bacteria spp, the fungi spp also produces esterases and lipase as the hydrolyzing enzymes though it was reported that no enzymes were identified in *C. ethanolica, F. solani* in PUR metabolism (Ru et al., 2020; Shimao, 2001).

1.5.4 Polystyrene

PS is a thermoplastic formed from the polymerization of styrene monomers (C_8H_8).

PS products are mostly used in the solid form for making coffee cups, trays, etc, or in the expanded form, as insulation materials in building, foam, or in its high-density form as liquid containers. Production of PS globally is increasing highly, and in 2013 accounted for about 7.1% of global plastic production (Maharana et al., 2007; Yang et al., 2018).

According to reports no known enzyme has been reported for the degradation of high molecular weight PS. The bacterium *Gloeophyllum striatum* DSM 9592, *G. trabeum* DSM 1398, some white-rot fungi spp *Pleurotus ostreatus, Phanerochaete chrysosporium,* and *Trametes vasicolor* were shown to degrade PS but the enzymes involved are yet to be elucidated and weight loss of high molecular weight PS may be caused by the degradation of chemical additives. That being said, a larger number of bacterial genera such as *Pseudomonas*, *Xanthobacter*, *Rhodococcus*, *Corynebacterium* are capable of metabolizing the monomer styrene as a sole carbon source with the use of styrene monooxygenase, styrene oxide isomerase, phenylacetaldehyde dehydrogenase as hydrolyzing enzymes.

The mealworm (larva of *Tenebrio molitor*), dark mealworm (*Tenebrio obscures*), and super worms (*Zophobas atratus*) have shown great dexterity in eating and degrading Styrofoam a form of PS. *Exiguobacterium sp* YT2, *Citrobacter*, *Kosakonia* isolated from

the gut of *Tenebrio molitor* showed capabilities of degrading PS and PE, this is a pointer of a mutualistic relationship between this larva and bacteria in their gut. More research can be directed into this aspect to identify more of this type of bacteria than can degrade plastic.

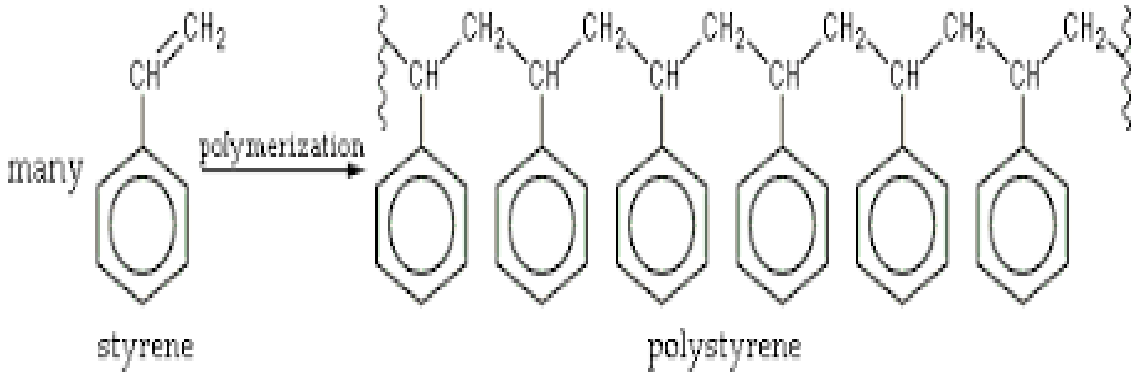


Figure 5: Chemical formula of polystyrene (adapted, Ho et al., 2018)

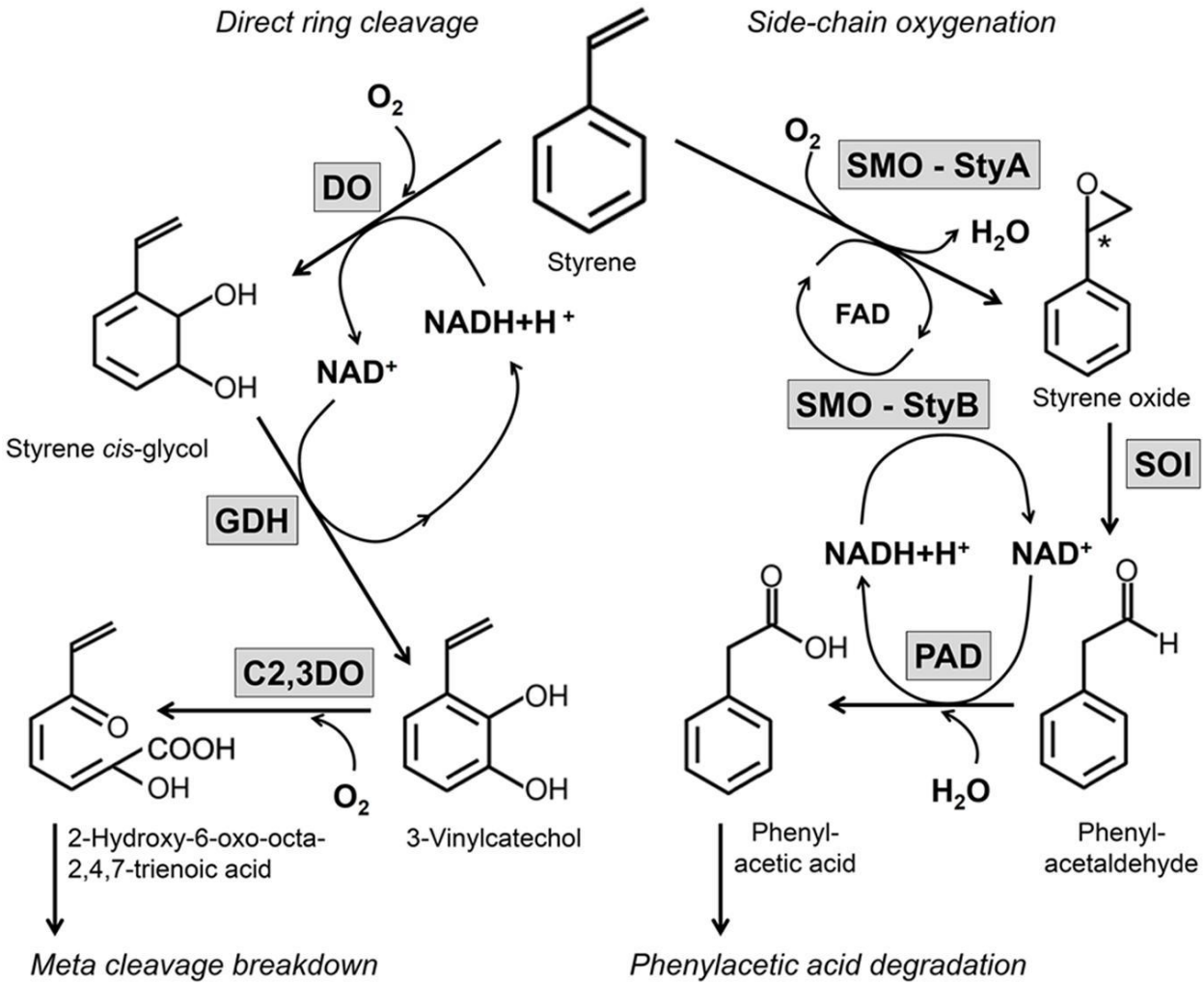


Figure 6: Pathway for the aerobic degradation routes of styrene by microorganisms (adapted from Oelschlägel et al., 2018)

1.5.5 Polyvinyl Chloride and Polypropylene

PVC and PP are amongst the most produced plastics. They are composed of repeating vinyl chloride and propene units respectively. Reports of biodegradation of PVC and PP are very few; though there has been a report of PVC biodegradation by white-rot fungi and PP degradation by fungal such as *Aspergillus niger* and bacteria such as *Pseudomonas* and *Vibrio* have been reported to degrade PP with a decrease in viscosity, carbonyl, and carboxyl group formation.

Most of these report available have only described the biodegradation of the polymer with evidence on weight loss, with no defined pathways or enzymes responsible for the degradation process (Alshehrei, 2017; Danso et al., 2019; Fesseha & Abebe, 2019)

1.5.6 Polyamides

Polyamides consist of repeating units of aliphatic, semi-aromatic, or aromatic molecules linked by amide bonds, typical examples are nylon and Kevlar. No known microorganism has been recorded to fully degrade high-molecular-weight polymer PA, but there have been reports of bacterial acting on the linear or cyclic nylon oligomers. For example, *Achromobacter guttatus* K172, *Arthrobacter sp* strain K172 have been able to employ some hydrolases in the metabolism of oligomers of PA. diverse marine bacterial known to also act on nylon include *Bacillus cereus*, *Bacillus sphaericus, Vibrio furnisii*, *Brevundimonas vesicularis* (Danso et al., 2019).

1.6 Environmental Factors Aiding Bioremediation of Plastic Litters

The synergy between other environmental factors and microbial degradation of synthetic plastic should be considered in the degradation of high molecular weight plastic. There have been reports of some considerable success of this type of plastic treatment. For example, most studies of PE biodegradation have reported that bacteria such as *Rhodococcus spp*, *Pseudomonas spp*, *Bacillus spp*, *Cupriavidis necator,* and fungi such as *Aspergillus* and *Fusarium* can hydrolyze PE only after pre-treatment with UV light and or thermal energy which render the carbon chains of the polymer sensitive to biodegradation by depolymerization of long-chain plastic as well as the formation of low molecular products (Ru et al., 2020; Zahra Montazer;). Also, *Thermobifida fusca* was able to degrade 50% of the initial weight of low crystalline PET at 55^{0}C in a space of 3 weeks (Ru et al., 2020).

1.7 Aim

In this study, I will explore different strains/isolates of bacterial with the capabilities of degrading low-density Polyethylene (LDPE). Polyethylene is intrinsic to our everyday life and accounts for the highest produced synthetic plastic. It can be categorized into low-density polyethylene (LDPE), high-density polyethylene (HDPE), medium-density polyethylene (MDPE), very low-density polyethylene (VLDPE). The choice for LDPE in this experiment is the fact that it is the PE of choice for making a lot of items especially

daily and single items like grocery bags, disposable cups, food wraps, hence posing as one of the worst litters of plastics in the environmental (Grover et al., 2015; Pramila & Ramesh, 2015.).

2. **Methods**

Pre-identified bacterial strains used in this study were isolated by (Abiriga et al., 2021) from the Revdalen Aquifer, a glaciofluvial deposit located in Vestfold and Telemark County in southeast Norway at coordinate 59°25′58.26″N and 9°06′1.53″E. The bacterial Strains used are given in the table below.

Table 1: List of bacterial strain used in the experiment

Bacteria Strains	Oxygen Requirement	Code
Pseudomonas silensiensis	Aerobic	R4-2g
Rhodococcus degradans	Aerobic	R1-1a
Pseudoarthrobacter phenanthrenivorans	Aerobic	R4-3d
Microbacterium hydrocarbonoxydans	Aerobic	R4-2h
Pseudoarthrobacter sulfonivorans	Anaerobic	R1-4b
Paraburkholderia xenovorans	Aerobic	R4-2c

2.1 Preparation of Erlenmeyer flask

All 250ml Erlenmeyer flasks used in this experiment were plugged with cotton wool and autoclaved at 121^{O}C for 20 min.

All nutrient, agar, and virgin LDPE pellets used in this experiment were weighed using a Bosch DMS 560 balance (Gebr Bosch, Jungingen, Germany)

2.2 Growth of pure bacterial strain on solid media

Molten TSA is poured on Petri-dish to about one-third full and allowed to set. Pure bacterial strain to be used were streaked on the plates to obtain isolated colonies

2.3 Preparation of liquid culture for plastic degradation

150ml of fortified M_9 minimum salt (5X) medium and 1.5g of virgin LDPE polymer were added to Erlenmeyer flasks. Each flask was inoculated using a sterile loop with a colony of the different bacterial strains taken from pure TSA cultured Petri dishes. The flasks were labeled, sealed with sterile cotton wool, and completely wrapped with aluminum foil to cut off sunlight and prevent the growth of algae. Negative control was maintained with pre-weighted LDPE plastics in Erlenmeyer flask containing 150ml of fortified M_9 minimum salt medium (5X) with no bacteria colony added, each flask is placed on a rotatory shaker (Ika-Schüttler MTS 4) and maintained at 100 rpm.

2.4 Preparation of stimulated liquid culture for LDPE polymer degradation

Materials

5.6g of Fortified M_9 minimum salt medium (5X)

1.5g of virgin LDPE polymer

10µl of baby oil by Johnson and Johnson (liquid paraffin, Isopropyl palmitate, perfume)

Method

150ml of fortified M9 minimum salt (5X) medium, 10µl of baby oil by Johnson and Johnson, and 1.5g of virgin LDPE polymer were added to Erlenmeyer flasks. Each flask was inoculated using a sterile loop with a colony of *Rhodococcus degradans* R1-1a and *Pseudomonas silensiensis* R4-2g taken from pure TSA cultured Petri dishes. The flasks were labeled, sealed with sterile cotton wool, and completely wrapped with aluminum foil to cut off sunlight and prevent the growth of algae. Negative control was maintained with pre-weighted LDPE plastics and 10µl of baby oil in Erlenmeyer flask containing 150ml of fortified M_9 minimum salt medium (5X) with no bacteria colony added, each flask is placed on a rotatory shaker (Ika-Schüttler MTS 4) and maintained at 100 rpm.

2.5 Assessing the growth of microbes on LDPE Polymer

2.5.1 Total ATP test for bacterial growth

Material

Total ATP kits

Eppendorf tubes (1.5ml)

Syringe tubes (1.0ml)

Nylon membrane filter

Method

Bacterial growth rates were determined by measuring the total ATP of the media containing bacterial isolates. The increase in total ATP is a reflection of the increase in growth and metabolic rate. 900µl of the sample was filtered through nylon membrane into 1.5ml Eppendorf tubes in other to remove the residue LDPE material from the sample. The total ATP of the filtrate was determined using the Aqua Snap Total ATP testing kit (Hygiena, USA). All procedures for testing were followed as directed in the kit manual. Readings were determined from the luminometer. ATP readings appeared after 15s for each sample and results were given in relative light units (RLU).

2.5.2 Enumeration of number of cells using the pour plate method

Materials

Pipette

Pipette tips (100-1000 ml)

Petri- dish plates

Molten TSA media

Plain M9 media

Tubes

Methods

Cultures to be tested were serially diluted in 1000-fold dilutions. 100ml of bacterial culture growing in a medium containing LDPE polymer is added to 900ml of M_9 minimum salt medium (5X) in a tube and mixed using a rotator creating a 10^{-1} dilution. Then, 100ml from the 10^{-1} dilution culture is added to 900ml of M_9 minimum salt

medium (5X) in another tube and then mixed creating a 10^{-2} dilution. This procedure is repeated to create 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions.

For each dilution tubes, 100ml of the diluted bacterial cultures were taken and pipette into the center of a Petri-dish plate. Half-strength TSA molten agar is poured on each of the plates to about one-third full. Gently swirl the half-strength TSA and bacteria culture for proper mixing and then allow the media to solidify. Incubate at room temperature for 24-72 hours.

$$Dilution = \frac{\text{volume of culture}}{\text{the vol of culture} + \text{vol of diluent}}$$

$$Dilution = \frac{100\ \text{ml}}{100\ \text{ml} + 900\ \text{ml}}$$

$$Dilution = \frac{1}{10}$$

Dilution = 10^{-1}

2.5.3 Enumeration bacterial densities

After incubating, count the number of colonies on each plate and expressed the number of colonies as colony-forming units per milliliter (CFU/ml).

CFU/ml = number of colonies × dilution factor

2.6 Measuring Biodegradation

2.6.1 Weight loss experiment

Materials

250ml Erlenmeyer flasks

Fortified M9 medium

Virgin LDPE

Eppendorf biophotometer (Eppendorf AG. 22331 Hamburg, Germany)

Method

I50ml M_9 minimum salt medium 5X fortified with solution 2 (1000x) and 2.0g of virgin LDPE polymer pellets were added into 250ml Erlenmeyer flasks poured into each flask.

Bacterial cultures of each isolate were placed into 1.5ml Eppendorf tubes containing 900µl of M_9 minimum salt medium 5X culture cells were vortex by placing Eppendorf tubes on a Heidolph REAX 2000 (Heidolph, Germany), tubes were spin at the highest speed this is to ensure proper dispersal of cells. Bacterial cultures were concentrated by calibrating at OD_{600} to 0.8nm as measured from the Eppendorf biophotometer (Eppendorf AG. 22331 Hamburg, Germany) and 100µl of the calibrated concentrate was pipette into each of the flasks. The flasks were made in triplicates (replication) to determine whether response differences were due to the treatments or just chance differences between the treatments caused by other factors. Flasks were placed on a rotatory shaker (Ika-Schüttler MTS 4) and maintained at 100 rpm for 60 days.

2.6.2 CO_2 Measurement experiment

Materials

150ml Erlenmeyer flasks

LDPE polymer (1.0g)

Rubber bung

Method

50ml M_9 minimum salt medium 5X fortified with solution 2 (1000x) and 1.0g of virgin LDPE polymer pellets were added into 150ml Erlenmeyer flasks. Colonies of bacterial isolates used were scooped from Petri dish plates into the culture. The flasks were sealed with a rubber bung and kept on a rotatory shaker at 100 rpm for 20days

2.7 Statistical analysis

A T-statistical test was carried out to test the statistical significance of the colony count of the stimulated cultures and the unstimulated cultures as well as for the weight loss experiment.

Plate count, (H_0): there is no significant difference in means between the plate count of the unstimulated cultures of *Rhodococcus degradans* R1-1a and *Pseudomonas silensiensis* R4-2g and the stimulated cultures ($p > 0.05$).

Weight loss, (H_0): there is no significant difference in mass (2.0g) between treated LDPE and the control samples ($p > 0.05$)

3. Results

The degradative potential of these strains was investigated on LDPE granules in laboratory conditions.

3.1 Growth of bacteria strains on Tryptic Soy Broth (TSB)

Bacteria strains produced gelatinized substances in TSB, although this was not further investigated, it would be worth a while to look into this in further studies. Hence, during the assessment or quantification of bacteria growth, the pour plate technique rather than the spread plate was used.

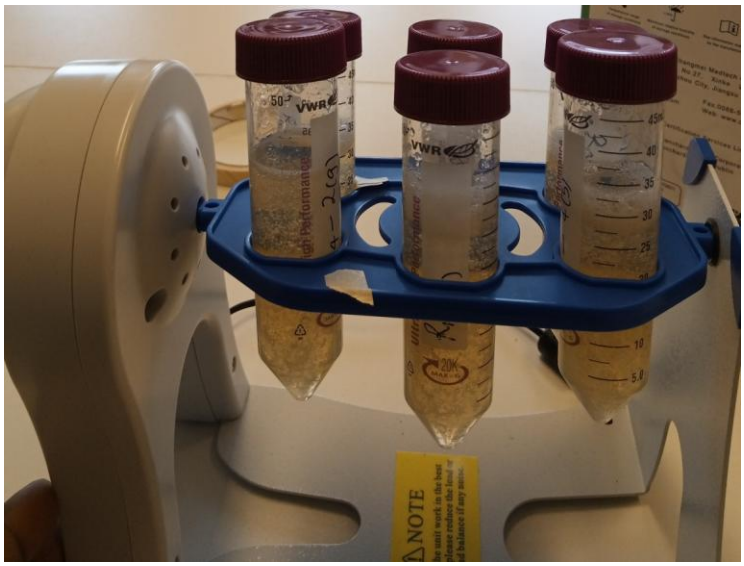


Figure 7: Bacterial strain in TSB

3.2 Assessing Bacteria Growth on LDPE.

Microbial growth on LDPE was assessed basically by two means, 1. By monitoring metabolic activities via ATP measurements and 2. Via viable plate count.

3.2.1 ATP measurements

All ATP measurements were done over 38 days and measurements are given in relative light units (RLU) via an illuminometer.

Table 2: Total ATP Values of the bacterial strains after 38 days inoculation with LDPE

Total ATP Values (RLU)					
Species	14days	20days	26days	32days	38days
R1-1(a)	409	457	503	754	867
R4-2(g)	242	284	383	487	432
R4-3(d)	22	14	23	26	28
R4-2(h)	14	11	15	25	26
R4-2(c)	65	78	52	18	11
R1-4(b)	15	12	17	24	28
Negative control	1	1	1	1	2

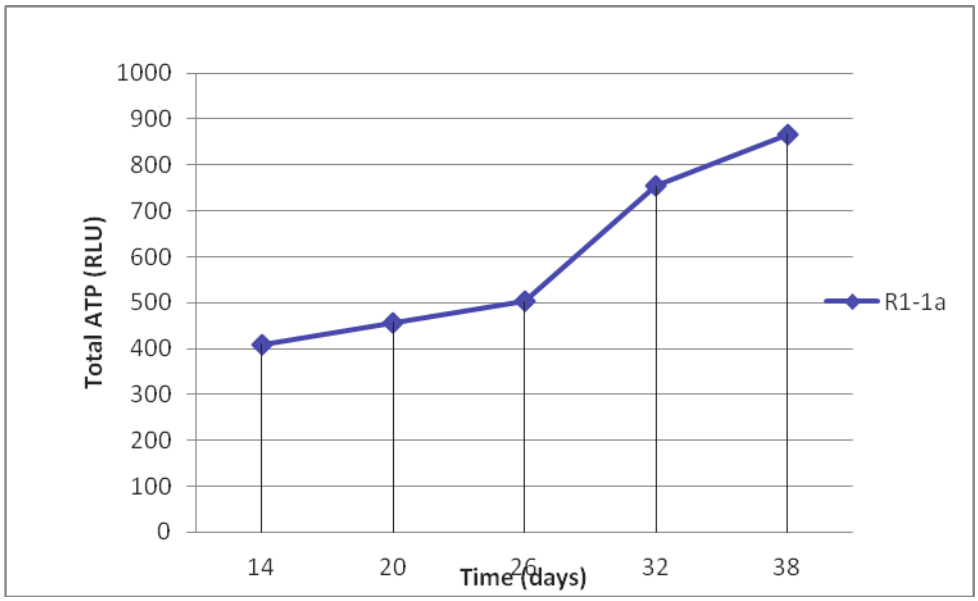


Figure 8: Total ATP values of *Rhodococcus degradans (R1-1a)*

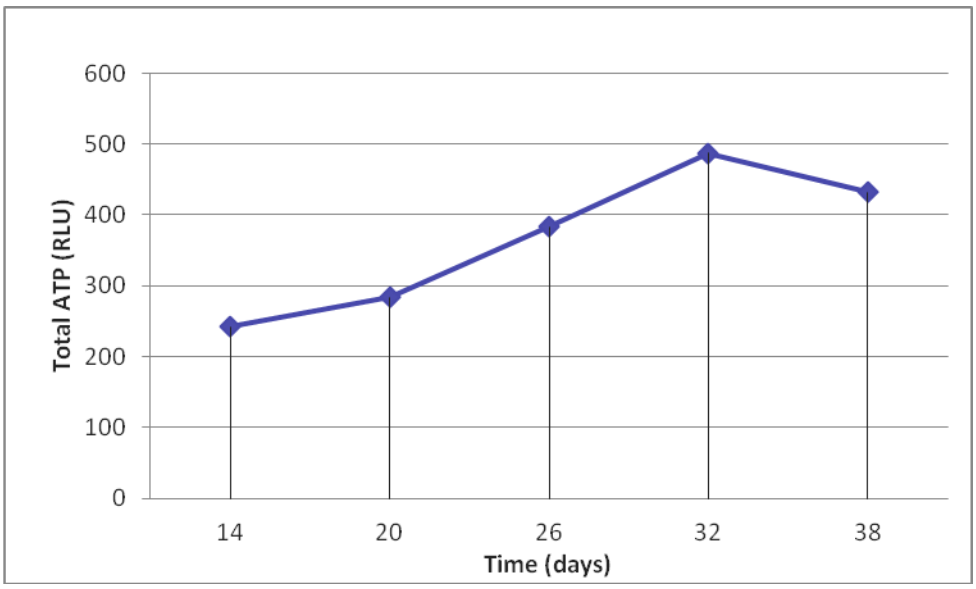


Figure 9: Total ATP value of *Pseudomonas silensiensis (R4-2g)*

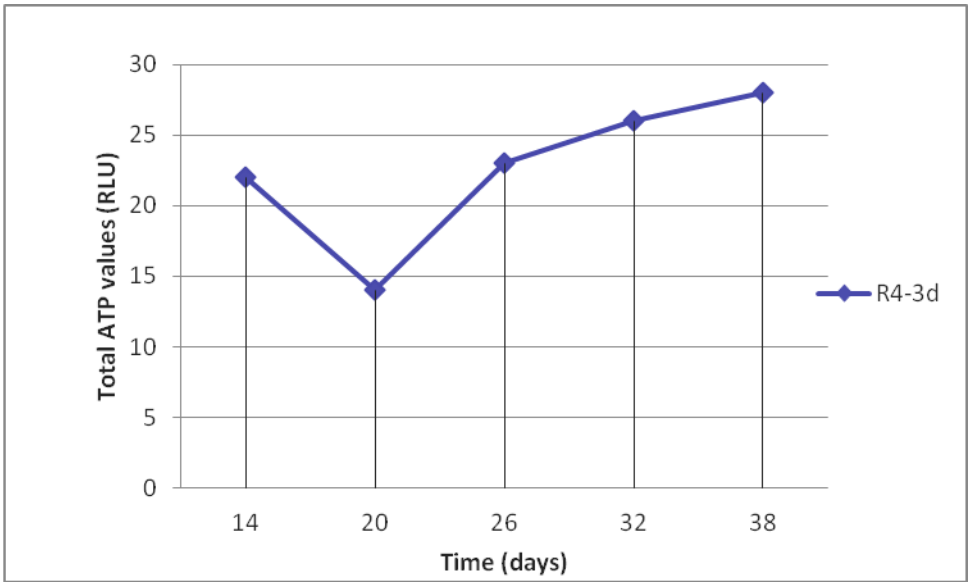


Figure 10: Total ATP values for *Pseudoarthrobacter phenanthrenivorans (R4-3d)*

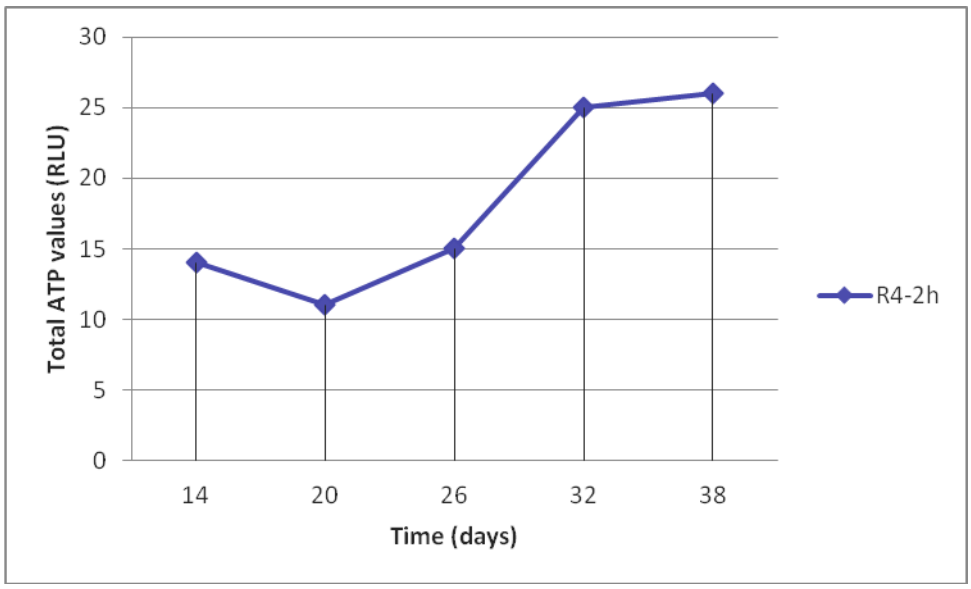


Figure 11: Total ATP values for *Microbacterium hydrocarbonoxydans (R4-2h)*

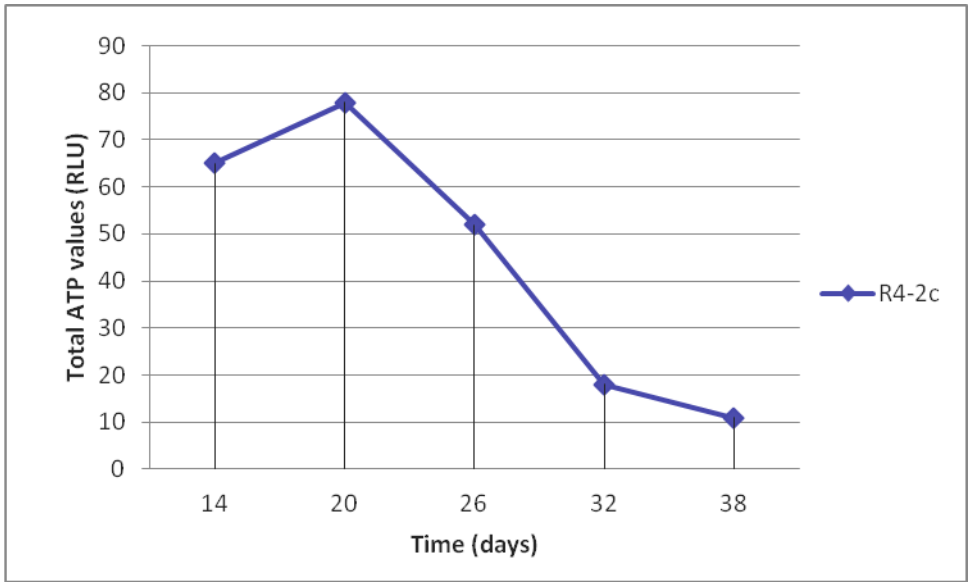


Figure 12: Total ATP values for *Paraburkholderia xenovorans (R4-2c)*

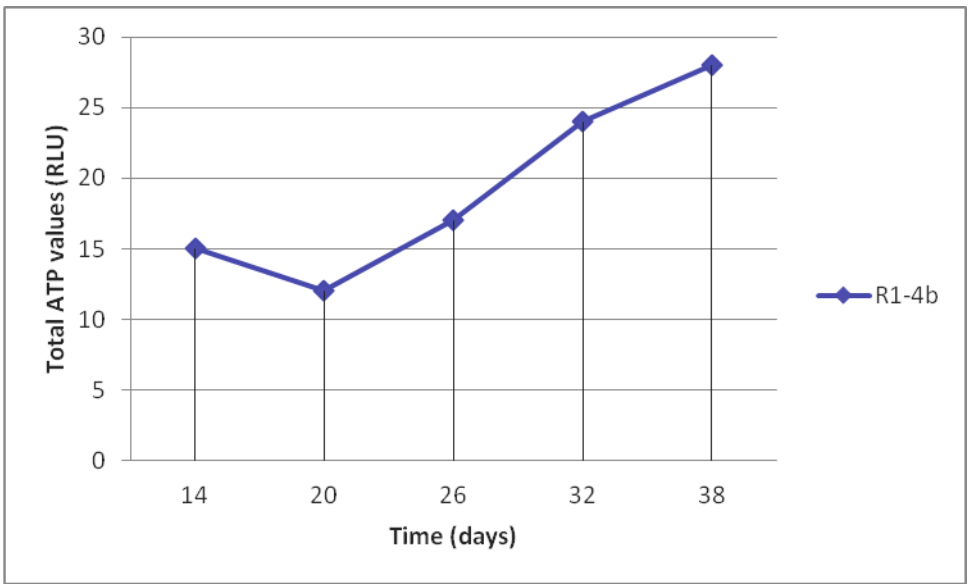


Figure 13: Total ATP values for *Pseudoarthrobacter sulfonivorans (R1-4b)*

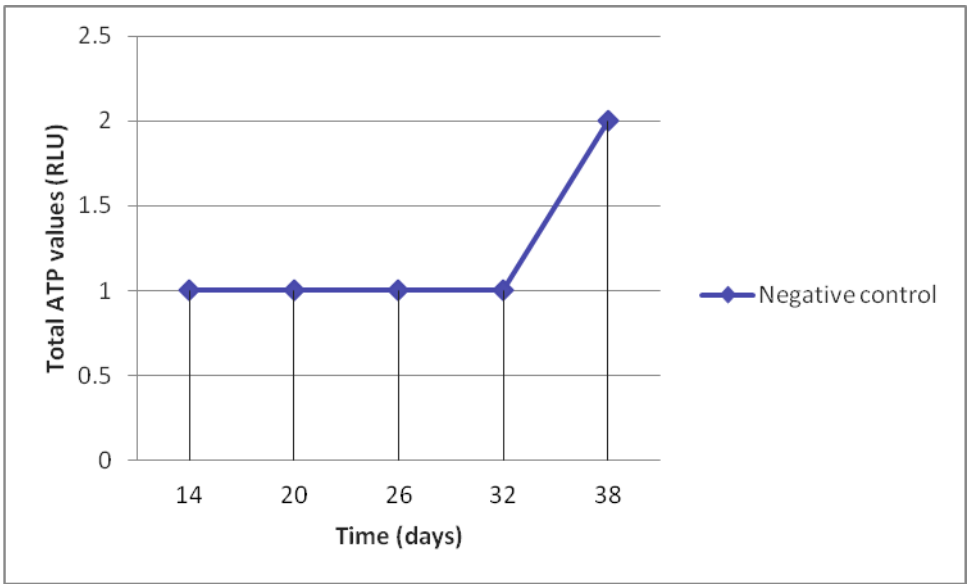


Figure 14*: Total ATP values for Negative control*

3.2.2 Viable Plate Count

Plate counts were carried over 175 days. All values are given in colony-forming units per milliliter (CFU/ml).

Table 3: Viable Colony count for unstimulated cultures

Time (days)	***Rhodococcus degradans***	***Pseudomonas silensiensis***	**Negative control**
41	670	11700	0
46	710	8500	0
87	1870	870	0
97	90	0	0
99	140	0	0
105	600	210	0
112	680	0	0
116	300	940	0
120	300	270	0
123	120	260	0
126	120	0	0
130	10	810	0
134	10	0	0
137	160	140	0
141	110	0	0
148	120	1390	0
151	10	0	0
164	200	1340	0
168	350	2100	0
175	440	2500	0

Table 4*: Viable colony count for stimulated cultures*

Time (Days)	*Rhodococcus degradans* R1-1a (Stimulated)	*Pseudomonas silensiensis* R4-2g (stimulated)	Negative control
41	370000	245000	0
46	450000	11500	0
87	253000	18700	0
97	107000	130000	0
99	175000	233000	0
105	192000	201000	0
112	179000	193000	0
116	201000	52000	0
120	170000	76000	0
123	135000	63000	0
126	68000	510000	0
130	102000	640000	0
134	67000	123000	0
137	102000	840000	0
141	29500	650000	0
148	54000	180000	0
151	92000	239000	0
164	20300	10300	0
168	288000	1510000	0
175	192000	840000	0

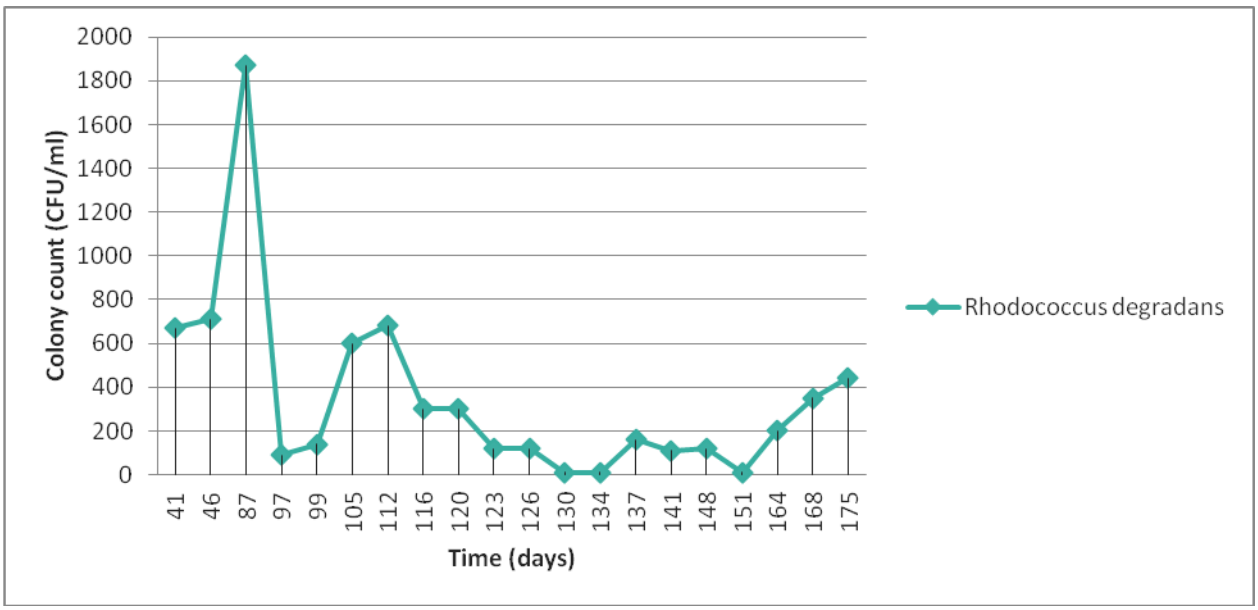


Figure 15: Viable colony count curve for unstimulated cultures of *Rhadococcus degradans* R1-1a

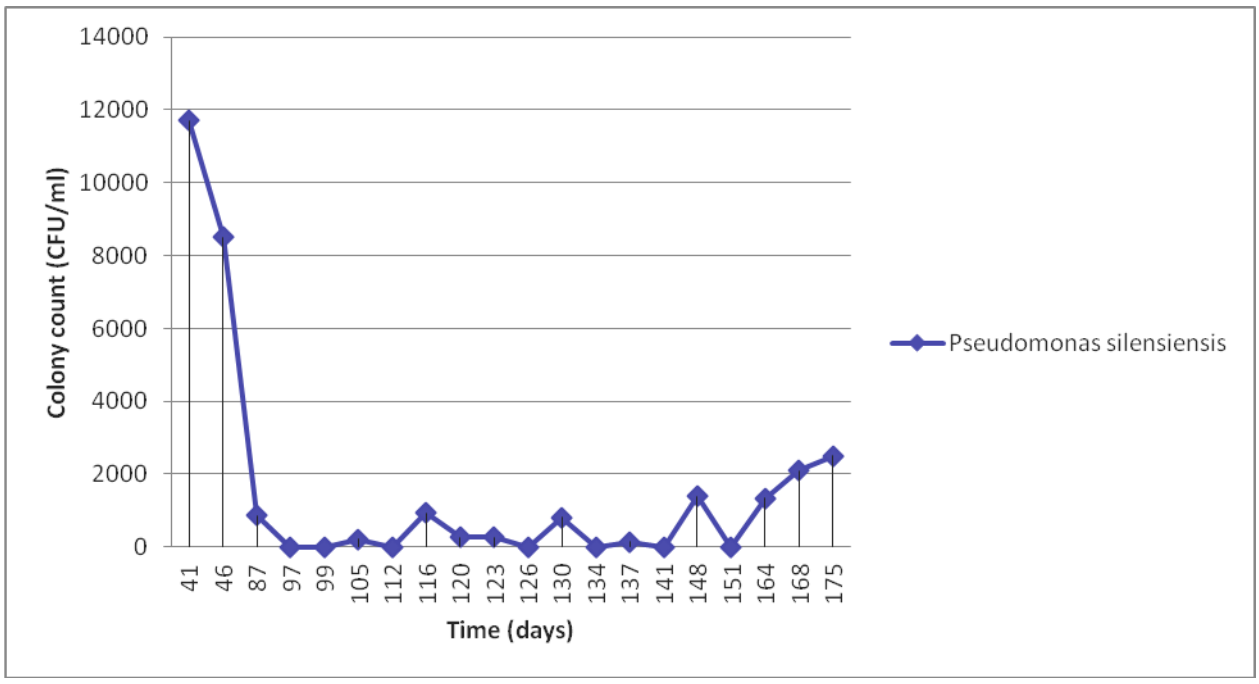


Figure 16*: Viable colony count curve for unstimulated cultures of Pseudomonas silensisnsis R4-2g*

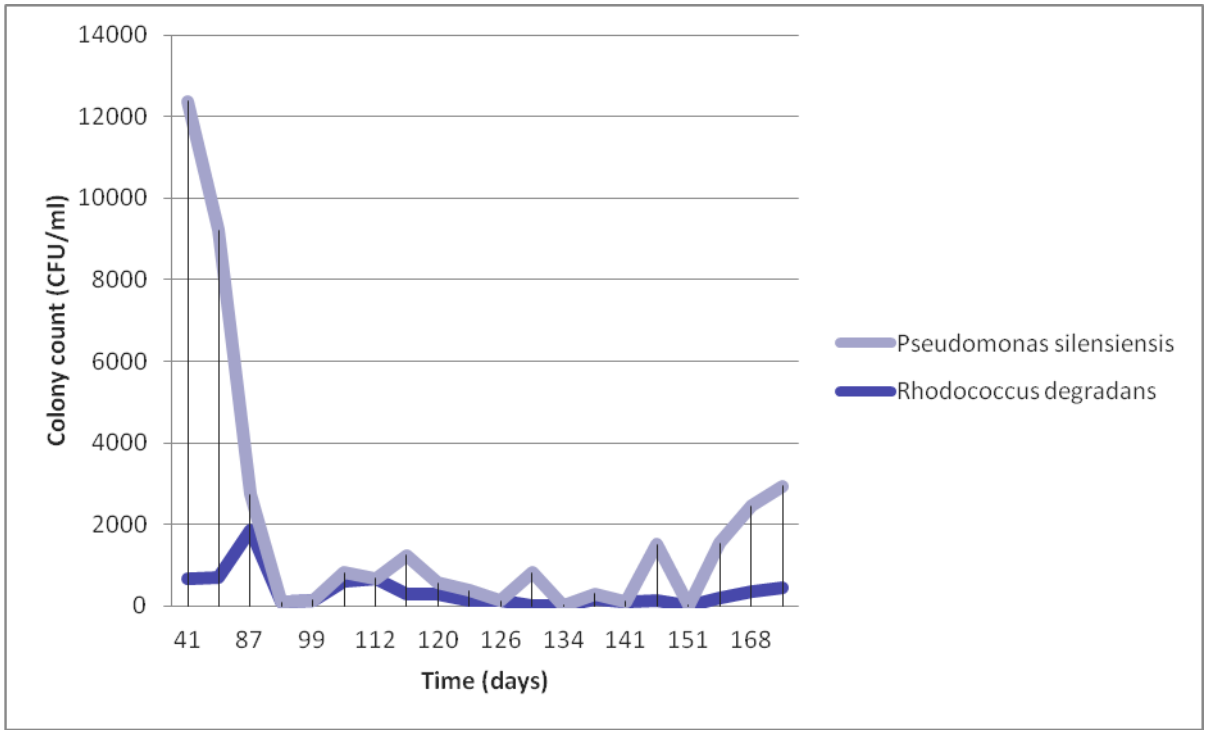


Figure 17: Viable colony count curve for unstimulated cultures of *Rhadococcus degradans* R1-1a and *Pseudomonas silensisnsis* R4-2g

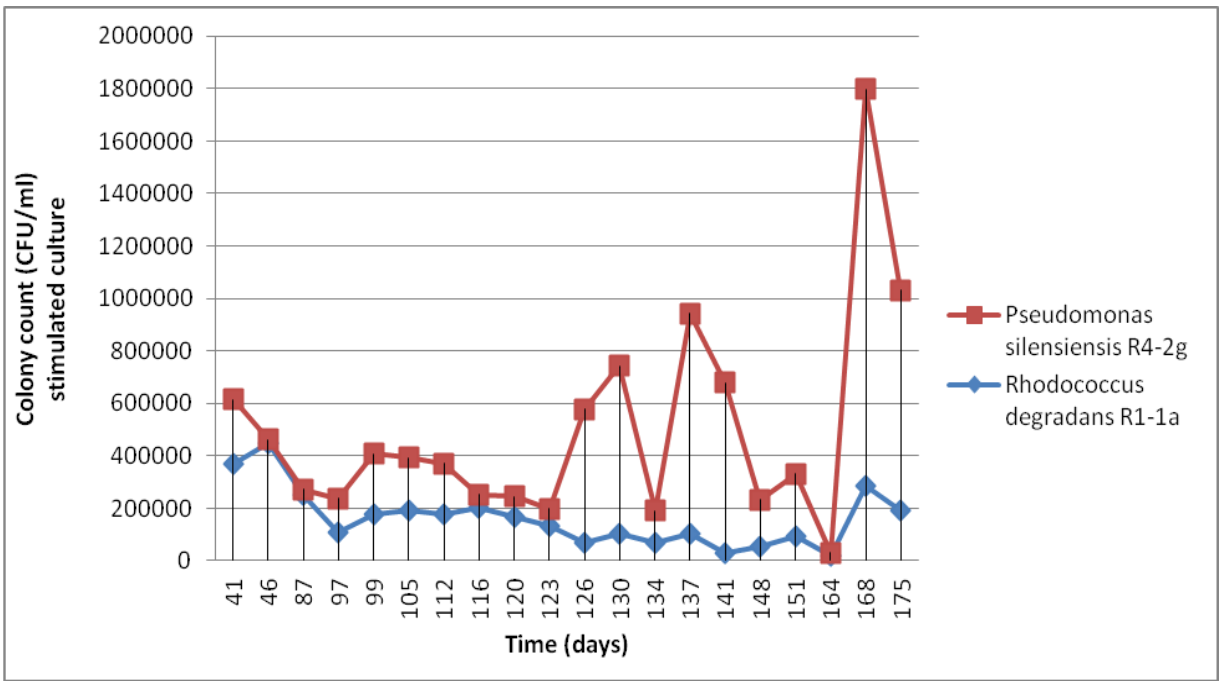


Figure 18: Viable colony count curve for stimulated cultures of *Rhodococcus degradans* R1-1a and *Pseudomonas silensisnsis* R4-2g

3.3 Statistical Analysis on weight-loss experiment

Table *5*:Two-Sample t-Test assuming unequal variance for mixed cultures and R4-2g and Negative control *of R1-1a*

	Rhodococcus degradans/Pseudomonas silensiensis	*Negative control*
Mean	1.566666667	2
Variance	0.003333333	0
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	-13	
P(T<=t) one-tail	0.002932577	
t Critical one-tail	1.49048E-08	
P(T<=t) two-tail	0.005865153	
t Critical two-tail	0.816496581	

Table 6:Two-Sample t-Test assuming unequal variance cultures of R1-1a and Negative control

	Rhodococcus degradans	*Negative control*
Mean	1.866666667	2
Variance	0.003333333	0
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	-4	
P(T<=t) one-tail	0.028595479	
t Critical one-tail	1.49048E-08	
P(T<=t) two-tail	0.057190958	
t Critical two-tail	0.816496581	

Table 7:Two-Sample t-Test assuming unequal variance of R4-2g and Negative control

	Pseudomonas silensiensis	*Negative control*
Mean	1.933333333	2
Variance	0.003333333	0
Observations	3	3
Hypothesized Mean Difference	0	
df	2	
t Stat	-2	
P(T<=t) one-tail	0.09175171	
t Critical one-tail	1.49048E-08	
P(T<=t) two-tail	0.183503419	
t Critical two-tail	0.816496581	

3.4 Statistical Analysis on plate count between the stimulated cultures of R1-1a and R4-2g

Table 8: Two-Sample t-Test assuming unequal variance of plate count between stimulated and unstimulated cultures of *Rhodococcus degradans*

	Rhodococcus degradans R1-1a(s)	*Rhodococcus degradans R1-1a(n)*
Mean	162340	350.5
Variance	12485254105	182120.7895
Observations	20	20
Hypothesized Mean Difference	0	
df	19	
t Stat	6.483357987	
P(T<=t) one-tail	1.63345E-06	
t Critical one-tail	1.729132792	
P(T<=t) two-tail	3.2669E-06	
t Critical two-tail	2.09302405	

Table 9: Two-Sample t-Test assuming unequal variance of plate count between stimulated and unstimulated cultures of *Pseudomonas silensiensis R4-2g*

	Pseudomonas silensiensis R4-2g(s)	*Pseudomonas silensiensis R4-2g(n)*
Mean	338275	1551.5
Variance	1.48497E+11	9362392.368
Observations	20	20
Hypothesized Mean Difference	0	
df	19	
t Stat	3.907646232	
P(T<=t) one-tail	0.000473071	
t Critical one-tail	1.729132792	
P(T<=t) two-tail	0.000946141	
t Critical two-tail	2.09302405	

4. Discussion

4.1 Assessing growth on LDPE

Six bacterial strains were screened initially in laboratory conditions and room temperature for their ability to grow on and degrade LDPE as their sole carbon source. All testing for ATP values was done after 14 days of inoculating LDPE with the test isolates.

The ATP values from two strains R1-1a and R4-2g showed promising metabolic activities on the culture media containing LDPE. There was a steady increment of the ATP measurement indicating that these strains can form biofilms on the plastic polymer and hence use it as a carbon source for energy production. Although the ATP values for R4-2(c), R4-2(h), R1-4(b), and R4-3(d) showed a steady rise but their values were too low to pursue further investigative measures on their biodegradative potentials.

The plate count method was also used to assess the growth activity of the bacterial strains on the LDPE polymer. Cultures of R4-2c, R4-3d, R4-2h, and R1-4b were no longer used in this experiment because they showed no colony formation after several days of incubation, hence the continued use of these cultures was stopped.

R1-1a and R4-2g showed an intermittent growth curve, seen as a continuous rise and fall of colony numbers for the plate count experiment. The intermittent rise and fall in the growth curve seen could be attributed to the insertion of a functional group on the carbon atoms making it more hydrophilic which is consistent with the mechanism of polymer biodegradation as proposed by Arutchelvi et al, 2008. The strains attach to the hydrophilic carbon and start growing by using the carbon as an energy source and once this carbon becomes exhausted the energy source decreases and plate count drops considerably. Another consideration to this is the fact that when there is hydrophilic carbon available, the strains forms bio-films and attaches to the carbon, during this process viable colonies reduces in the culture media but as soon as the hydrophilic carbon is used up the polymer becomes more hydrophobic hence the surface becomes unavailable for biofilm formation or strain attachment, more viable colonies are found in the culture media hence increase the colony count. These are just claims but further

analysis like FTIR and SEM analysis needs to be done to further analyze functional group formation and polymer structure respectively to ascertain these claims.

The stimulated cultures of R1-1a and R4-2g also showed a similar intermittent growth curve pattern as the unstimulated cultures. The need for stimulating the cultures was to investigate how degradation can be accelerated with the view that LDPE polymer can be very slow to biodegradation due to its physical properties, hence the idea of a co-metabolite that can easily be degraded and have the same metabolic pathway as LDPE degradation can be used stimulate the bacterial strain metabolic activities which in turn can produce an increase in degradation of the LDPE polymer. According to Montazer et al, 2020, enzymes involved in alkane hydrolyses are the best-studied enzymes for PE degradation, hence with the use of baby oil which contains shorter hydrocarbon chains as a co-metabolite, their degradation produces enzymes that have the possibility of hydrolyzing the surface of LDPE.

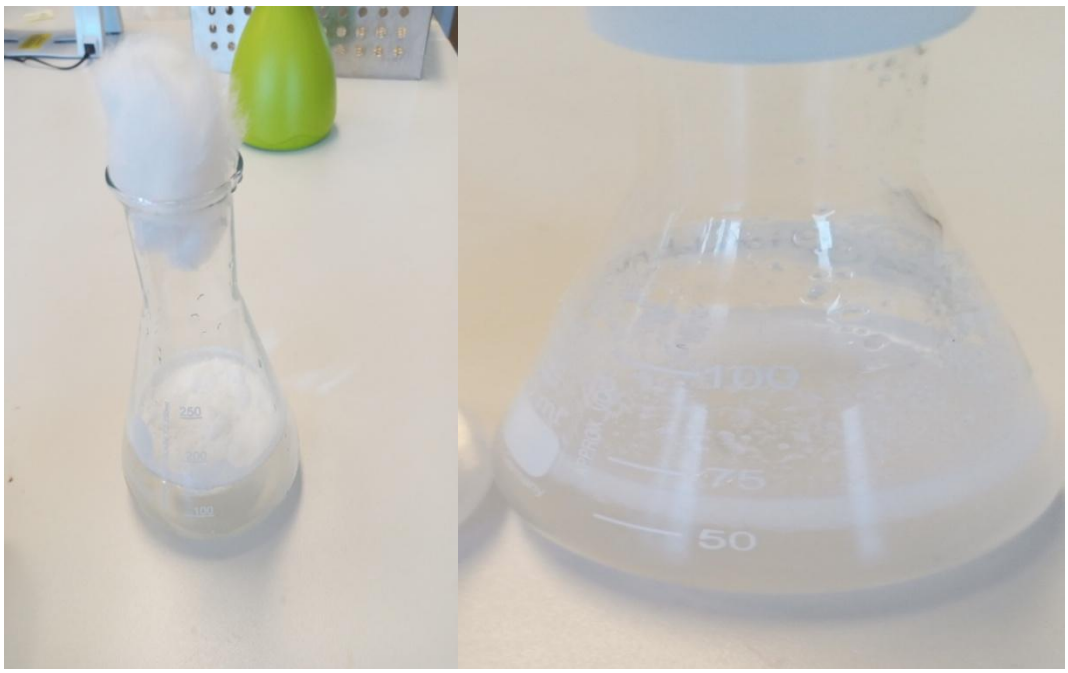
Plate count of R1-1a stimulated and R1-1a unstimulated showed that there is a significant difference in colony count ($p<0.05$), while there was also a significant difference in colony count between the stimulated cultures of R4-2g and unstimulated cultures of R4-2g ($p<0.05$). The stimulated cultures show a higher plate count as compared to the unstimulated cultures but these results of stimulation were inconclusive because a parallel culture of the bacteria strains on baby oil alone was not set up to compare their growth curves. It shows that the bacteria strains can grow on cultures of baby oil and further experiment can be carried out to ascertain this claim that stimulation can accelerate LDPE polymer degradation.

4.2 Biodegradability test

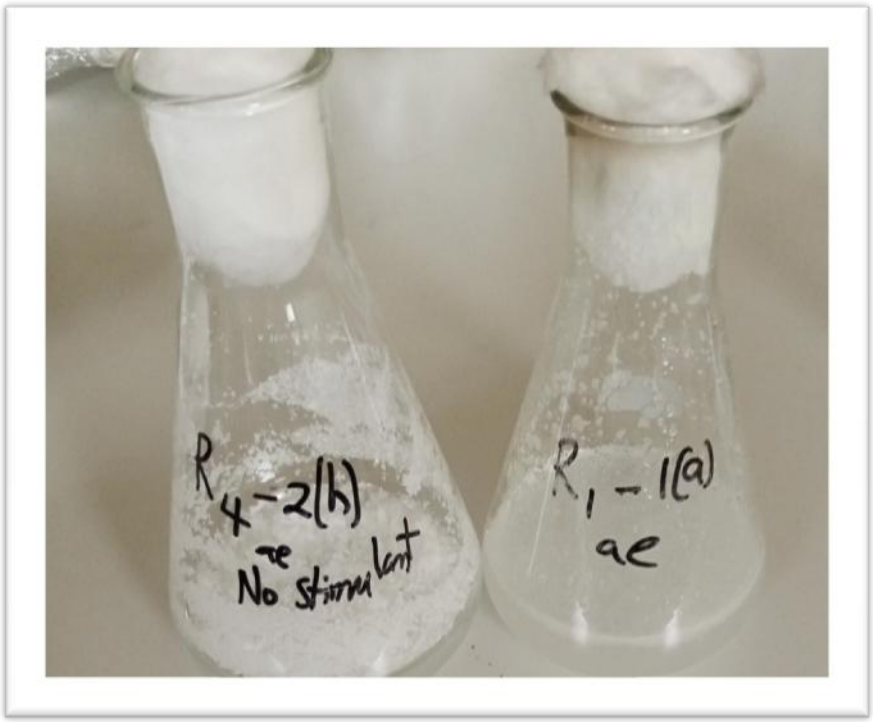
4.2.1 Visual observation

Evaluation of visual changes in the plastic polymer can be used as a test for biodegradation. Although these changes cannot be used to ascertain the metabolism of the polymer it indicates microbial attack (Alshehrei, 2017).

The LDPE polymer at the point of inoculation of the bacterial strains was whitish, viscose and highly hydrophobic, but throughout plate count, the plastic became more transparent, less viscose, and less hydrophobic as seen in the figure below



a b



c

Figure 19: (a) LDPE culture without microorganism (negative control), (b) LDPE cultured with R4-2(g), (c), Visual nature of LDPE after 148days of degradation. Flask 1 (left), contains LDPE inoculated with R4-2(h), plastic is still whitish, viscous, and hydrophobic, Flask 2 (right), inoculated with R1-1a, LDPE is transparent and more water-soluble.

4.2.2 Weight loss experiment

Weight loss is one way to measure the biodegradation of PE polymer. Polymer degradation has been shown to occur on the surface of the polymer, hence, weight loss is the proportionality of the surface of polymer degraded by microorganisms (Das & Kumar, 2015).

After 60 days of incubation, the bacterial biofilm was washed off from the surface of the LDPE with a 2% (v/v) aqueous Sodium dodecyl sulfate (SDS) for 4hrs (using a shaker), and this solution was drained off the plastic using a filter paper followed adequate rinsing with distilled water and finally with 70% ethanol to ensure maximum removal of cells and debris. The washed LDPE pellets were dried for 3 days at room temperature before weighing.

The mean weight was computed and the percentage change in weight was determined by the formula;

Percentage change in weight = Change in weight/Original weight × 100% (Muhonja et al, 2008).

Table 10:*Percentage change in weight of 2.0g of LDPE upon 60 days of incubation*

Species	Mean weight ± Standard error	Percentage change in weight
R1-1a	1.87 ± 0.033	6.5%
R4-2g	1.93 ± 0.033	3.5%
R1-1a/R4-2g	1.57 ± 0.033	21.5%
Negative control	0	100%

The results of the weight loss weight experiment were done not the compare the degradative ability of the bacterial isolates rather show their plastotrophic ability. The weight loss experiment shows that there was no significant difference in mean weight loss between the initial weight of LDPE and final weight of LDPE after 60 days of incubation at $p>0.05$ with cultures of *Rhodococcus degradans* and *Pseudomonas silensiensis.* But a mixed culture of both had a significant mean weight loss difference as compared to the initial weight of the LDPE used in the negative control ($p<0.05$). This marked a 6.5% and 3.5% mean weight loss respectively for *Rhodococcus degradans* and *Pseudomonas silensiensis*, while a mixed culture of both showed a higher percentage of 21.5% mean weight loss reduction. These results show that the bacterial isolates break down very slowly LDPE for their carbon and energy source and even do it faster as mixed cultures.

These results can be compared to a reported weight loss of PE sheets by (Ingavale & Raut, 2018) who reported a mean weight loss of PE sheets treated with *Bacillus weihenstephanensis* and observed a reduction of 7.02% and 7.08% for both LDPE and HDPE after 6 months of incubation. Muhonja et al (2018), also reported a mean weight loss of 35.72 ± 4.01%, 20.28 ± 2.30%, 2.80 ± 0.38% by *Bacillus cereus* strain A5, a(MG64264), *Brevibacillus borstelensis* strain B2, 2(MG645267) and *Pseudomonas putida* strain B1, 1a(MG645383) on a 30-micron polyethylene sheet following a 16 weeks incubation at 28^{o}C.

4.2.3 Carbon dioxide (CO_2) evolution test

The effervescence of CO_2 can be taken as a parameter for direct breakdown and mineralization of an organic substance, hence, CO_2 evolution can be used in the determination of biodegradation of a polymeric material (van der Zee, 2011).

The Dräger Accuro CO_2 measurement system was used. Flasks containing LDPE and bacterial cultures are connected to another chamber (Dräger tubes) holding a quantity of CO_2 sorbent. Pressure within the flasks is increased to force out CO_2 into the Dräger tubes containing CO_2 sorbent. The amount of CO_2 evolved is directly measured from the gradated tubes as indicated by the color change (from white to light violet/blue-violet) of the CO_2 sorbent.

Table 11: CO_2 Measurement in Parts per Millions (ppm)

Bacterial isolates	CO_2 measurement (ppm)
R4-2g (20days of incubation)	400
R4-2g (5 months of incubation)	100
R1-1a (20days of incubation)	150
R1-1a (5months of incubation)	100
R1-1a/R4-2g (4 months of incubation)	250
Negative control (20days of incubation)	<50

The result above shows CO_2 emission by the bacterial isolates used in the experiment. The evolution of CO_2 suggests that the carbon source (LDPE) is oxidized by the bacterial isolates for their energy requirement and metabolic activities.

Pseudomonas silensiensis R4-2g, recorded higher CO_2 concentration (400ppm) after 20days of culturing in LDPE as compared to the concentration (100ppm) evolved after 5months of culturing. The same pattern was also seen with *Rhodococcus degradans* (R1-1a) which recorded a concentration of CO_2 of 150ppm after 20days of incubation as compared to the concentration of 100ppm after 5months of culturing, while a mixed culture of R1-1a and R4-2g recorded a concentration of 250ppm of CO_2.

LDPE polymers consist of branched-chain regions (amorphous regions) which reduce the crystallinity and the packing density of the polymer (Arutchelvi et al., 2008; Fesseha & Abebe, 2017). These amorphous regions are easily degraded by microorganisms as compared to the crystalline region of the polymer. Hence, the higher CO_2 concentration of R4-2g and R1-1a within a shorter time of culturing (20days) as compared to the CO_2 concentration after 5 months of culturing might be due to the rapid biodegradation of these branched amorphous regions and as these regions diminish LDPE becomes even

more difficult to biodegrade hence the reduction of CO_2 concentration, though more samples need more testing to ascertain this claim.

Of the 6 isolates used in this study, 2 of the isolates (*Rhodococcus degradans and Pseudomonas silensiensis)* shows solid evidence of their ability to degrade LDPE polymer as shown from their continuous growth in LDPE cultures as seen in plate count, weight loss experiment, and effervescence of CO_2 from LDPE polymer. These microbes need a source of energy for their metabolic activities and because the LDPE is the only source of carbon present in the culture setup it must be that the carbon source for their metabolic activities might only be coming from the LDPE polymer. Although there might be a school of thought that viable microbes can use the nutrient from dead microbes for their energy source hence a possible source of CO_2 evolution hence it is quite imperative for the physical structure of the polymer to be analyzed by FTIR and SEM to confirm the biodegradation of this polymer.

5. **Recommendation**

Polymer usage has become intrinsic to our everyday activities, which has also led to a huge pile of plastic litters in the environment; hence we have seen this thesis that microorganisms can degrade PE in the environment but the real aim is to make this degradation even occur at a faster rate. One way to facilitate faster microbial degradation of polymer is to pre-treat this polymer. These pre-treatment could range from thermal, ultra-violet irradiation, gamma irradiation, etc. these treatment weakens the bonds of these polymer, reduces its hydrophobicity and viscosity of the polymer, and introduce functional groups on the polymer that makes them more accessible to microbial biofilms formation and biodegradation. *Rhodococcus ruber* C28 recorded weight loss of 8% of photo-oxidized PE after 4weeks which is higher than the rates already reported 3.5% to 8.4% after 10 years (Arutchelvi et al., 2008). Hence, it would be nice to pre-treat LDPE polymers first before culturing with *Rhodococcus degradans* R1-1a and *Pseudomonas silensiensis* R4-2g, hopefully, it will make for a fascinating result.

Bacterial degrade plastics with the help of secreted extracellular enzymes. These enzymes are coded for by the genes carried on plasmids (degradative plasmids).

Plasmids are circular double-stranded deoxyribonucleic acid molecules that can replicate autonomously in their host cell. The plasmid varies in length/size, copy number, or compatibility/incompatibility group (Miljković-Selimović et al., 2007; Thomas, 2014). The genes coding for by these plasmids may confer a special advantage to the host bacteria such as antibiotic resistance and degradation of xenobiotic/recalcitrant substances. Plasmids can be transferred from one bacterial cell to another via conjugation, transformation, or transduction. The transfer of plasmids between microorganisms via horizontal transfer aids the evolutionary development of microorganisms (Shintani et al., 2015). We can tap into this technique of plasmid transfer to increase the plasmid content of degradative bacteria in other isolates to increase the biodegradation of plastics in our environment.

6. **Conclusion**

LDPE polymer makes for one of the most single-use plastic materials worldwide thus also creating the most plastic litter problem around the world. This polymer due to its high molecular weight and hydrophobic surfaces makes them highly resistant to microbial degradation hence can persist in the environment for many years. Although various plastic degrading methods are available, the cheapest and most eco-friendly method is degradation using microbes, hence, the quest by researchers to investigate microbes with an evolved potential of degrading these recalcitrant polymers.

In this study, two isolated strains of microorganisms *Rhodococcus degradans and Pseudomonas silensiensis* were found to be useful for the biodegradation of LDPE, which is the first time reported with applicable evidence. However, further research is still needed to further equip these microorganisms to ensure the complete and faster mineralization of this recalcitrant polymer.

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Annexes

*Annex 1**: Procedure and Preparation of Stock Solution***

1. 5X M_9 Minimum Salt Solution **(Sigma-Aldrich, St Louis, USA)**

   Recipe

   5X M_9 minimum salt = 5.6g

   Distilled water = 500ml

   Pour into a 500ml bottle. Mix well and autoclave for 20 mins at 121^{o}C. Mix well by inversion after autoclaving and store at room temperature.

2. Fortified M_9 Minimum Salt Solution

   Recipe

   5X M_9 minimum salt = 5.6g

   Distilled water = 500ml

   Trace element solution 2 (1000x) = 500µl

   Pour into a 500ml bottle; thoroughly mix by shaking and then autoclave for 20 mins at 121^{o}C. Store at room temperature.

3. **Half-Strength Tryptic Soy Agar (TSA)** (Merck KGaA, Darmstadt, Germany).

Composition in g/litre: Pancreatic digest of casein (15.0), Papaic digest of soya bean meal (5.0), Sodium chloride (5.0), agar-agar (15.0).

Receipe

TSA = 10g

Agar = 5g

Distilled water = 500ml

Pour into a 500ml cylindrical bottle. Mix very well by shaking and inversion, autoclave for 20 minutes at 121^{o}C, and store at 50^{o}C in an incubator to prevent solidification of the media.

The receipe for the half-strength TSA agar used were as proposed by Abiriga et al., 2021.

*Annex 2****: Bacterial cultures in aluminum foil wrapped Erlenmeyer flasks on the rotor.***

*Annex 3****: The Accuro setup for CO2 Measurement***

*Annex 4**: descriptive statistics of R1-1a; R4-2g; and, R1-1a/R4-2g***

R4-2g	
Mean	1.933333333
Standard Error	0.033333333
Median	1.9
Mode	1.9
Standard Deviation	0.057735027
Sample Variance	0.003333333
Kurtosis	
Skewness	1.732050808
Range	0.1
Minimum	1.9
Maximum	2
Sum	5.8
Count	3
Confidence Level (95.0%)	0.143421758

R1-1a	
Mean	1.866666667
Standard Error	0.033333333
Median	1.9
Mode	1.9
Standard Deviation	0.057735027
Sample Variance	0.003333333
Kurtosis	
Skewness	-1.732050808
Range	0.1
Minimum	1.8
Maximum	1.9
Sum	5.6
Count	3
Confidence Level (95.0%)	0.143421758

Consortium *(R1-1a/R4-2g)*	
Mean	1.566666667
Standard Error	0.033333333
Median	1.6
Mode	1.6
Standard Deviation	0.057735027
Sample Variance	0.003333333
Kurtosis	
Skewness	-1.732050808
Range	0.1
Minimum	1.5
Maximum	1.6
Sum	4.7
Count	3
Confidence Level (95.0%)	0.143421758