Project write-up submitted to The University of Nottingham in partial fulfilment of the requirements for the degree of Bachelor of Science in Biotechnology.

Evaluation of Microbial Degradation of Polystyrene

Name : **Kwon Jun Lee**
Student ID : **4288856**
Supervisor : **Dr. Nagamani Bora**
Abstract length : **167**
Word count : **4994**

Keywords : **Polystyrene, Biodegradation, PHA**

Abstract

Polystyrene, a synthetic hydrocarbon made from styrene monomers, have been a key player in the development of modern society, being used everywhere from food packaging to shipping containers. However, the mass production of nonbiodegradable polymer such as polystyrene caused unrepairable damage to the environment. With 4.9 billion tons of polystyrene ending up in waste and into the environment. Although recycling is possible, it is not considered economical causing many countries to dispose waste polystyrene instead of recycling. In this study, microbial biodegradation of polystyrene in conjunction with production of biodegradable polymer were conducted to study the potentials of microbial bioconversion of synthetic, non-biodegradable polymer into an environmentally friendly biopolymer, polyhydroxyalkanoate (PHA) under minimal salt media. Although the biodegradation of polystyrene by isolated bacteria were not as high as previously known isolates, the accumulation of PHA was exhibited. The presence of intracellular PHA granules were examined using Sudan Black B staining. This study suggests that microbial bioconversion of polystyrene is possible and potential solution to polystyrene waste issue.

1 Introduction

1.1. Current status

Ever since the introduction of plastics in 1930, plastic became a key component in modern manufacturing process (Klump 2014). Plastics are synthetic hydrocarbon polymers that are lightweight, durable, moisture resistant, strong and non-biodegradable. The incredible strength, resistance to elements, and light weight gave the versatility to be used in many applications. In modern times, plastic is used everywhere, from food and chemical storage to our clothing. However, plastics are made by fossil fuel and mixture of other harmful chemicals. Due to the negative impact on the nature, it is widely known as a pollutant in the soil as well as oceans (Mondal *et al.* 2019). Between 1950 and 2015, it is estimated that out of 8.3 billion tons (Bt) of plastic was produced, 4.9 Bt was discarded to landfills, oceans, and other natural environments. Then on, the discarded plastics caused many issues such as microplastics being ingestion by wild animals, polluting the soil in landfills, and even creating a zone in the ocean called plastic island(Jambeck *et al.* 2015; Wang *et al.* 2019). With degradation taking up to 1000 years, the need for quick and clean method of

decomposition of plastic is needed. This study will focus on polystyrene which covers 8.96% of United States' municipal solid waste (Barnes *et al.* 2009).

1.2. Polystyrene

Polystyrene is a thermoplastic resin made from liquid hydrocarbon called styrene (figure 1). The length of the styrene polymer depends on the temperature of the polymerization and the methods used (Matyjaszewski 1989). Because of its good processing properties, it is the most widely used plastic in the world (Klump 2014). Polystyrene is widely used in packaging as it can be made into foam material called expanded polystyrene (EPS) and extruded polystyrene (XPS) with more than 95% air making it a great insulator for hot foods and they are used in other applications such as

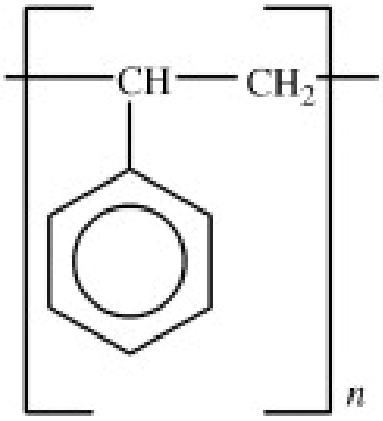


Figure 1
Structure of polystyrene

fillers for automobile industry. In Sutton Bonington campus, between October and February, weekly average usage of cup lids (polystyrene) were 675 totalling 2700 cup lids being used and disposed to garbage.

1.3. Current methods of polystyrene degradation and recycling

Current methods of disposal of plastics involve mechanical, photolytic, and chemical methods. Mechanical degradation will break down the plastic to smaller pieces so that it will become quicker and easier for photolytic degradation to work. Photolytic degradation utilises the sun's ultraviolet rays to break the bonds within the plastic polymers to reduce them to more vulnerable monomers. And finally, chemical, currently there are two forms of chemical degradation being used; using solvents to dissolve EPS to reduce the size, and to burn the plastic to get energy from their bonds

releasing CO_2 and H_2O when they are incinerated at suitable temperature (Abdel-Raouf *et al.* 2013; Mathalon and Hill 2014). However, these happen only if the polystyrene was collected and sorted before they end up in the landfills.

The collected polystyrene goes through multiple steps to be recycled. Initially all polystyrene is washed to remove all food residues, dried, crushed then melted to become a new material (Thakur *et al.* 2018). Although polystyrene is 100% recyclable, it is very difficult due to the complexity of the recycling process. Also, recycling of polystyrene is also considered economically inefficient as government regulations prevent recycled polystyrene from being used for food packaging highly reducing the market value of recycled polystyrene. As well as not being food safe, recycled polystyrene showed increased concentration of hexabromocyclododecane a chemical listed as a persistent organic pollutant under the UN Environment Programme's Stockholm Convention (UNEP 2014). Making recycling more difficult to promote.

The biodegradation of plastic is specifically related to the functional group, chemical structures and molecular weights and many polymer-degrading microorganisms have been isolated (Gu 2003). The monomers (Velasco *et al.* 1998; Santos *et al.* 2000) and oligomers(Tsuchii *et al.* 1977) of polystyrene is susceptible to biodegradation, however, fresh/ undisturbed polystyrene is considered not biodegradable due the time it takes for complete degradation to happen due to it's high stability and molecular weight (Mor and Sivan 2008). The method for the environmental biodegradation of polystyrene is needed because degradation of polystyrene under environmental exposures may produce more harm than good. Natural degradation of polystyrene may take hundreds of years depending on the conditions, monomer of polystyrene, styrene is a well-known genotoxic for mammals and considered a carcinogen for human (Gibbs and Mulligan 1997; Marczynski *et al.* 2000). And the by-products of polystyrene degradations include benzene, acetophenone, benzaldehyde, formic acid, benzoic acid, etc which are all toxic to mammals (Faravelli *et al.* 2001; Wypych 2012). These toxins are one of the biggest reasons why the need for environmentally friendly way of removal is needed.

1.4. Current Status of bacterial degradation

The attempts to identify polystyrene degrading microorganisms has been going on since 1979, and there are many organisms that has been identified to have the ability to degrade polystyrene. These microorganisms were isolated from various environments such as activated sludge, silt loam, cow manure, mealworm intestine, etc (Tang *et al.* 2017; Kaplan [n.d.]). Currently there are many bacterial isolates with potential capability of using polystyrene as carbon source. They are *Rhodococcus ruber (Mor and Sivan 2008), Exiguobacterium* sp. Strain YT2 and various fungi. The microbial degradation of polystyrene varied from 0.001% to 3% in 4 months (Yang *et al.* 2015). Also the regulatory genes responsible styrene degradation has been identified to be *styS* and *styR*(Santos *et al.* 2000).

1.5. Bioplastic

Bio-based materials like polyhydroxyalkanoate (PHA) are produced by various bacteria under stress as a storage compounds of carbon and energy source because of its structure, it has the property of biodegradable thermoplastic (Albuquerque *et al.* 2007; Chee *et al.* 2010). With some bacteria having the capability to produce PHA as much as 90% of dry cell under controlled environment (Madison and Huisman 1999). With 40% of the production cost of PHA (Choi and Lee 1997) going into the substrate costs, cheaper alternatives for substrates e.g., Starch, tapioca, whey, and molasses(Albuquerque *et al.* 2007). The preferred alternative substrates being food crops, the ethical concerns of food security are raised. In order to minimise food wastage and to reduce pollution, the substrate for PHA synthesis should be focused on waste products.

Despite having to isolate many bacteria with the capability of utilizing polystyrene as carbon source, none of the past experiments attempted to test for assimilation of bioplastic in conjunction with degradation of polystyrene.

1.6. Aim of the Study

In this study, environmental bacterial isolates were used to test for the degradation of polystyrene and the ability to accumulate bioplastic specifically PHA. In order to understand the potentials of the microbes, multiple growth and staining were done. These include growth in basal minimal salt media with polystyrene, Sudan Black B staining, and Nile Red staining (Zhang *et al.* 2005; Mesquita *et al.* 2015). It is

hypothesised that small number of bacteria capable of degrading polystyrene will have the capability to produce PHA granules under stress which will become visible after the Sudan Black B staining and Nile red staining.

2. Materials and Methods

2.1. Purification and 16S rRNA identification of environmental isolates

2.1.1. Purification and biobanking

A collection of isolates was provided by Dr Nagamani Bora isolated from previous studies. These environmental isolates contained mixture of microbial communities and required purification steps to yield pure strains to be banked in the culture collection. The environmental isolates were plated onto Tryptone soya (TS) agar and were incubated at 28°C for 72 hours. After the incubation, each distinct colony morphologies were selected to be further purified. After each colony were purified, they were inoculated in baffled flask with 25ml of TS broth for 24 hrs. After 24 hrs, all bacteria were gram stained and visualised to categorise them broadly into gram positive and gram negatives. This quick screen is necessary for grouping and also for subsequent DNA extraction procedures.

2.1.2. Genetic Isolation

Five ml of culture samples were pelleted at 5000x g for 10 minutes then resuspended in de-ionised water twice to remove any media and exoproteins produced that may affect the DNA extraction. The purified isolates were diluted to meet the OD_{600} of 1 to ensure that there were no excess cells potentially reducing the purity of the product. DNA extraction was done using Qiagen DNeasy Blood & Tissue Kit. Three main steps of DNA extraction are lysis/ extraction, purification, and elution. Extraction of DNA from bacteria is done with various chemicals and enzymes. In the case of Qiagen kit, Buffer ATL (Sodium Dodecyl sulphate) was used in conjunction with Proteinase K a wide broad-spectrum serine protease. SDS denatures nuclease and other protein while Proteinase K will lyse various protein reducing the chance of unwanted protein affecting the PCR. Specifically, for gram-positive, pre-treatment is required with additional enzymatic lysis buffer. The lysis buffer contains lysosome that will lyse peptidoglycan layers present in gram-positive cell membrane (Nash *et al.* 2006) and

Buffer AL which is mainly guanidine hydrochloride (GuHCl), a chaotropic salt that destabilises the protein bonds leading denaturisation of protein such as nucleases and RNase. With the cellular material exposed, Ethanol is added to precipitate the DNA and salt is removed allowing. The supernatant of the extraction is the moved to a collection column. The Qiagen DNA collection column uses silicas-based adsorption. Although the mechanism of DNA adsorption is not fully understood, it is believed that GuHCl acts as a salt bridge between negatively charged silica and negatively charged DNA(Vandeventer *et al.* 2013) and dehydrating the DNA backbone. Additional Guanidine salts in the AL buffer and Buffer AW1 makes the DNA bind to the column even strongly. Once all the silica has been bonded with DNA, all the unwanted materials are forced down by centrifuge. AW1 buffer is added to remove contaminates such as residual proteins. The washing is done by centrifuging the buffer through the column. Then AW2 buffer which as Ethanol is added to remove salts from the column. Then column is let to dry to allow ethanol to evaporate. As soon as column is free of ethanol, AE buffer is added. AE buffer contains Tris-base and EDTA which helps rehydration of DNA to remove it from the silica it was bounded to. The final centrifuge releases the DNA from the silica column leaving the DNA in the new collection tube.

After the DNA extraction, all samples were subjected to rep-PCR to de-replicate the strains (appendix 3). Rep- PCR generates DNA fingerprinting profiles that is specific to strains (Spigaglia and Mastrantonio 2003). REP elements are usually 33 and 40 bp in length having about 500 to 1000 copiers per genome. rep-PCR amplifies non-coding repetitive sequence in the bacterial genome (Versalovic *et al.* [n.d.]). The recipe for rep-PCR is listed on table 1 below

Table 1: REP PCR Reagent Mix (for one sample)

Ingredient	1X reaction
PCR Master Mix (10x)	5 µl
REP Forward primer	2 µl
REP Reverse primer	2 µl
$MgCl_2$	1.5 µl
dNTPs	1 µl
DNA	1 µl
PCR dH_2O	37.25 µl

Taq	.25 µl
Total	50 µl

The master mix was made by adding PCR grade dH_2O, PCR (buffer), $MgCl_2$, dNTPs, REP forward primer (REP F), REP reverse primer (REP R), and Taq polymerase was added just before the PCR It is crucial that PCR dH_2O (RNase free H_2O) is used in PCR because, any contaminations such as RNase could denature Taq polymerase inhibiting the PCR. $MgCl_2$ acts as a substrate for Taq polymerase allowing dNTPs to bind. The cycle for rep PCR is explained below.

1. Initial denaturation for 10 minutes at 95°C
2. 28 cycles
    a. Denaturation for 1 minutes at 95°C
    b. Annealing for 1 minutes at 40°C
    c. Extension for 8 minutes at 65°C
3. Final extension for 16 minutes at 65°C
4. Storage at 4°C

The amplified rep-PCR products were electrophoresed in 1% agarose gel containing 1x Tris-acetate-EDTA (TAE) and 5ul of ethidium bromide and visualised under the ultraviolet light (appendix 5). By electrophoresis, the amplified genes are separated depending on the size. From the electrophoresis, distinctly different strains were selected to be identified using 16S rRNA gene.

16S PCR sequencing was selected because it is considered an effective and rapid primary identification method (Jenkins *et al.* 2012). 16S PCR amplifies highly conserved region of the 16S ribosomal subunits (Patel *et al.* 2017). 16S ribosomal subunits consists of variable regions that is distinct to each species allowing quick identification by comparing to GenBank after sequencing. The reagents for 16S PCR are same as REP PCR but slightly different amounts of reagents are used. The primers used for 16S PCR were 27F and 1492R. For the PCR, the reagent mix was made following as per the table 2 below.

Table 2: 16S PCR Reagent Mix.

Ingredient	Amount for 1
PCR Master Mix (10x)	5 µl
27 F	1 µl
1492 R	1 µl
$MgCl_2$	1.5 µl
dNTPs	1 µl
PCR dH_2O	37 µl
Taq	.5 µl
Total	49 µl

The Taq was added right before the start of the 30 cycle 1µl of DNA was added to 49 µl of Cocktail mix. The cycle used for 16S PCR are as followed:

1. Initial denaturation for 10 minutes at 95°C. This is called hot start PCR where you add Taq before doing the PCR
2. 30 cycles
    a. Denaturation for 1 minutes at 95°C
    b. Annealing for 1 minutes at 55°C
    c. Extension for 1 minutes at 72°C
3. Final extension for 16 minutes at 72°C
4. Storage at 4°C

To purify the PCR products before sequencing, Qiagen QIAquick PCR Purification Kit was used. The kit functioned the same as the column purification used in DNeasy kit, silica binding to rRNA while removing salts and other impurities. With the target band (brightest band), PCR products were sent for sequencing to external company.

2.1.3. **Analysis of Sequences**:

The sequences for both leading and lagging strands were trimmed to remove aberrant data using Bioedit. With raw sequence trimmed, the sequence was reviewed base by base to replace base pairs (n) that the program could not interpret. 1492R sequences were flipped as sequenced data is in reverse. With both sequences edited, point of intersection is found using ClusterX function in Bioedit where it searches for base pairs

that match to form a more accurate whole sequence. The consensus sequence was assembled. Assembled consensus sequence is reviewed yet again to look for any uncertain sequence and is edited after reviewing the chromatograph and choosing the base pair with highest peak. Consensus sequence was saved in fasta format. A Blast analysis was performed on the aligned sequences on NCBI website to search for similar sequences.

2.2. Growth of Isolates on Polystyrene plastic

The degradation of polystyrene was done in a glass shake flask with magnetic stirrer bar to aerate the media on the stirrer plate at 600 RPM placed inside the incubator set at 28°C. Polystyrene was collected from local café's hot beverage cup lids. Polystyrene was cut in to small pieces avoiding crevices and corners which are thicker than flat surfaces to give uniform surface. All the pieces were sterilised in 99% ethanol for 24 hrs before measuring. The pieces of plastics were dried and weighted. 30ml of Basal Minimal Salt Media (BMSM) media was used as a growth media. The degradation took place over the course of 30 days.

Once microorganisms were identified and purified, they were introduced to polystyrene and Basal Minimal Salt Media (BMSM) specified by Zhang *et al*. 2005. Bacteria that cannot degrade polystyrene and utilise it as carbon source will not be able to survive in this media. The composition of the culture was as explained in table 3.

Table 3: Basal Minimal Salt Media (Zhang *et al.* 2005).

Ingredient	g/L
$NaNO_3$	4.0
NaCl	1.0
KCl	1.0
$CaCl_2 \times 2H_2O$	0.1
KH_2PO_4	3.0
$Na_2HPO_4 \times 12H_2O$	3.0
$MgSO_4$	0.2
$FeSO_4 \times 7H_2O$	0.001
Trace Metals	g/L

$FeCl_2$ x $6H_2O$	0.08
$ZnSO_4$ x $7H_2O$	0.75
$CoCl_2$ x $6H_2O$	0.08
$CuSO_4$ x $5H_2O$	0.075
$MnSO_4$ x H_2O	0.75
H_3BO_3	0.15
Na_2MoO_4 x $2H_2O$	0.05

2ml of trace metal solution was added post sterilization and the initial pH was adjusted to 6.8.

2.2.1. Pre-treatment of Polystyrene

Using sterile techniques, sterilised plastic was placed in the glass flask with magnetic stir bar and sponge cap to allow airflow. After ensuring the ethanol has all evaporated, 30ml of BMSM was poured. The samples were diluted with media to 0.5 OD_{600} then inoculated in the media with polystyrene. The flask was moved to an incubator with stir plate set at 600 rpm. The control was maintained (no inoculation) simultaneously this was to test the effect of BMSM on plastic without the aid of bacteria. The OD and purity of the samples were checked throughout one month to ensure the sterility of the sample and to create profile of the growth curve to confirm the growth of bacteria. After one month, polystyrene was removed from the media and washed in DW and in Ethanol to remove any biosurfactant (Satpute *et al.* 2010). If the bacteria is capable of producing biosurfactant, it will be attached to the plastic increasing the weight. Hence, it is important to ensure that plastic does not have any foreign materials attached for accurate data. Polystyrene samples were dried with filter paper then airdried to be weighted (Asmita *et al.* 2015). The colonies with capability to biodegrade polystyrene was indicated by the weight loss. Also, the degradation could be extrapolated from the growth profile. Colonies that shows continual growth indicates that the colonies are obtaining necessary nutrients from the only source of nutrients, added polystyrene. To identify colonies with PHA production, 1ml of the sample was stored to be stained with Sudan Black B (SBB) to allow bright-field microscopy to visualise PHA granules (Mesquita *et al.* 2015). Also, streak plates were stained with Nile Red to specifically visualise PHA granules under the ultraviolet transilluminator.

2.2.2. Ultraviolet treated Polystyrene

Another set of polystyrene were treated with ultraviolet to see if it increases the biodegradability of polystyrene. Vimala and Mathew, 2016 indicated that ultraviolet rays act as an initiator of polyethylene oxidation which was proven with increased degradation compared to untreated polyethylene. Similar to polyethylene, ultraviolet causes loss of the mechanical and tensile properties of polystyrene (Kiatkamjornwong *et al.* 1999). ultraviolet treated polystyrene should be easier for bacteria to utilise as carbon source as longer chains of styrene is broken down into shorter styrene by increasing the production of free radicals by photooxidation(Yousif and Haddad 2013). Styrene degradation was seen by many bacteria as it is naturally occurring compound(Shirai and Hisatsuka 1979; Duetz *et al.* 2001). To test the biodegradion of ultraviolet exposed polystyrene, it was cut in the pieces and was subjected to ultraviolet treatment using a ultraviolet transilluminator for 72hours. After the ultraviolet treatment, they were sterilised wit 99% ethanol. Once they have been sterilised for 24 hrs to remove contaminates, the rest of the steps are identical as non-ultraviolet treated samples as explained in 2.2.1.

2.3. Sudan Black B staining 3%Growth of Isolates on Polystyrene plastic

Under the nutrient lacking conditions, PHA are produced as energy and carbon source (Bhuwal *et al.* 2013). As the minimal media lacks various nutrients, if bacteria were able to produce PHA, they should have accumulated PHA granulates. The accumulated PHA could be visualised using phase contrast light microscopy, however, the use of SBB was chosen as SBB stains the intracellular granulates dark blue, allowing easy contrast (Johnston *et al.* 2018). SBB is a lipophilic stain known for high sensitivity to PHA granulates (Wei *et al.* 2011). In order to test for formation of PHA, 1ml of samples were taken at the end of the degradation. The samples were pelleted to only leaving the biomass. The pellet was washed in 1ml DW and resuspended in 1ml of DW. 100µl of DW was placed on microscope slide then 50 µl of the resuspended sample was added. The diluted sample was spread into thin layer by using sterile plastic 10µL inoculation loop. After the sample covered the whole slide, it was left to air dry under the Bunsen burner. Once the slide was dry, it was heat fixed by passing the slide through the flame 5 times. With the samples fixed, SBB (3% w/v om 70% ethanol, modified from Mesquita *et al*. 2015 was applied to the fixed slide for 10 minutes. The

slides were thoroughly washed using DW then decolouriser was used to remove access stain (<5 seconds). The decolouriser was washed off using DW again. In order to visualise the structure of the cell, counter stain (safranine) was used for 30 seconds. After the 30 seconds, the slides were washed with DW and let to dry. Dried slides were examined by means of a microscope at 1000x total magnification under oil submersion. If the staining was successful and samples had intracellular PHA granulates, cell wall would have turned pink for gram negative cells and PHA granulates would be coloured dark blue. The images of the slides were acquired by Galaxy S8 + (SM-G955n) with 3d printed adaptor modified from OpenOcular V1.

2.4. Nile Red Staining

Nile red (9-diethylamino-5-benzo [α] phenoxazinone), is a lipophilic stain like SBB. However unlike SBB, Nile red is more specific for poly(3-hydroxybtyrate) PHB, the most prominent member of bacterial PHA (Jendrossek 2005). Nile red solution (1% w/v) was prepared in 50ml tube, then wrapped with aluminium foil to prevent exposure to light. Nile red solution was then added to prepared streak plates flooding the plate for 10 minutes (covered with aluminium foil). After the incubation, excess stain was drained off the it was visualised under the ultraviolet transilluminator.

3. Results

3.1. Physiological Determination

3.1.1. Gram staining results

The mix colony bacterial isolates from the waste water streams were purified. On average about 2 different colony morphology was seen in each mix colony isolates producing 16 distinct colonies from 8 isolates (appendix 1). The isolated colonies were gram stained using methods explained above. The results of the gram staining are shown below and in appendix 2. As seen on figure 2 below, only C4 and L5 are gram positive bacteria.

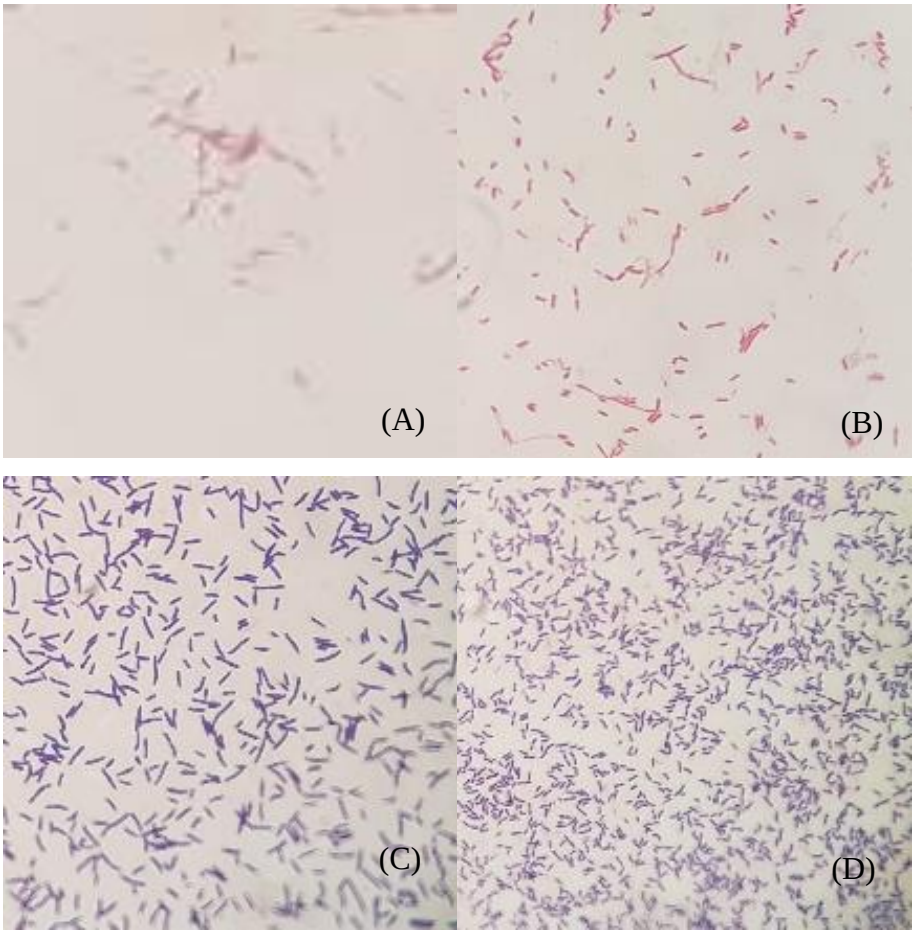


Figure 2
Gram staining results of C-1(A), C-7(B), C-4(C), L-5(D)

3.1.2. Colony Morphology

Once the gram staining and colony morphology (appendix 3) identification was complete as seem on table 4, the colonies with same morphology were categorised into distinct groups. After sorting, there were ten distinct groups. Ten selected microbes were C1-1, C2-2 C6-1, C6-2, C7-1, C7-1-3, C7-3, C8-1, C4-1, and C4-1-2 (figure 3). This was done in order to reduce duplicates from happening during the DNA extraction step.

Table 4: Colony morphology / characteristics of the Isolates

Bacterial isolates.	Colour	Margin	Elevation	Opaque / translucent	Colony Shape	Gram nature	Cell shape
C1-1	Yellow	Entire	Raised	Opaque	Circular	Negative	*Bacilli*
C1-2	Yellow	undulated	Flat	translucent	irregular	Negative	*Bacilli*
C2-1	Yellow	Entire	Raised	Opaque	Circular	Negative	*Bacilli*

C2-2	Yellow	undulated	Flat	translucent	irregular	Negative	*Bacilli*
C3	Yellow	Entire	Flat	translucent	Circular	Negative	*Bacilli*
C4-1-1	Yellow	undulated	Raised	Opaque	irregular	Positive	*Bacilli*
C4-1-2	White	Entire	convex	Opaque	Circular	Positive	*Bacilli*
C5-1	Yellow	undulated	Flat	translucent	irregular	Negative	*Bacilli*
C5-3	Yellow	undulated	Raised	Opaque	Circular	Negative	*Bacilli*
C6-1	Yellow	Entire	Raised	translucent	Circular	Negative	*Bacilli*
C6-2	Yellow	undulated	Raised	translucent	irregular	Negative	*Bacilli*
C7-1	Yellow	Flat	Raised	translucent	circular	Negative	*Bacilli*
C7-2	Yellow	undulated	Raised	translucent	irregular	Negative	*Bacilli*
C8-1	Yellow	undulated	Raised	translucent	irregular	Negative	*Bacilli*
C8-2	Yellow	Entire	Raised	translucent	Circular	Negative	*Bacilli*
C8-3	Yellow	Entire	Raised	Opaque	Circular	Negative	*Bacilli*

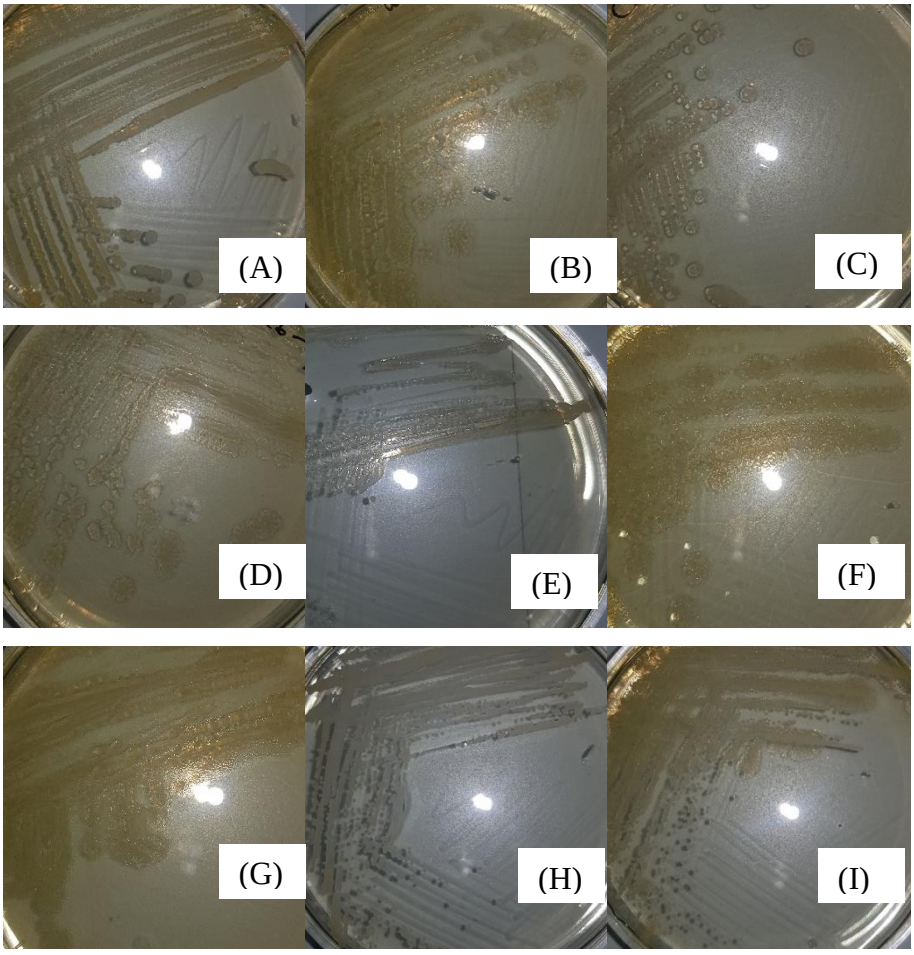


Figure 3
Colony morphology of C1-1(A), C2-2(B), C6-1(C), C6-2(D), C7-1(E),C7-3(F),C8-1(G),

C4-1(H),C4-1-2(I)

3.2. Genomic Determination

3.2.1. DNA extraction results

As C4 series were found to be gram positive, they required additional lysis buffer and enzymes. With the DNA extracted using the Qiagen DNeasy Kit, gel electrophoresis was done to test if the extraction was success. The figure 4 below shows 8 of ten extraction excluding C4, as C4 required additional steps taking longer time for extraction.

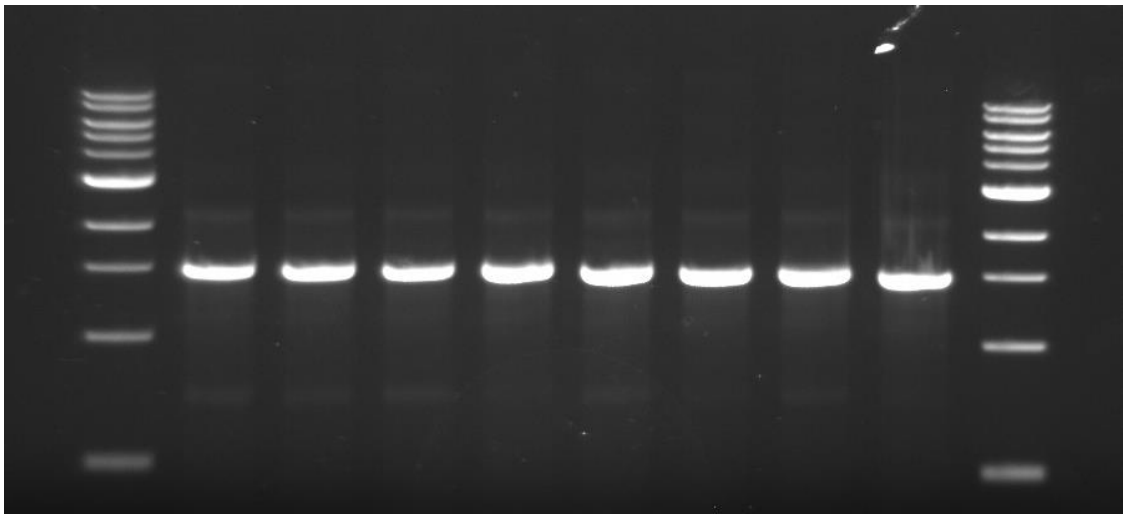


Figure 4: DNA Extraction results:
100bp Ladder, C1-1, C2-2 C6-1, C6-2, C7-1, C7-1-3, C7-3, C8-1, 100bp ladder

3.2.2. Rep-PCR results

As seen on the figure 5 below, there were replicates of the strains that exhibited different colony morphology; C2-2, and C6-2, C6-1 and C7-1, C7-1-3 and C7-3, and C4-1-1 and C4-1-2 were found to be same strains.

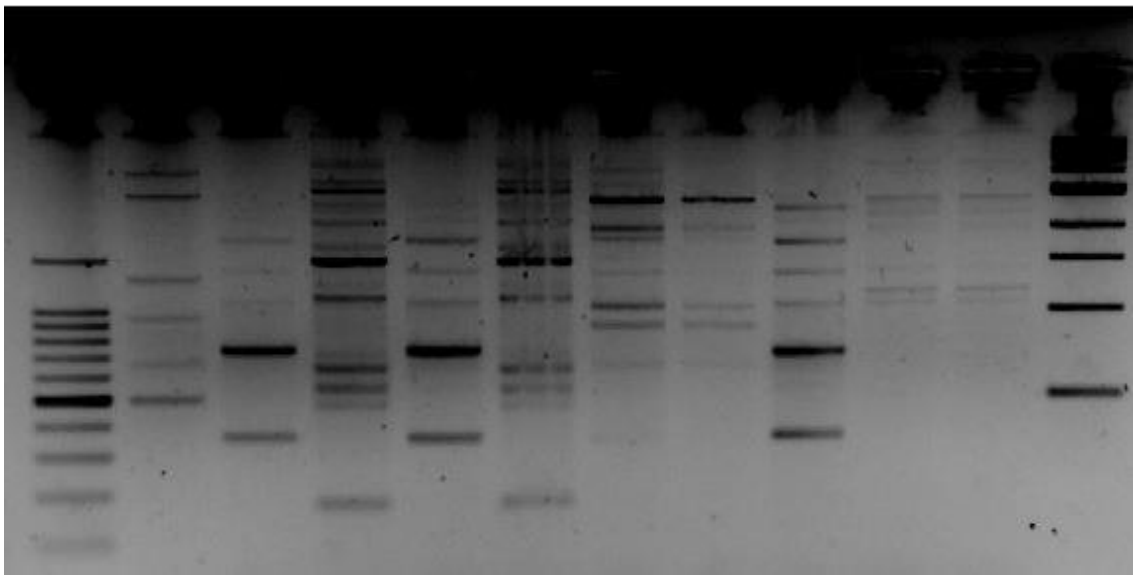


Figure 5: Negative contrast of REP PCR gel electrophoresis
100bp Ladder, C1-1, C2-2, C6-1, C6-2, C7-1, C7-1-3, C7-3, C8-1, C4-1-1, C4-1-2, 1kbp Ladder

Based on de-replication, 6 samples were selected to be amplified using 16S PCR (27F, 1498R).

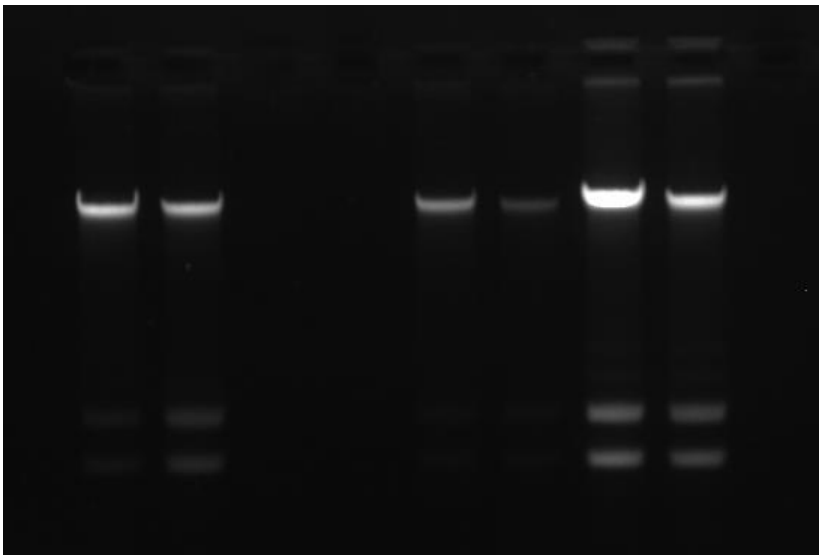


Figure 6: Gel electrophoresis of 16S PCR before the PCR purification.
C1-1, C2-2, blank, blank, C6-1, C7-1-3, C8-1 and C4-1.

After the amplification, as seen on figure 6 above, 3 bands were present which are the 16s target and two unwanted primer dimers and secondary structures of the primers, that had to be removed. The primer dimers were removed using Qiagen QIAqick purification Kit. Resulting in a clear single band seen in figure 7.

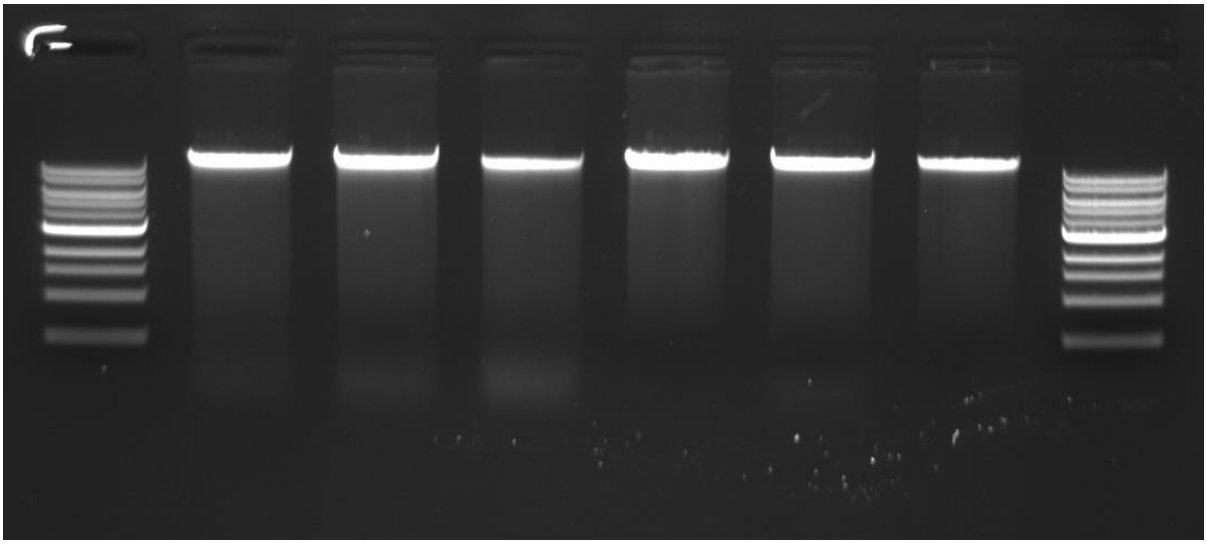


Figure 7: The purified 16S PCR product**.**
100bp Ladder,C1-1, C2-2, blank, blank, C6-1, C7-1-3, C8-1 and C4-1, 100bp ladder

3.2.3. Analysis of 16S gene sequence

These selected samples were, C1-1, C2-2, C6-1, C7-1-3, C8-1 and C4-1. The sequenced results indicated that all but one (C4-1) were *Pseudomonas spp* and C-4-1 came out inconclusive with *Strenotrophomonas* with Ident score of 90% (table 5). However, these results do not mean that the samples are the strains as the NCBI is only capable of searching through the known strain that they have the sequence for.

Table 5: 16S Sequence Results.

	C1-1	C2-2	C6-1	C7-1-3	C8-1	C4-1
1	*Pseudomonas reidholzensis* ID3 Ident: 99%	*Pseudomonas turukhanskensis* strain IB1.1 Ident: 99	*Pseudomonas marginalis* ICMP3553 Ident: 100%	*Pseudomonas turukhanskensis* strain IB1.1 Ident: 99	*Pseudomonas turukhanskensis* strain IB1.1 Ident: 99%	*Stenotrophomonas*
2	*Pseudomonas oryzihabitians* L-1 Ident: 99%	*Pseudomonas rhizosphaerae* IH5	*Pseudomonas extremaustralis* 14-3	*Pseudomonas punonensis* strain LMT03	*Pseudomonas Argentinensis* CH01 Ident: 97%	*Pseudomonas geniculata*

		Ident: 97	Ident: 99%	Ident: 98%		ATCC 19374=JC M13324 Ident: 89%
3	*Pseudomonas cremoricolorata* IAM 1541 Ident: 99%	*Pseudomonas coleopteurm* Esc2Am Ident: 97%	*Pseudomonas orientalis* CFML96-170 ident: 99%	*Pseudomonas argentinensis* CH01 Ident: 98%	*Pseudomonas Straminea* CB-7 Ident: 97%	*Stenotrophomonas pavanii* LMG 25348 Ident: 89%

3.3. Initial Growth from the sample C- series.

Four samples (C1-1, C2-2, C6-1, C4-1.1) were inoculated using the steps mentioned in 2.2.2 in duplicates. Over the period of 329 hours, the OD_{600} of the samples were collected. After the growth for 329 hrs, only C1-1 and C2-2 showed continuous growth for a longer period of time when compared with other samples seen in figure 8 below. The continuous growth indicated the ability to live on BMSM and potential for degrading PE. Based on the observation made, the C1 and C2 were selected to go to in to extended controlled experiments. During the initial experiment, one of C2 and C4 showed signs of contamination and the data from C2 and C4 is from one experiment.

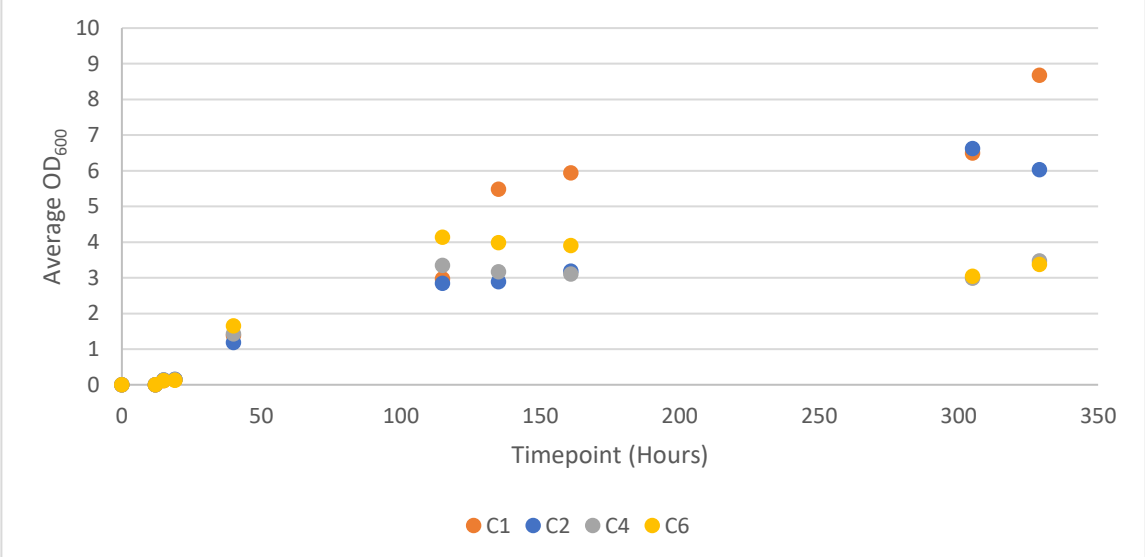


Figure 8:Initial growth Profile of C series

3.4. Microbial degradation of Polystyrene

The degradation of the plastics was carried over the span of 30 days for both ultraviolet treated polystyrene and for untreated polystyrene. Initially, 50mg of polystyrene were used for untreated polystyrene, however due to lack of polystyrene left, ultraviolet treated polystyrene had different weight which were all recorded. Because degradation of ultraviolet treated polystyrene is an experimental test, the growth curve of the samples was not made. However, growth curve for degradation of untreated polystyrene was made. In order to expand the variety of bacteria for degradation, additional 5 isolates (L1, L3, L4, L5, L6), which are endophytic bacteria isolated from environment.

The potential of biodegradation of non-treated polystyrene, could be extrapolated from the table 6. L4 had the maximum percentage of biodegradation of polystyrene with 3.3% over the course of 30 days. Followed by L1 and L5 as seen on figure 9. L4 was a gram negative - endophytic bacteria. Visually, after draining excess media, in the L4 flask, there were a lot of specs of plastic which were not present before potentially indicating mechanical/ physical degradation.

Table 6: Degradation of Untreated Polystyrene after 30 days

Sample no.	Polystyrene (average)		Total Weight Loss	Percent loss in weight
	Before	After		
Control	50	49	1	2%
C1	50	48.55	1.45	2.9
C2	50	48.8	1.2	2.4
L1	50	48.4	1.6	3.2
L3	50	49	1	2
L4	50	48.6	1.65	3.3
L5	50	48.4	1.6	3.2
L6	50	49.45	0.55	1.1

**L3-1, L4-1 showed signs of contamination and was removed from the average.

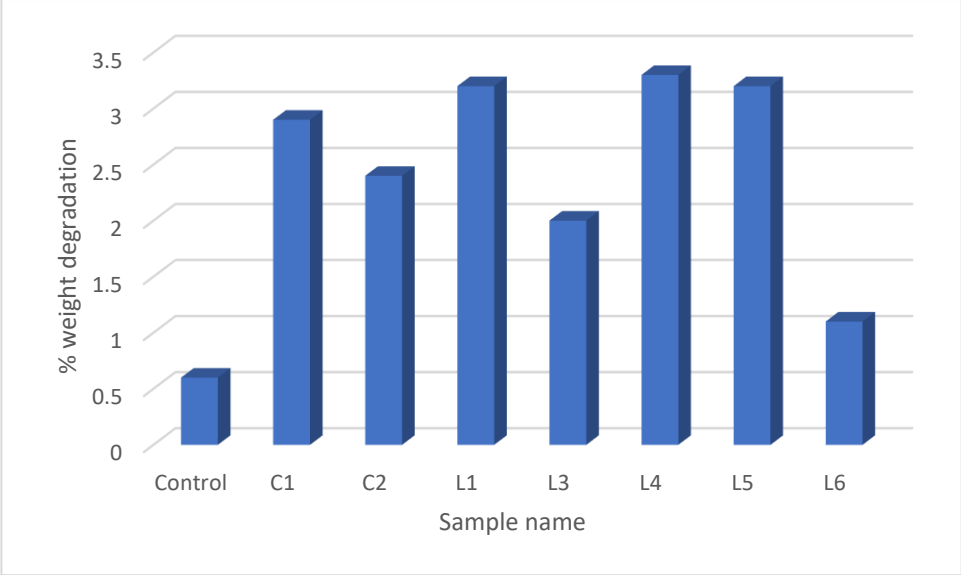


Figure 9: percent weight loss of untreated (non-ultraviolet) polystyrene

However, only C1 and C2 showed continued growth as seen in figure 10 below, which indicates that they are able to continuously consume and utilise carbon from polystyrene to grow.

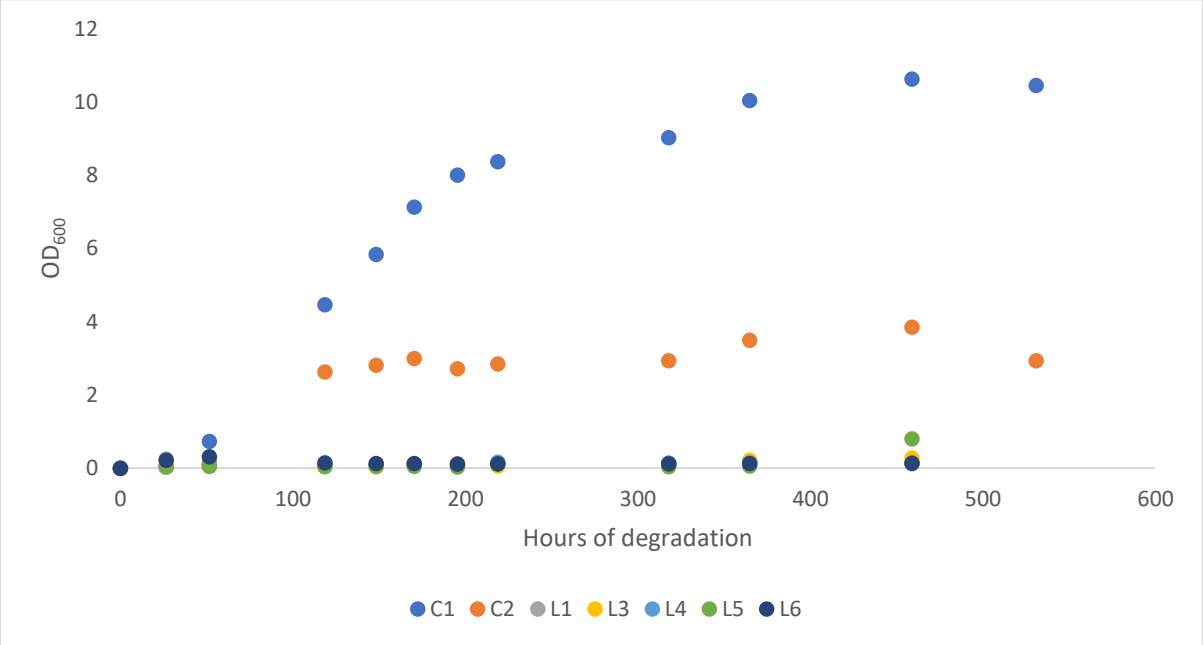


Figure 10: Growth profile of environmental isolates in Basal media with Untreated (non-ultraviolet) Polystyrene

On the contrary to the expectation that ultraviolet treatment of polystyrene will promote the biodegradability of polystyrene(Yousif and Haddad 2013). Although physical integrity of polystyrene decreased significantly (qualitative observation) based on the strength required to rip polystyrene before the degradation, the max percentage of

weight decrease was 0.623% by L5 which is still 2.57% lower than untreated polystyrene. And C1 had the highest percent weight loss (figure 11)

Table 7: Degradation of ultraviolet treated Polystyrene after 30 days

Sample no.	Polystyrene (average)		Total Weight Loss	Percent loss in weight
	Before	After		
Control	46.3	46	0.3	0.64794816
C1	49.3	49.1	0.2	0.40567951
C2	50.55	50.4	0.15	0.29673591
L1	45.95	45.8	0.15	0.32644178
L3	53.35	53.25	0.1	0.18744142
L4	52.2	52	0.2	0.38314176
L5	48.15	47.85	0.3	0.62305296
L6	48.4	48.4	0	0

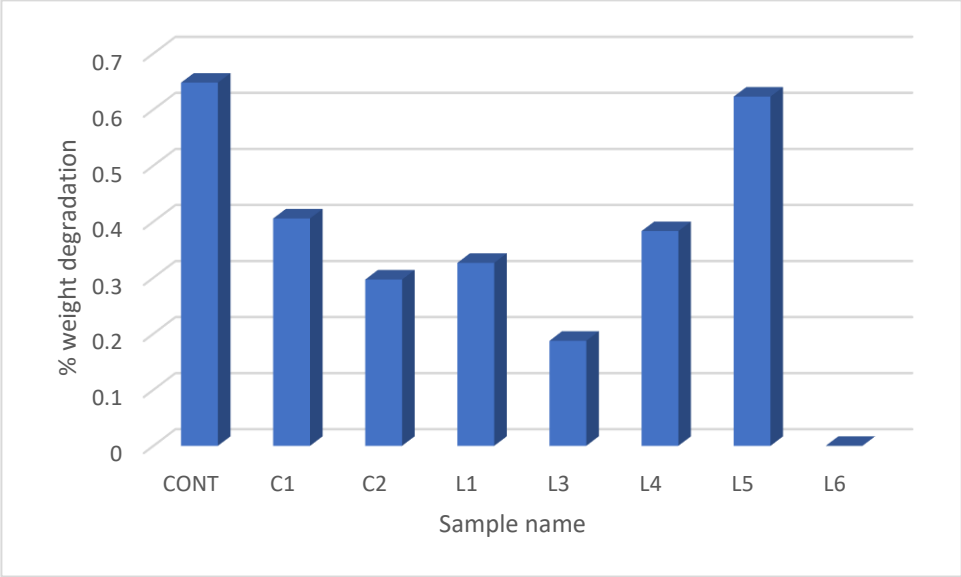


Figure 11: Percent Weight loss of ultraviolet Treated PS

3.5. Staining to identify the strains accumulating PHA's or PHB's

SBB staining showed that C1 contained intracellular PHA granules visible as dark blue colour under the bright-light microscopy. Figure 12 below shows the comparison between C1 (PHA +) and L4 (PHA-). From this we can extrapolate that C1 is capable of storing carbons taken up from the polystyrene in a PHA form. The dark blue (purple) is

indication of stained intracellular PHA granules. The stains were repeated 3 times to ensure that they were not dye residues.

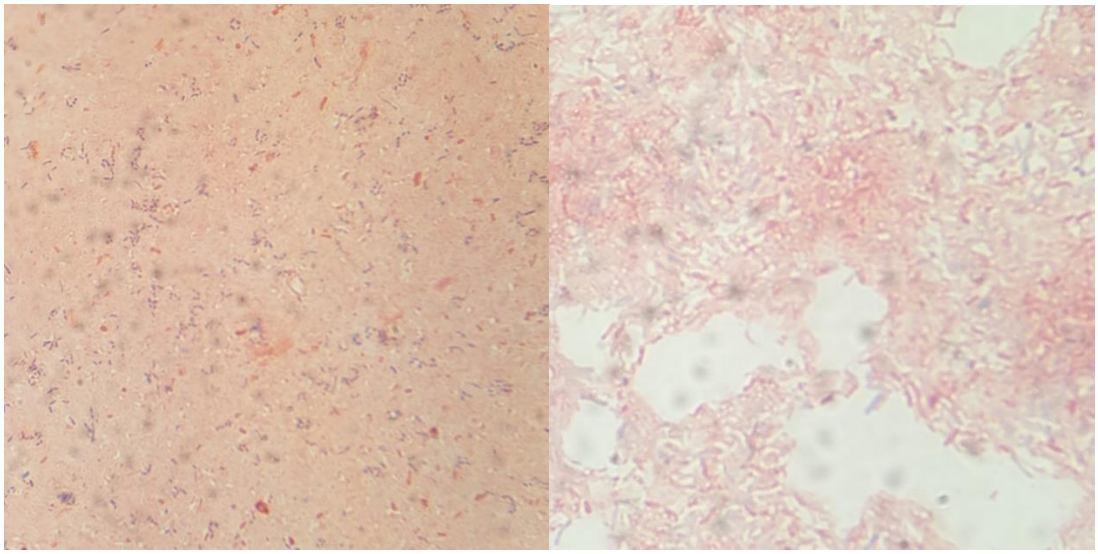


Figure 12: Sudan Black B staining of C1 and L4.
Dark blue (purple) specs are stained PHA granules.

Nile red staining was attempted however, the results were inconclusive as the time of the experiment did not permit the usage of fluorescent microscope. As seen on figure 13 below, plate that has been stained with Nile red it changed the colours of all the samples. Some samples such as C1, L1, L6 became darker in colour while C2, L4, L5 became translucent.

Figure 13a: Nile Red staining under ultraviolet thransluminater

Not stained with Nile Red Stained with Nile Red

C2	C1-1	C1	Control

L3	L1-1	L1	C2-1
L5	L4-1	L4	L3-1
	L6-1	L6	L5-1

Figure 13b: Order of samples on the plate:

4. Discussion

This study examined and isolated microbes from waste water streams and various endophytic bacteria from plants on their potentials in degrading polystyrene and synthesis of PHA. Many previously isolated microorganisms have shown better degrading abilities (Yang *et al.* 2015; Tang *et al.* 2017), none have examined the ability to convert petrol-based polystyrene into biodegradable PHA. The degradation of polystyrene offers end-of-cycle approach to polystyrene. Microbial degradation may not release harmful toxins or take hundreds of years, but it will be releasing waste into the environment in forms of CO_2 and other by products. The approach to produce bioplastics from biodegradation of polystyrene offers environmentally friendly methods of current issues of plastic waste.

During this experiment, the focus of the screening was split into two, the ability to degrade polystyrene and produce and accumulate PHA granules. The screening was done in two steps. Initially, environmental samples were grown in BMSM with polystyrene for degradation of polystyrene. Then, after 30 days, the cells were collected and stained with SBB and Nile Red, two preferred stains for PHA and PHB.

4.1. Biodegradation of Polystyrene

The biodegradability of non ultraviolet treated polystyrene and ultraviolet treated polystyrene was studied respectively. All selected isolates were able to degrade small amounts of polystyrene (1.1%-3.3%). It has been studied in the past that biodegradation of polystyrene is possible by many microorganisms (Yang *et al.* 2015; Kaplan [n.d.]). And it was previously shown that exposure of the polystyrene to ultraviolet induced degradation by promoting formation of free radicals between the styrene molecules (Yousif and Haddad 2013).

Results with ultraviolet treatment of polystyrene showed minimal degradation of polystyrene (0%~0.648%). The result was not as expected from the results from other papers. The reason for decrease in degradation is unknown however, there are few possibilities on why this could have happened. It was hypothesised that as photooxidation of polystyrene is caused by ultraviolet exposure, chemicals such as benzaldehyde was released as a by-product of polystyrene degradation. Benzaldehyde have shown potential antimicrobial properties in the past. (Wypych 2012; Ullah *et al.* 2015).

Future work could include longer time for biodegradation of polystyrene to obtain more information about the full capability of each strains. And perform gas chromatography mass spectrometry (GC/MS) of the supernatant after the degradation to understand what the final products and by-products of the degradation were (Arutchelvi *et al.* 2008; Alshehrei 2017). The optimal condition of the biodegradation should also be identified to understand what are the limiting factors of the biodegradation and how they would react to polystyrene in nutrient rich environment as well (Hoffmann *et al.* 1997; Gartiser *et al.* 1998; Asmita *et al.* 2015)

4.2. Accumulation of PHA

After the Sudan black B staining, C1, *Pseudomonas reidholzensis* exhibited the potential to not only degrade polystyrene (figure 4) but to be able to synthesise intracellular PHA granules (figure 11). The ability to convert and consume polystyrene into PHA was not seen in previous researches. Although this experiment was able to successfully to use Sudan black B as a PHA, Nile Red staining was not reliable. If the Nile Red staining was successful, when examined under the fluorescent microscope at wavelength of 570 nm, the stained PHB should give off red glow (Greenspan 1985). Due to inconclusively of result, the Nile red staining will require fluorescent microscopy to identify the HA/PHB accumulation.

4.3. Potential process of Bioconversion of Polystyrene into Bioplastic

Based on the results of the experiment, the potential for environmental bioconversion of polystyrene was exhibited. Past research focused on the biodegradation of polystyrene to perform efficient degradation, however, with the results obtained from this experiment, it was proven that it is possible to convert polystyrene into bioplastic.

Rapid biodegradation of polystyrene is crucial as it was shown to be extremely stable in the environment and to release toxic chemicals as it decomposes (Marczynski *et al.* 2000; Yousif and Haddad 2013). More recently, the advancement and usage of environmentally friendly biodegradable bioplastics such as PHA are being promoted. The bioconversion of polystyrene will not only reduce polystyrene waste but provide an alternative for the plastic current society relies on.

5. Conclusion

To summarise the experiment done in this paper, the isolation of microorganisms from environmental sample with the capability to degrade polystyrene and utilizing the carbon and nutrients from the polystyrene to produce polyhydroxyalkanoate. The main task of the research was achieved with successful isolation of *Pseudomonas reidholzensis (99% ident)*, Gram negative, *bacillus,* organism isolated from waste water streams. With further analysis and understanding of the pathway of polystyrene degradation and polyhydroxyalkanoate synthesis, this study may be fundamental in order to develop a more environmental and economical solution to plastic waste in the future.

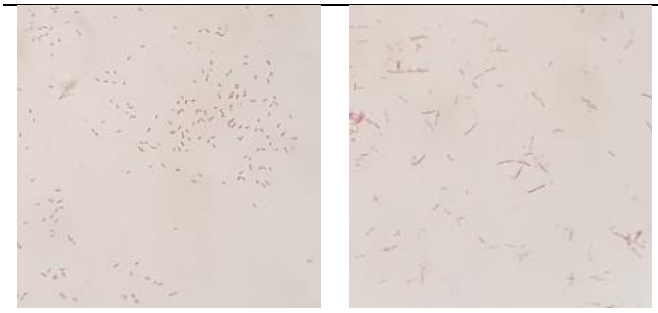
6.Appendices

Appendix 1: Number of colonies isolated from each mix colony tubes.

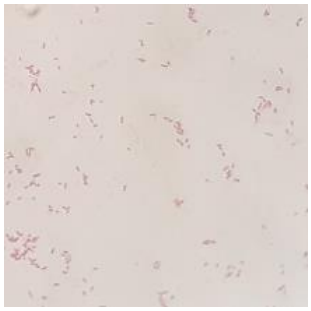
Sample	C1	C2	C3	C4	C5	C6	C7	C8
colonies	2	2	1	2	2	2	2	3

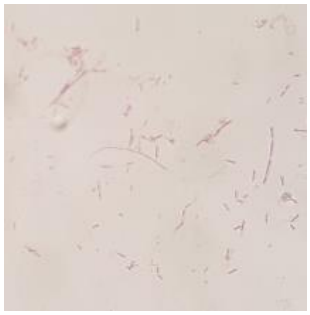
Appendix 2: Gram staining results

C1-1 C1-2

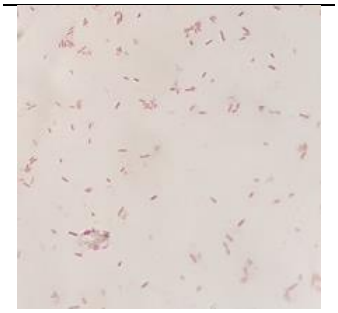


C2-1 C2-2



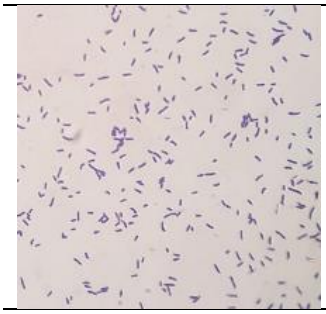


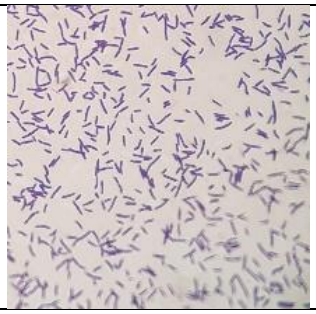
C3



C4-1-1

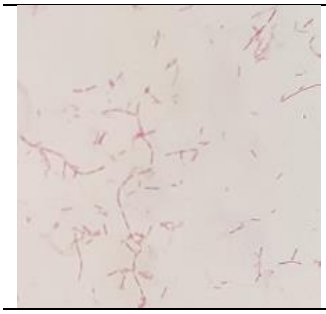
C4-2

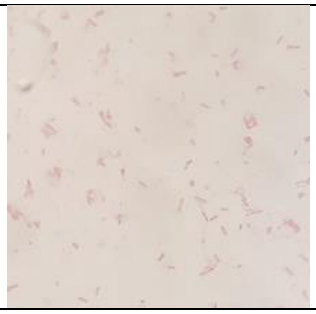




C5-1

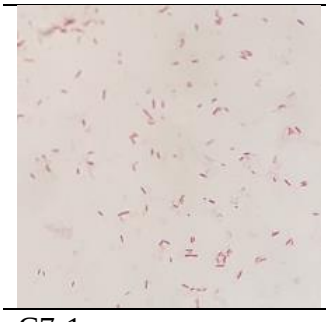
C5-2

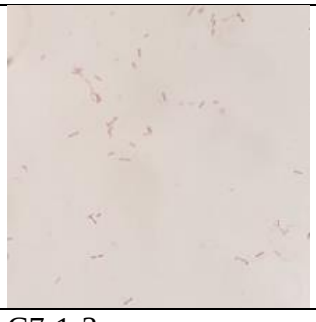




C6-1

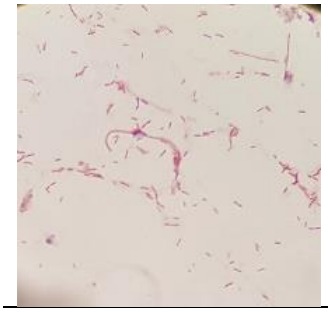
C6-2



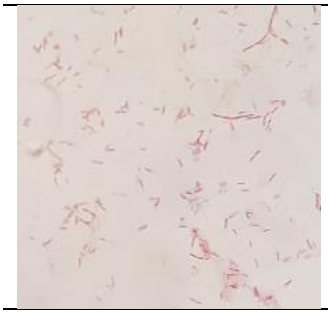
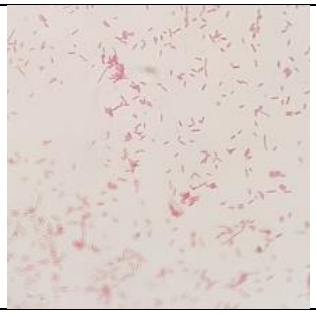
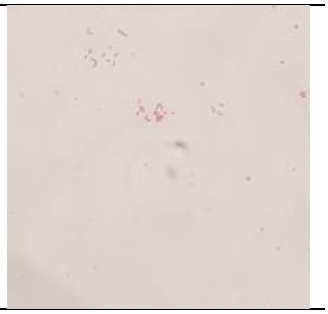


C7-1

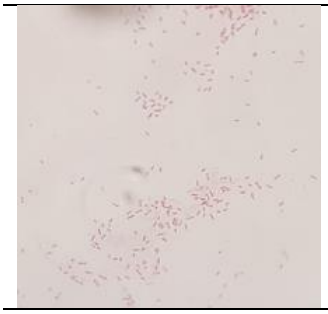
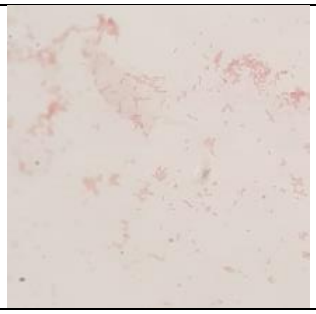
C7-1-3



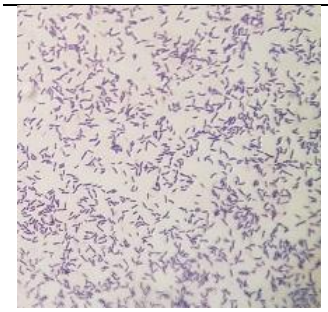
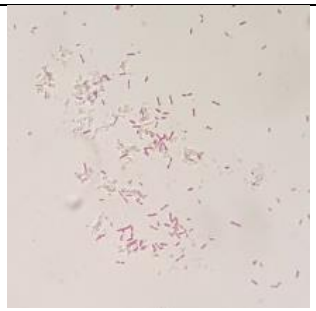

C8-1 C8-2 C8-3

L3 L4

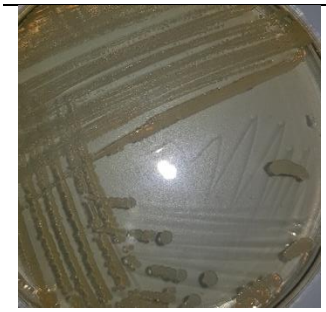



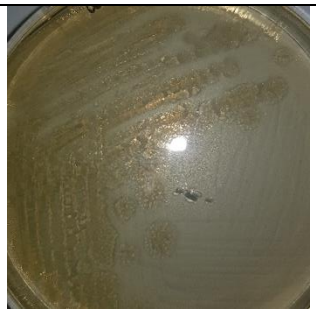
L5 L6

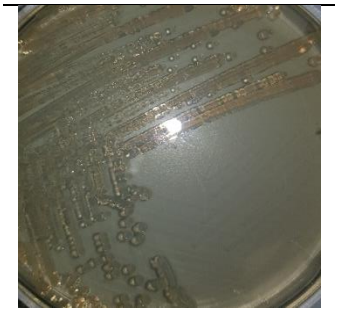



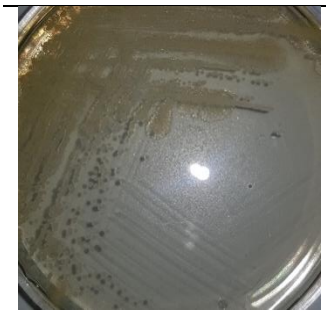
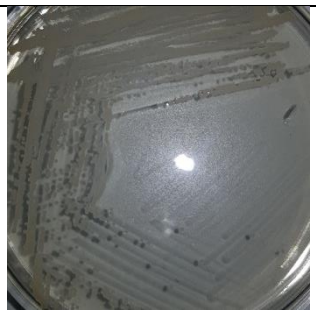
Appendix 3: Colony morphology

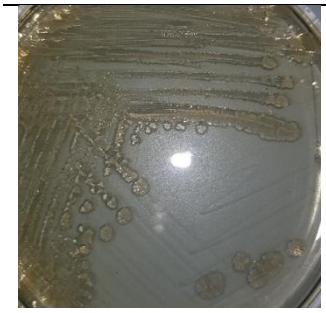
C1-1 C1-2



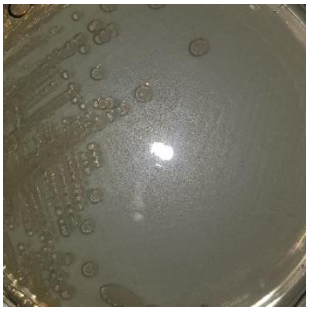

C2-1	C2-2
	

C3


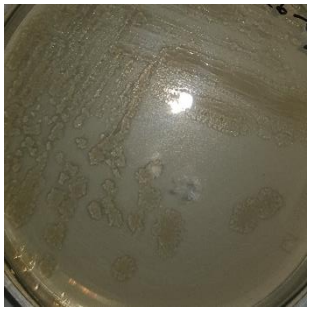
C4-1	C4-1-2
	

C5-1	C5-3
	

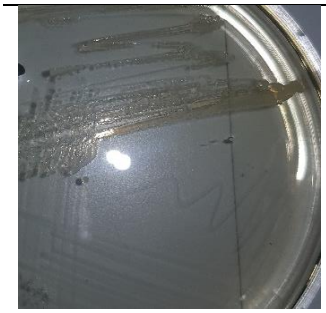
C6-1	C6-2



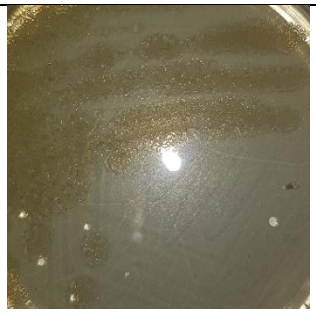
C7-1



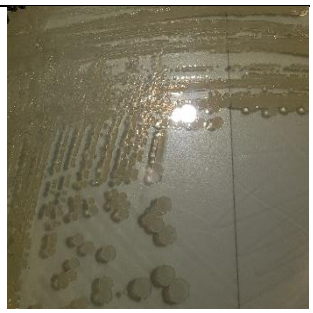
C7-1-3



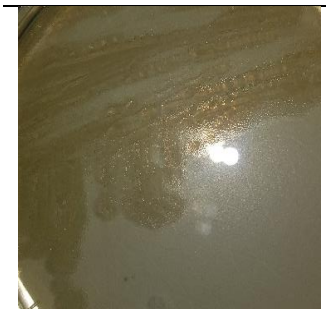
C8-1



C8-2



C8-3



Acknowledgment

I would like to thank Dr Nagamani Bora, my supervisor for her guidance and support throughout this project. I would also like to express my gratitude to all the lab technicians who provided with the assistant I needed in this project.

In addition , I would like to thank my parents for their support, love, and guidance. For being there for me and supporting me emotionally and mentally.

7. Reference

Abdel-Raouf, M.E.-S., Mahmoud Abdel-Raheim, A.R. and El-Saeed, S.M. (2013). Thermo-Catalytic Versus Thermo-Chemical Recycling of Polystyrene Waste. *Waste and Biomass Valorization* **4**(1):37–46. doi: https://doi.org/10.1007/s12649-012-9136-4.

Albuquerque, M.G.E., Eiroa, M., Torres, C., Nunes, B.R. and Reis, M.A.M. (2007). Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses. *Journal of Biotechnology* **130**(4):411–421. doi: https://doi.org/10.1016/j.jbiotec.2007.05.011.

Alshehrei, F. (2017). Biodegradation of Synthetic and Natural Plastic by Microorganisms. *Environmental Microbiology*:12.

Arutchelvi, J., Sudhakar, M., Arkatkar, A., Doble, M., Bhaduri, S. and Uppara, P.V. (2008). Biodegradation of polyethylene and polypropylene. *INDIAN J BIOTECHNOL*:14.

Asmita, K., Shubhamsingh, T. and Tejashree, S. (2015). Isolation of Plastic Degrading Micro-organisms from Soil Samples Collected at Various Locations in Mumbai, India. **4**:9.

Barnes, D.K.A., Galgani, F., Thompson, R.C. and Barlaz, M. (2009). Accumulation and fragmentation of plastic debris in global environments. *Philosophical Transactions of the Royal Society B: Biological Sciences* **364**(1526):1985–1998. doi: https://doi.org/10.1098/rstb.2008.0205.

Bhuwal, A.K., Singh, G., Aggarwal, N.K., Goyal, V. and Yadav, A. (2013). Isolation and Screening of Polyhydroxyalkanoates Producing Bacteria from Pulp, Paper, and Cardboard Industry Wastes. *International Journal of Biomaterials* **2013**:1–10. doi: https://doi.org/10.1155/2013/752821.

Chee, J.-Y., Yoga, S.-S., Lau, N.-S., Ling, S.-C., Abed, R.M.M. and Sudesh, K. (2010). Bacterially Produced Polyhydroxyalkanoate (PHA): Converting Renewable Resources into Bioplastics. :10.

Choi, J. and Lee, S.Y. (1997). Process analysis and economic evaluation for Poly(3-hydroxybutyrate) production by fermentation. *Bioprocess Engineering* **17**(6):335. doi: https://doi.org/10.1007/s004490050394.

Duetz, W., Dobson, A.D.W., O'Leary, N.D. and O'Connor, K.E. (2001). Transcriptional regulation of styrene degradation in Pseudomonas putida CA-3. *Microbiology* **147**(4):973–979. doi: https://doi.org/10.1099/00221287-147-4-973.

Faravelli, T., Pinciroli, M., Pisano, F., Bozzano, G., Dente, M. and Ranzi, E. (2001). Thermal degradation of polystyrene. *Journal of Analytical and Applied Pyrolysis* **60**(1):103–121. doi: https://doi.org/10.1016/S0165-2370(00)00159-5.

Gartiser, S., Wallrabenstein, M. and Stiene, G. (1998). Assessment of Several Test Methods for the Determination of the Anaerobic Biodegradability of Polymers. *Journal of Polymers and the Environment* **6**(3):159–173. doi: https://doi.org/10.1023/A:1021869530253.

Gibbs, B.F. and Mulligan, C.N. (1997). Styrene Toxicity: An Ecotoxicological Assessment. *Ecotoxicology and Environmental Safety* **38**(3):181–194. doi: https://doi.org/10.1006/eesa.1997.1526.

Greenspan, P. (1985). Nile red: a selective fluorescent stain for intracellular lipid droplets. *The Journal of Cell Biology* **100**(3):965–973. doi: https://doi.org/10.1083/jcb.100.3.965.

Gu, J.-D. (2003). Microbiological deterioration and degradation of synthetic polymeric materials: recent research advances. *International Biodeterioration & Biodegradation* **52**(2):69–91. doi: https://doi.org/10.1016/S0964-8305(02)00177-4.

Hoffmann, J., Řezníčèková, I., Vaňòková, S. and Kupec, J. (1997). Manometric determination of biological degradability of substances poorly soluble in aqueous environments. *International Biodeterioration & Biodegradation* **39**(4):327–332. doi: https://doi.org/10.1016/S0964-8305(97)00033-4.

Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., … Law, K.L. (2015). Plastic waste inputs from land into the ocean. *Science* **347**(6223):768–771. doi: https://doi.org/10.1126/science.1260352.

Jendrossek, D. (2005). Fluorescence Microscopical Investigation of Poly(3-hydroxybutyrate) Granule Formation in Bacteria †. *Biomacromolecules* **6**(2):598–603. doi: https://doi.org/10.1021/bm049441r.

Jenkins, C., Ling, C.L., Ciesielczuk, H.L., Lockwood, J., Hopkins, S., McHugh, T.D., … Kibbler, C.C. (2012). Detection and identification of bacteria in clinical samples by 16S rRNA gene sequencing: comparison of two different approaches in clinical practice. *Journal of Medical Microbiology* **61**(Pt_4):483–488. doi: https://doi.org/10.1099/jmm.0.030387-0.

Johnston, B., Radecka, I., Hill, D., Chiellini, E., Ilieva, V., Sikorska, W., … Kowalczuk, M. (2018). The Microbial Production of Polyhydroxyalkanoates from Waste Polystyrene Fragments Attained Using Oxidative Degradation. *Polymers* **10**(9):957. doi: https://doi.org/10.3390/polym10090957.

Kaplan, D.L. Biodegradation of Polystyrene, Poly(methyl methacrylate), and Phenol Formaldehyde. :3.

Kiatkamjornwong, S., Sonsuk, M., Wittayapichet, S., Prasassarakich, P. and Vejjanukroh, P.-C. (1999). Degradation of styrene-g-cassava starch filled polystyrene plastics. *Polymer Degradation and Stability* **66**(3):323–335. doi: https://doi.org/10.1016/S0141-3910(99)00082-8.

Klump, M. (2014). Polystyrene. *ICIS Chemical Business* **14**(286):47.

Madison, L.L. and Huisman, G.W. (1999). Metabolic Engineering of Poly(3-Hydroxyalkanoates): From DNA to Plastic. *MICROBIOL. MOL. BIOL. REV.* **63**:33.

Marczynski, B., Peel, M. and Baur, X. (2000). New aspects in genotoxic risk assessment of styrene exposure – a working hypothesis. *Medical Hypotheses* **54**(4):619–623. doi: https://doi.org/10.1054/mehy.1999.0907.

Mathalon, A. and Hill, P. (2014). Microplastic fibers in the intertidal ecosystem surrounding Halifax Harbor, Nova Scotia. *Marine Pollution Bulletin* **81**(1):69–79. doi: https://doi.org/10.1016/j.marpolbul.2014.02.018.

Matyjaszewski, K. (1989). Carbocationic Polymerization: Styrene and Substituted Styrenes. *Comprehensive Polymer Science and Supplements*. Elsevier, pp. 639–671.

Mesquita, D.P., Amaral, A.L., Leal, C., Oehmen, A., Reis, M.A.M. and Ferreira, E.C. (2015). Polyhydroxyalkanoate granules quantification in mixed microbial cultures using image

analysis: Sudan Black B versus Nile Blue A staining. *Analytica Chimica Acta* **865**:8–15. doi: https://doi.org/10.1016/j.aca.2015.01.018.

Mondal, M.K., Bose, B.P. and Bansal, P. (2019). Recycling waste thermoplastic for energy efficient construction materials: An experimental investigation. *Journal of Environmental Management* **240**:119–125. doi: https://doi.org/10.1016/j.jenvman.2019.03.016.

Mor, R. and Sivan, A. (2008). Biofilm formation and partial biodegradation of polystyrene by the actinomycete Rhodococcus ruber: Biodegradation of polystyrene. *Biodegradation* **19**(6):851–858. doi: https://doi.org/10.1007/s10532-008-9188-0.

Motta, O., Proto, A., De Carlo, F., De Caro, F., Santoro, E., Brunetti, L. and Capunzo, M. (2009). Utilization of chemically oxidized polystyrene as co-substrate by filamentous fungi. *International Journal of Hygiene and Environmental Health* **212**(1):61–66. doi: https://doi.org/10.1016/j.ijheh.2007.09.014.

Nash, J.A., Ballard, T.N.S., Weaver, T.E. and Akinbi, H.T. (2006). The Peptidoglycan-Degrading Property of Lysozyme Is Not Required for Bactericidal Activity In Vivo. *The Journal of Immunology* **177**(1):519–526. doi: https://doi.org/10.4049/jimmunol.177.1.519.

Patel, A., Harris, K.A. and Fitzgerald, F. (2017). What is broad-range 16S rDNA PCR? *Archives of disease in childhood - Education & practice edition* **102**(5):261–264. doi: https://doi.org/10.1136/archdischild-2016-312049.

Santos, P.M., Blatny, J.M., Di Bartolo, I., Valla, S. and Zennaro, E. (2000). Physiological Analysis of the Expression of the Styrene Degradation Gene Cluster in Pseudomonas fluorescens ST. *Applied and Environmental Microbiology* **66**(4):1305–1310. doi: https://doi.org/10.1128/AEM.66.4.1305-1310.2000.

Satpute, S.K., Banpurkar, A.G., Dhakephalkar, P.K., Banat, I.M. and Chopade, B.A. (2010). Methods for investigating biosurfactants and bioemulsifiers: a review. *Critical Reviews in Biotechnology* **30**(2):127–144. doi: https://doi.org/10.3109/07388550903427280.

Shirai, K. and Hisatsuka, K. (1979). Isolation and Identification of Styrene Assimilating Bactiria. *Agricultural and Biological Chemistry* **43**(7):1595–1596. doi: https://doi.org/10.1080/00021369.1979.10863668.

Spigaglia, P. and Mastrantonio, P. (2003). Evaluation of Repetitive Element Sequence-Based PCR as a Molecular Typing Method for Clostridium difficile. *Journal of Clinical Microbiology* **41**(6):2454–2457. doi: https://doi.org/10.1128/JCM.41.6.2454-2457.2003.

Tang, Z.-L., Kuo, T.-A. and Liu, H.-H. (2017). The Study of the Microbes Degraded Polystyrene. **2**(1):5.

Thakur, S., Verma, A., Sharma, B., Chaudhary, J., Tamulevicius, S. and Thakur, V.K. (2018). Recent developments in recycling of polystyrene based plastics. *Current Opinion in Green and Sustainable Chemistry* **13**:32–38. doi: https://doi.org/10.1016/j.cogsc.2018.03.011.

Tsuchii, A., Suzuki, T. and Takahara, Y. (1977). Microbial Degradation of Styrene Oligomer. *Agricultural and Biological Chemistry* **41**(12):2417–2421. doi: https://doi.org/10.1080/00021369.1977.10862874.

Ullah, I., Khan, A.L., Ali, L., Khan, A.R., Waqas, M., Hussain, J., … Shin, J.-H. (2015). Benzaldehyde as an insecticidal, antimicrobial, and antioxidant compound produced by Photorhabdus temperata M1021. *Journal of Microbiology* **53**(2):127–133. doi: https://doi.org/10.1007/s12275-015-4632-4.

UNEP (2014). Stockholm convention on persistent organic pollutions (POPs).

Vandeventer, P.E., Mejia, J., Nadim, A., Johal, M.S. and Niemz, A. (2013). DNA Adsorption to and Elution from Silica Surfaces: Influence of Amino Acid Buffers. *The Journal of Physical Chemistry B* **117**(37):10742–10749. doi: https://doi.org/10.1021/jp405753m.

Velasco, A., Alonso, S., Garcia, J.L., Perera, J. and Diaz, E. (1998). Genetic and Functional Analysis of the Styrene Catabolic Cluster of Pseudomonas sp. Strain Y2. *J. BACTERIOL.* **180**:9.

Versalovic, J., Koeuth, T. and Lupski, J.R. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. :9.

Wang, M.H., He, Y. and Sen, B. (2019). Research and management of plastic pollution in coastal environments of China. *Environmental Pollution* **248**:898–905. doi: https://doi.org/10.1016/j.envpol.2019.02.098.

Wei, Y.-H., Chen, W.-C., Huang, C.-K., Wu, H.-S., Sun, Y.-M., Lo, C.-W. and Janarthanan, O.-M. (2011). Screening and Evaluation of Polyhydroxybutyrate-Producing Strains from Indigenous Isolate Cupriavidus taiwanensis Strains. *International Journal of Molecular Sciences* **12**(1):252–265. doi: https://doi.org/10.3390/ijms12010252.

Wypych, G. (2012). PS polystyrene. In: Wypych, G. (ed.). *Handbook of Polymers*. Elsevier, pp. 541–547.

Yang, Y., Yang, J., Wu, W.-M., Zhao, J., Song, Y., Gao, L., … Jiang, L. (2015). Biodegradation and Mineralization of Polystyrene by Plastic-Eating Mealworms: Part 2. Role of Gut Microorganisms. *Environmental Science & Technology* **49**(20):12087–12093. doi: https://doi.org/10.1021/acs.est.5b02663.

Yousif, E. and Haddad, R. (2013). Photodegradation and photostabilization of polymers, especially polystyrene: review. *SpringerPlus* **2**(1):398. doi: https://doi.org/10.1186/2193-1801-2-398.

Zhang, G., Wu, Y., Qian, X. and Meng, Q. (2005). Biodegradation of crude oil by *Pseudomonas aeruginosa* in the presence of rhamnolipids. *Journal of Zhejiang University SCIENCE* **6B**(8):725–730. doi: https://doi.org/10.1631/jzus.2005.B0725.