ISOLATION AND IDENTIFICATION OF PLASTIC DEGRADING MICROORGANISM

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ABSTRACT

Increased usage of plastic is leading to plastic pollution in the environment. Whilst plastics are believed to be non-biodegradable, it is believed that with long exposure to the environment, the microbiome in the environment may evolve to metabolise plastic. Many genera of microbes have been isolated and tested for degradation of polyethylene terephthalate and polystyrene however, deeper understanding of the mechanism and the demand for novel plastic degrading microorganisms is higher than ever. In this work, TPA degrading *Streptomyces* sps. was isolated from soil samples collected near human sewage treatment plant by utilizing selective enrichment on the isolates. Identification of the strain was done using molecular approaches and phenotypic assays showed the plastic degrading potential of the isolate. Metabolic profiles of the isolate were assessed by LCMS with the results indicating difference in the culture grown in protocatechuic acid and terephthalic acid.

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ABBREVIATIONS

°C	Degrees Celsius
µL	Microliter
L	Litre
mL	Millilitre
OD	Optical density
sp.	Species
g	Grams
PET	Polyethylene terephthalate
PCL	Polycaprolactone
PS	Polystyrene
PLA	Polylactic acid
PVC	Polyvinyl Chloride
PE	Polyethylene
PP	Polypropylene
TPA	Terephthalic acid
PCA	Protocatechuic acid
EDTA	Polystyrene
bp	Base pair
LCMS	Liquid chromatography and mass spectrum
HPLC	High Pressure Liquid Chromatography
RPM	Rotation Per minute
M	Molar
HST	Human Sewage Treatment

CHAPTER ONE

Introduction

CHAPTER 1 INTRODUCTION

1.1 Pandora's Box of the 20th century

1.1.1 Brief history of plastic

"A substance hard as horn, but as flexible as leather, capable of being cast or stamped, painted, dyed, or carved" was the description Alexander Parkes used to describe his invention during the International Exhibition of 1862 in London (Buchanan, 2019). His invention, Parkesine, was the first man-made synthetic polymer which we call plastic (Patent, 1857, Chu et al., 2015). Since the first discovery, many different types of plastic were developed with much desired properties such as strength, flexibility, chemical inertness, and resistance to environmental degradation (Nagalakshmaiah et al., 2019). Most of the petroleum-based material plastics such as Polyethylene terephthalate (PET), Polyethylene (PE), Polystyrene (PS), polyvinylchloride (PVC), and polypropylene (PP) exhibit the above desired traits. These developments and mass production of different plastics allowed plastics to become the most widely used and produced synthetic polymer in the world. Plastics are omnipresent and are present in all forms of our daily usage, from food packaging to our computers. As the demand for plastic increased, the annual production of plastic grew more rapidly than ever with an annual production of three hundred sixty million tonnes excluding PET and PP (Singh et al., 2017). The massive increase in production lead to inappropriate disposal increasing the amount of plastic waste.

It is estimated that about 79% of all plastic ends up in the landfill and environment. To reduce the environmental impact of plastic waste, Society of Plastic Industry (SPI) constructed a resin identification code to aid in disposal and recycling. Although a small number of plastics are recycled, about 50% of the produced plastic is single use plastic that cannot be reused or recycled into high value products. Currently, it is estimated that about one hundred and sixty-five million tons of plastic is in the ocean and about 4600 million tonnes in the landfill. Waste that ends up in the environment is known to cause the death of birds, fish, and other mammals as their intestines are blocked by ingested pieces of plastic. Some of this plastic waste is disposed by incineration releasing toxic chemicals such as 'Dioxins' into the atmosphere (Jayasekara et al., 2005). As plastics are synthetic molecules, the natural environment was only introduced to plastic in the last 100 years. It is not known if natural evolution have designed or will be able to modify enzyme structures capable of degrading synthetic polymers (Mueller, 2006). The chemical stability of plastic and resistance to microbial degradation causes the environmental degradation of plastic to potentially take up to thousands of years (Zheng et al., 2005). Plastic waste became a bigger issue as more and more communities began to realize the harmful impact of plastic affecting all realms of life and environment.

As people began to realize the dangers of plastic's resistance to microbial and fungal degradation in nature in the 1980's, many countries including the United States began to explore production of biodegradable plastics. "Bio" Plastics with blends of natural compounds such as cellulose,

starch, and different biodegradable polymers to petroleum-based polymer to aid in degradation were explored heavily. However, this momentum in the 1980's did not make a huge difference in the current market. Now, the research has shifted more towards using renewable materials such as Polylactic acid, furan -2, 5- dicarboxylic acid, and polyhydroxy butyrate in order to replace current plastics. While the work is still on-going, it has not been fully successful to replace the non-biodegradable plastics.

The characteristics we desired made the plastics non-biodegradable. Because of the lack of microbial and fungal interaction, degradation of plastic is believed to be heavily depended on environmental factors such as UV, temperature, and moisture. UV, and temperature accelerates oxidation causing components of the long chain backbone of the polymer to separate and react with one another to cause changes to the property of the polymer (Shah et al., 2008). However, the degradation by environmental factors is not enough to degrade 4600 million tonnes of discarded plastics (2015) already in the environment (Ritchie and Roser, 2020).

1.2 Current solution to Plastic waste

From 1950 to 2015, 6.3 billion tonnes of plastics were discarded as waste and only 9% of the waste was recycled. 12% was burnt with or without the energy recovery. The rest was dumped into the environment or buried in the landfills (Geyer et al., 2017). Despite exponential increase in the plastic waste, current method of end-life solution for such waste is not viable due to

detrimental impact on the environment and their limited uses. Widely used solutions for tackling plastic waste is described below.

1.2.1 Landfill disposal

The disposal of synthetic polymers into landfill causing environmental and human health issues. Not only it takes hundreds to thousands of years to decompose (Barnes et al., 2009), plastics exposed to the photo-oxidation, thermooxidation, hydrolytic, and microbial degradation (Webb et al., 2012) in the landfill, and in the environment may cause the chemicals within the plastic such as plasticizers, monomers, and other additives to become more readily available to the surrounding environment e.g., underground water systems, soil, and to the wildlife(Asakura et al., 2004). The landfill leachates with plastic originated contaminates cause plethora of problems to the chemical balance of the environment and to microbiome such as but not limited to, toxic effects to the life, growth impairment (Hahladakis et al., 2018, Wang et al., 2020, Li et al., 2021, Tetu et al., 2019). Furthermore, dumping plastic waste into the landfill not only pollutes the environment, but it is waste of natural resources and energy that was used to produce the plastics.

1.2.2 Incineration

As a n alternative end-of-life method of disposal of waste plastic, plastic wastes are incinerated to minimize the chemical pollution to the soil and waters while regenerating energy(Lea, 1996). When compared to non-combined heat and powerplants that utilize fossil fuel, efficient combustion of plastic material waste gives a net negative contribution of greenhouse gases

(Eriksson and Finnveden, 2009). With complete combustion of the plastic waste, the plastic material is reduced down to carbonic acid, CO_2, and water. While incineration of polyvinylchloride releases HCL (Verma et al., 2016). Incomplete combustion not only produces CO and smoke, but dioxins, and other hazardous substances may be formed and released into the atmosphere (Verma et al., 2016). Although recovery of energy via incineration is a better alternative to landfills it is crucial that complete incineration is done in efficient manner to minimize release of toxic pollutants.

1.2.3 Recycling

The most economically stable solution to plastic waste is to recycle the plastic materials. There are 2 types of recycling that does not destroy the value of the original material: chemical and mechanical (Schyns and Shaver, 2020).. Chemical and mechanical recycling is main form of recycling for primary, secondary, and tertiary recycling (Schyns and Shaver, 2020).. Primary and secondary recycling utilizes mechanical where plastic is melted and turned into another plastic product while tertiary recycling depolymerizes the polymer to its subsequent monomers (Schyns and Shaver, 2020). These recycling methods maximize the lifespan of plastic however, the requirements for the process are very tricky as plastics have to be sorted into their groups and washed prior to being recycled and the fact that it may be recycled finite number of times makes it even more difficult to keep track.

1.3 Why PET and Why PS

1.3.1 PET

Polyethlyene terephthalate(PET) is considered highly recyclable plastic resin which widely used in packaging for due to its costs, lightweight, resealable, shatter resistance and “recyclable” properties(Welle, 2015). However the requirements for recycling PET to be reused as food packaging includes “super-clean” recyclate which should be indistinguishable from virgin PET which makes it economically and practically impossible for the manufacturers to recycle as producing virgin PET is cheaper and easier(Welle, 2015, Welle, 2011). And as end market for post-consumer recycled PET ends up as fibre, PET sheets, and secondary packaging materials which inevitably ends up not being able to be further recycled in standard settings due to difficulties of separating various materials embedded within the product. And with recollection rate in the United Kingdom being 32.7% in 2009, further increases the seriousness of environmental damage as the numbers indicate that from 102,000 tonnes of PET produced, 64,000 tonnes were thrown away to be incinerated or to damage the environment just in 2009(Welle, 2011).

1.3.2 Polystyrene

While PET is mainly used as food packaging, polystyrene (PS) is widely used as insulator and even as food packaging as well. PS can be used as solid and as well as expanded foam which in practice can be recycled. However, due to the difficulty and the danger that arise from recycling and melting polystyrene, the recovery process becomes more expensive than using virgin material. So in many developed countries, PS foam is treated as municipal solid waste and disposed off into the environment (Maharana et al., 2007). In the process of disposal, the PS is often mechanically broken down due to weathering further making collection difficult. Another massive issue regarding PS is the use of PS foam as fishing buoys. These buoys are subjected to harsh sea environments releasing millions of Polystyrene foam shavings which sea creatures such as fish and birds mistakenly consume and die from poisoning and blockage in their systems.

As it is currently financially and technologically unfavourable to recycle the PET and PS for the industry, it is crucial to investigate novel process to find a method to tackle plastic pollution in the direction of microbial degradation.

1.4 Biological degradation of PET and PS

Biological degradation of Plastics has been a heavily researched topic in the 21st century as many began to realize the extent and deleterious effects of plastic pollution in the environment. Biodegradation of plastic usually begins

with conversion of large and heavy plastic molecules into smaller components to be water-soluble or can be taken up for metabolism. This form of degradation is called mineralization where the compound is converted into minerals by microorganisms which can be converted into CO_2 or other naturally occurring compounds (Benzerara et al., 2011). This is however very difficult due to the size/molecular weight of the polymer, many times, the extracellular enzyme is exported to break up the surface layer of the plastic slowly breaking down the polymer into smaller more water-soluble breakdown products. However, this is not as easy as it seems, because within the plastic, sometimes the plastic is not made of one compound but with multiple compounds such as PET. PET is composed of ethylene molecules and terephthalate bonded together making it difficult for the enzyme to find the "correct" orientation to attack the molecule.

1.4.1 Current knowledge of plastic biodegradation by microorganisms

The Isolation and characterization of microorganisms capable of biodegrading various synthetic plastics is still ongoing. Recent advances in technology and awareness have highlighted potential microbial degradation for commonly used plastics such as polyethylene, polystyrene, polypropylene, polyvinyl chloride, polyethylene terephthalate, and poly urethane (Mohanan et al., 2020).

1.4.2 Microbial degradation of PS

PS have been heavily researched. Recently Kim et al. (2020) has successfully isolated a strain of *Pseudomonas aeruginosa* DSM 50071 from

the gut of larvae of *Zophobas atratus* commonly known as superworms (Kim et al., 2020).The researchers fed super worms Styrofoam in order to perform selective pressure on the gut microbe of the super worms. The selective pressure caused a massive increase in the growth of PS degrading *Pseudomonas sp*. which they were able to confirm by isolating and inoculating the isolate in liquid carbon free basal medium. The surface examination via SEM and XPS confirmed that PS film (99.9% pure, purchased from Goodfellow) was indeed degraded by *Pseudomonas* sp*.* Although they were not able to identify specific enzymes nor metabolic pathway the isolate utilized to biodegrade PS film, they were able to confirm the degradation to be highly involved with the presence of biofilm as the film exhibited high levels of oxygenation which is an indication of biological degradation of polymer. The identification of enzyme responsible for biodegradation of plastic is still considered very difficult due to lack of confirmed biodegradation of virgin plasticizer free plastic. As well as *Pseudomonas* sp., *Rhodococcus ruber* was also showed potential degrading capabilities on Polystyrene(Kim et al., 2020, Mor and Sivan, 2008). However, some enzymes were identified to be capable of breaking down PET.

1.4.2 PET Biodegradation

Enzymes capable of hydrolysing PET was first identified by Yoshida and his team in 2016 from a PET-contaminated sediment near a plastic recycling centre in Japan (Hiraga et al., 2019, Yoshida et al., 2016). This finding triggered an interest in research towards the identification and isolation of plastic degrading microorganisms. The *Idonella sakaiensis*

isolated by Oda and his team, excretes a special esterase group enzyme called PETase. Because of the crystalline structure of PET, it is believed that the accessibility of the ester bonds is very difficult. However, the identification of PETase allowed a new hope for plastic degradation. The PETase hydrolyses the bond present between ethylene and terephthalate producing mono and bis ethylene terephthalate (MHET/BHET), ethylene, and terephthalate (Tanasupawat et al., 2016). Being able to release the MHET and BHET molecules means that ethylene and terephthalate become more readily available for various microorganisms to mineralize and utilize it as sole carbon source.

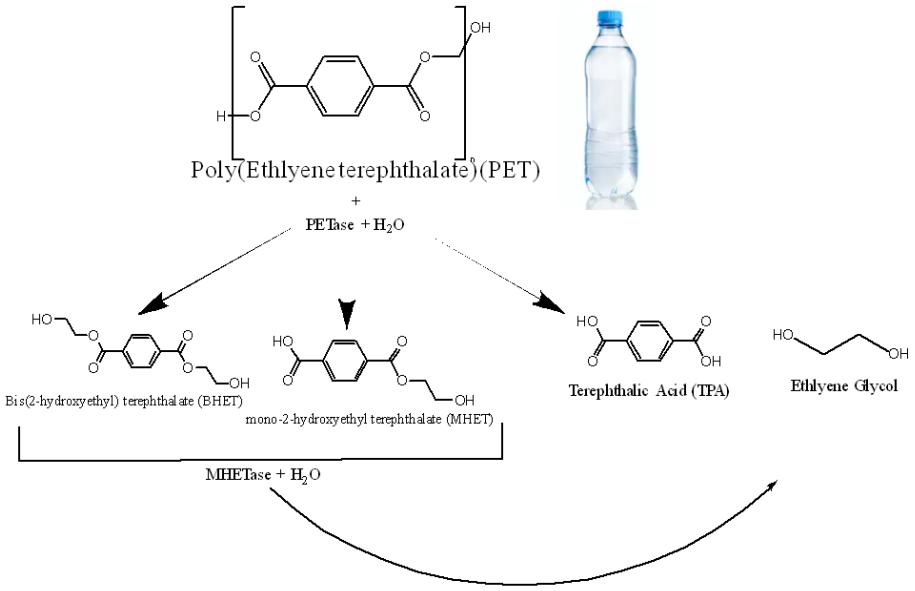


Figure 1: Enzymatic degradation of PET plastic. PET polymer is first depolymerized by PETase into Bis(2hydroxyethyl) terephthalate (BHET), Mono-2hydroxyethyl terephthalate (MHET) and into TPA. BHET and MHET is then degraded further into Ethylene glycol and Terephthalic Acid which can be more readily bioassimilate.

However, release of terephthalate poses difficulties for microorganisms in the long term. The structure of TPA makes mineralisation difficult due to its double carboxyl group. So, despite being able to break open the PET molecule, *Idonella sakaiensis* can only utilize the “easier” ethylene molecule while leaving more difficult terephthalate molecules behind. The concept of using TPA degrading chassis such as *Comamonas* sp., *Delftia* sp., *Paraburkholderia* sp., and *Rhodoccus* sp. (Sasoh et al., 2006, Shigematsu et al., 2003, Hara et al., 2007, Chain et al., 2006, Knott et al., 2020) has been mentioned by Salvador and his group but there is no evidence that they explored this idea further (Salvador et al., 2019) .

1.4.3 Degradation of TPA

While ethylene glycol (one of the products of PET degradation), is more readily mineralised by various microorganisms, the other half of the product, terephthalate, is not easily degraded. Currently there are only 6 groups of

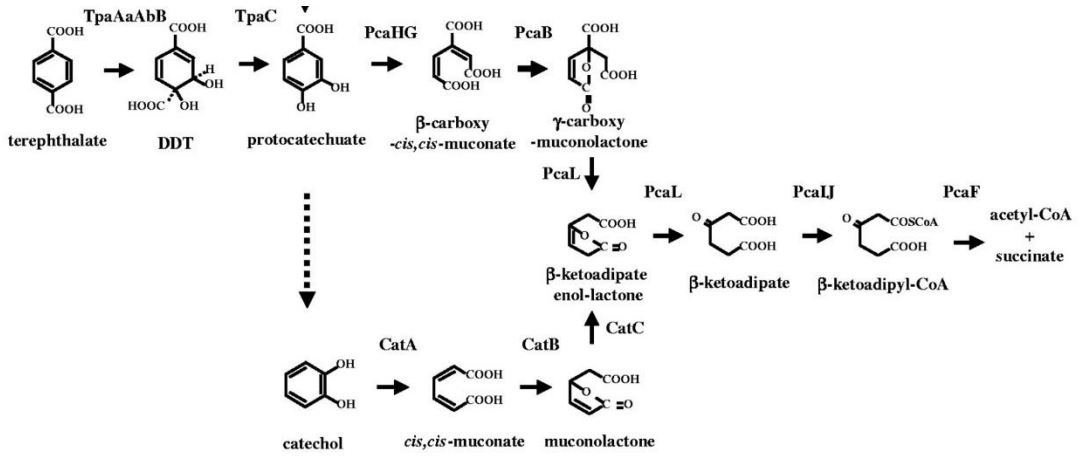


Figure 2: Proposed degradation pathway of TPA by Rhodoccus sp. (Hara et al., 2007). The process involved decarboxylation and carboxylation of TPA into PCA which is broken down into more simple components finally being

known genera and few strains from these genera capable of utilizing TPA as sole carbon source. These genera are *Rhodococcus, Comamonas, Pseudomonas, Bacillus, Arthrobacter,* and *Dietzia* (Sasoh et al., 2006, Shigematsu et al., 2003, Hara et al., 2007, Chain et al., 2006, Knott et al., 2020) . The metabolic pathway of TPA degradation is believed to involve protocatechuate (PCA). The breakdown of TPA involves oxygenation and dehydrogenation of TPA to produce PCA which is more readily degraded figure 2. PCA is an important intermediate metabolite in microbial degradation of aromatic compounds such as 4-hydroxybenzoic acid, vanillic acid, and phthalates.

1.5 Screening/ Selective Enrichment techniques

Based on Kim et al. (2020) and Yoshida et al. (2016) works, many researchers followed similar routes to isolate and identify potential plastic degrading microorganisms. To increase the likelihood of successful isolation of microorganisms with the capability to degrade target materials from environmental samples or organic samples like done by Kim et al. (2020), it is crucial to increase the selective pressure on the sample by increasing the nutrient availability. For Kim et al. (2020), his group increased the presence of PS inside the gut of the superworm while Yoshida et al (2016) intentionally sampled in areas with high PET contaminations and inoculated dilutions of samples in carbon free medium with PET film to screen for potential PET degraders. These two methods were effective in isolating from environmental

samples however, these methods are prone to be either miss or hit and can be time consuming. For example, Yoshida and his group worked on 250 different samples with only one microbe showing degrading capabilities. Also, it is proven that biodegradation in some instances can happen when there is a consortium of microbes in the environment to breakdown the plastic polymers (Meyer-Cifuentes et al., 2020). If this is distorted into individual strains, degradation may not happen causing difficulty in identifying the key microorganisms responsible for the breakdown.

Some researchers such as Jumaah (2017) isolated the individual microbes prior to assessing the biodegradation of plastic for post screening the strains for their plastic degrading capabilities.

On top of the nutrient limitation, many plate-based assays have been developed to screen for screening of potential microorganisms capable of degrading plastics. One widely used plate-based assay for plastic degrading microorganisms is polycaprolactone (PCL) emulsion plate. PCL is a synthetic polyester that has been utilized by many researchers as a model substrate for assessment of PETase and cutinase enzymatic activities (Nyyssola et al., 2013, Almeida et al., 2019, Danso et al., 2018). This screening method showed activity against various polyesterases which are a crucial group of enzymes required for plastic degradation.

Another potential group of enzymes responsible for the degradation of synthetic polymers are esterases, cutinases, and lipase(Krakor et al., 2021, Kaushal et al., 2021, Austin et al., 2018). More specifically serine hydrolases like cutinases (EC.3.1.1.74) have shown effective degradation of PET to TPA

and Ethlyene glycol. All above mentioned group of enzymes are naturally found do hydrolyze large macromolecules. Large polymer degrading enzymes such as cellulases and chitinases were screened as a part of characterization as well. The screening was done with emulsion plate with emulsified cellulose and chitin.(Gupta et al., 2012, Kumkum and Garg, 2019).

1.6 Proposed solution

The chemical and mechanical recycling methods are viable solution however, with limitation of economics and the low rate of recycling indicates that majority of plastic waste ends up in the environment where plastic and chemicals within the plastic contaminates the environment. To minimize the harm done to the environment, it is crucial to identify and figure out a method to not only remove plastic waste from the environment but to reduce the harm done to the environment. Whilst most research have been done on degradation of commercial plastics with well-known strains such as *Bacillus sp, Pseudomonas* sp., *Streptococcus* sp., and even animals such as mealworms and silk worms (Bombelli et al., 2017, Kumar et al., 2007, Satheeshkumar. S. et al., 2016). It is crucial to identify and isolate potential microorganisms capable of degradation of polymeric structures not only the plasticizers and other contaminants found in. the commercial plastics.

1.7 Aim and Objectives

Aim

The aim is to isolate novel strains of bacteria which are capable of growing on PS and PET from the environment.

Objectives

1.7.1 Evaluation of the biodegradation of virgin PS and PET by *Pseudomonas* sp.

1.7.2 Isolation and enrichment of novel PET degrading *Streptomyces* from the environment

1.7.3 Microbiological characterization of the HST strain.

CHAPTER TWO

Materials and Methods

CHAPTER 2 MATERIALS AND METHODS

2.1 Chemicals

Except where otherwise stated, all chemicals used in this study were purchased from Sigma-Aldrich.

2.1.1 Buffers

Phosphate buffer solution and Tris-EDTA buffer were prepared according to the manufacturer's guidance.

2.2 Plastics

Except where otherwise stated, all plastics used in this study were purchased as virgin and plasticiser free from Goodfellow company.

2.3 DNA Oligonucleotides (Primers used)

These primers were used for 16S rRNA PCR for identification of isolates in species level (Wawrik et al., 2005). 16S rRNA sequencing is considered to be a gold standard in identification of unknown micro-organisms as it is a conserved gene (Janda and Abbott, 2007)

Table 1: Primers used in this study (Wawrik et al., 2005)

Primer Name	Sequence (5' – 3')

27_F	AGAGTTTGATCMTGGCTCAG
1492R	TACGGYTACCTTGTTACGACTT

2.4 Strains used in this study

Table 2: Bacterial strains used in this study. The isolates are obtained as the consequence of the methods outlined in the materials and methods section.

Strains	Description	Source/Reference
C1	*Pseudomonas* sp. isolated from wastewater	Previous study
C2	*Pseudomonas* sp. isolated from wastewater	Previous study
C18	*Pseudomonas* sp. isolated from wastewater	Previous study
C20	*Pseudomonas* sp. isolated from wastewater	Previous study
B18	*Pseudomonas* sp. isolated from wastewater	Previous study
B22M1	*Pseudomonas* sp. isolated from wastewater	Previous study
CV1	*Streptomyces sp.* isolated from Coventry streets.	This study
CV2	*Streptomyces sp.* isolated from Coventry streets.	This study

CV3	*Streptomyces sp.* isolated from Coventry streets.	This study
CV4	*Streptomyces sp.* isolated from Coventry streets.	This study
CV5	*Streptomyces sp.* isolated from Coventry streets.	This study
CV6	*Streptomyces sp.* isolated from Coventry streets.	This study
CV8	*Streptomyces sp.* isolated from Coventry streets.	This study
CV10	*Streptomyces sp.* isolated from Coventry streets.	This study
CV11	*Streptomyces sp.* isolated from Coventry streets.	This study
CV13	*Streptomyces sp.* isolated from Coventry streets.	This study
CV15	*Streptomyces sp.* isolated from Coventry streets.	This study
CV16	*Streptomyces sp.* isolated from Coventry streets.	This study
CV17	*Streptomyces sp.* isolated from Coventry streets.	This study
HST	*Streptomyces sp.* isolated from human sewage area	This study

4.5M	*Streptomyces sp.* isolated from Staples Landfill site	This study
21.5M	*Streptomyces sp.* isolated from Staples Landfill site	This study

The urban samples were collected in 50 mL falcon tubes with alcohol washed spatula to ensure no crossover of the samples occurred. Samples were taken from wide range of areas such as London, Bridlington, Coventry, Landfill, and a Sewage treatment centre to maximize the potential for successful isolation of potential plastic degrading microorganisms. Error! R eference source not found. shows all the strains and location of the sampling used in this study.

2.4.1 Pseudomonas culture

The *Pseudomonas sp.* cryostock from the previous study was used to assess the biodegradation of plastic in the first instance. Fresh glycerol stocks were prepared and stored at -80 ºC until usage.

2.4.2 Strain Storage

For the method of storing of the strains for long term, CryoBeads™ was used for *Pseudomonas* sp. and 20% glycerol spore stock was prepared for isolated *Streptomyces* sp.

The isolated strain was inoculated in 10 mL of TSB medium for 3 days. 100 µL of the culture was plated onto oatmeal agar. The plate was grown at 30 ºC for 14 days. 1 mL of sterile 80% glycerol was added to sporulated plate. Using a sterile L spreading stick, glycerol was spread around with light pressure.

2.5 Growth conditions of strains

Unless stated otherwise, *Pseudomonas* sp. and isolated *Streptomyces* cultures were grown in 250 mL baffled flasks sealed with foam bongs and incubated at 30ºC at 120 rpm. Cultures in 50 mL falcon tube were incubated at 30ºC at 200 rpm, and plates were incubated at 30ºC incubator. The chemical composition of medium used, and BG-11 trace elements is listed in**.**

2.6 Media composition

2.6.1 Stock solution of Benzoic Compounds

To reduce the breakdown of the compound containing a benzoic ring, catechol vanillic acid, 4-HBA, PCA, TPA, benzoic acid, and toluic acid were first dissolved in water prior to being filter sterilized. To make 0.5 M stock solution of the compound, corresponding amount of the compound was added to 70 mL of dH_2O. With continuous stirring, pellets of NaOH were dropped one at a time to maintain a pH 8. When most of the powder was dissolved, using a pipette, small amounts of 10M and 1M NaOH solution was used to dissolve the rest. Once all the powder was dissolved, the volume was brought up to

100 mL by addition of dH_2O. The stock solution was filter sterilized using a 0.2µm sterile syringe filter. Post sterilization appropriate amounts of the stock solution were added to medium or agar prior to being used/ poured.

2.6.2 Liquid medium

2.6.2.1 International Streptomyces Project medium #4 (ISP4)

International Streptomyces protocol #4 (inorganic Salt Starch) medium also known as ISP4, recommended by Shirling and Gottlieb was used (Shirling and Gottlieb, 1966). It is composed of 10 g L^{-1} soluble starch, 1 g L^{-1} K_2HPO_4, 1 g L^{-1} $MgSO_4$, 1g L^{-1} NaCl, 2 g L^{-1} $(NH_4)_2SO_4$, 2g L^{-1} $CaCO_3$ with addition of BG-11 trace salts to supplement all the necessary trace salts that is not included in ISP4 trace solution such as H_3BO_3, $CuSO_4$, and $Co(NO_3)_2$ to ensure all the necessary cofactors for potential enzymes are present. 10g of soluble starch was first dissolved in 300 mL of water to prevent clumping before adding rest of the media components.

2.6.2.2 Glucose, Yeast extract, Malt extract (GYM) / +S

Glucose yeast malt extract is a rich medium used for subculturing and for pH optimization. The ingredients of the media are as follows; 4 g L^{-1} glucose, 4 g L^{-1} yeast extract, 10 g L^{-1} malt extract, pH 7.2 (Vetrovsky et al., 2014). On certain occasions, 20 g L^{-1} of soluble starch was added to make GYM+S medium (DSMZ media 214).

2.6.2.3 LB

Lysogeny broth was used for streak plating of the culture to check for any signs of contamination. The medium was prepared by dissolving 25g L^{-1} in distilled water and autoclaved

2.6.2.4 TSB

Tryptic Soya Broth is another rich medium used in this experiment. and This media was used for propagating the strains and for extraction of genomic-DNA. The medium is composed of 30 g L^{-1} of Tryptic Soya broth powder (Sigma).

2.6.2.5 Minimal Medium (M9)

M9 minimal mineral media was used for checking biodegradation of plastic. The composition of M9 media comprises of 6 g L^{-1} Na_2HPO_4, 3 g L^{-1} KH_2PO_4, 1 g L^{-1} NH_4Cl, and 1 mL L^{-1} of 1000x BG11 Trace Metals. Desired concentration of carbon source ,1 mL of filter sterilized 1M $MgSO_4.7H_2O$ and 300µl of 1M $CaCl_2$ were added post sterilization after autoclaving.

2.6.3 Solid medium

Solid medium was used to isolate and to subculture from broth cultures to be visualized for morphology and to perform contamination tests.

2.6.3.1 ISP4 Agar

To make ISP4 agar plates, 2X working concentration of ISP4 liquid media (34 g L^{-1}) was made as well as 2X working concentration agar solution

(24 g L^{-1}) was autoclaved separately. After autoclaving, equal volumes of two solutions were mixed thoroughly and about 20 mL of mixed solution was poured into petri dishes.

2.6.3.2 GYM Agar

GYM solid medium was made by adding 3 g L^{-1} of $CaCO_3$ and 12 g L^{-1} of agar into GYM liquid media. After sterilization by autoclaving, the media was poured into petri dishes.

2.6.3.3 Potato Dextrose Agar (PDA)

PDA was used for morphological differentiation of the isolates to further characterize the strain. To make PDA, 39 g L^{-1} of Potato Dextrose agar powder (Millipore) was dissolved in H_2O. The solution was then autoclaved and poured into petri dishes.

2.6.3.4 Oatmeal Agar

Oatmeal agar was prepared by dissolving 72.5 g L^{-1} of Oatmeal agar powder (Millipore) in H_2O. The solution was autoclaved and poured into petri dishes.

2.6.3.5 Soya-flour Mannitol Agar (SFM)

Soya-flour mannitol plates were prepared by separately dissolving and autoclaving 20 g/L of Soya flour (purchased from Holland & Barrett™) with 20 g L^{-1} Mannitol and 14 g L^{-1} of agar in 500 mL of H_2O. After autoclaving, equal volume of two independently autoclaved solutions were mixed thoroughly prior to being poured into petri dish.

2.6.4. Selection and assay Plate for rapid screening of potential plastic degrading microorganisms and metabolite consumption.

Various assay plates were prepared and used to further screen, isolate, and characterize isolates that have been obtained during this work with potential plastic degrading capabilities. The M9 minimal medium with BG11 trace metals were used as the base for the assay plates.

2.6.4.1 M9 solid medium

M9 solid media was prepared by mixing 2x working concentration M9 solution with BG11 trace and 2x working concentration agar solution. 1 mL /L of 1M $MgSO_4$ and 300 µl/L of 1M $CaCl_2$ was added after combining two solutions. For TPA assay, sterile TPA stock solution was added prior to pouring. The mixture was allowed to cool and poured into petri dishes.

2.6.4.2 Polycaprolactone assay plate

Polycaprolactone (PCL) emulsion was made by dissolving 5 g of PCL in 15 mL of Dichloromethane at 50°C with continuous stirring. To this 35 mL of acetone was added while stirring at max speed, by adding 50 mL of H_2O. 2x agar was prepared by adding 14 g of agar to 400 mL of water. Double concentration of M9 solution was made by dissolving 10.92 g of M9 powder in 500 mL of H_2O. To this 1 mL of BG11 trace metal solution and 1 g of yeast extract was added. The 2x M9 and agar solutions were sterilized by autoclaving. After cooling the agar to 50 °C, the emulsion was added to the 2x agar solution while stirring constantly under the fume hood. The solution was stirred and heated to 60°C for 2 hours to remove solvent. 2x M9 solution was

added to the 2x agar + PCL solution. While stirring, 1 mL of 1M $MgSO_4$ and 300 µL of 1M $CaCl_2$ were added and poured.

2.6.4.3 Cellulase activity assay plate

2 g/L of crystalline Cellulose (microcrystalline powder, Sigma-Aldrich) was added to 2x agar solution and autoclaved. And the rest was prepared as described in 2.6.4.1.

2.6.4.4 Chitin plate

Colloidal chitin was prepared according to the methods described by Murthy and Bleakley (2012) (Murthy, 2012).Five grams of chitin powder from shrimp sells (Sigma-Aldrich) was dissolved in 50 mL of concentrated HCl under the fume hood, for 2 hrs at room temperature. After, Chitin + HCl solution was added slowly to 500 mL of H_2O with constant stirring to precipitate chitin. The precipitated chitin was aliquoted into 5 different centrifuge safe bottles and centrifuged at 25,000 rpm for 10 minutes. The chitin precipitate was washed 3 times using H_2O before resuspending in 50 mL of H_2O. The final pH of the media was adjusted to 5.5 using NaOH. The stock solution was sterilized by autoclaving (Yildirim-Aksoy et al., 2018). 10 mL of chitin solution was added to 2x agar and autoclaved. The rest is as described in 2.6.4.1.

2.7 Screening of *Pseudomonas sp.* for potential plastic degradation.

2.7.1 Strain revival

The cryostock of *Pseudomonas* sp. was revived by inoculating it on LB broth and LB agar. Initially, the cryostock was defrosted in ice to minimize the temperature shock that may occur from rapid defrosting. The defrosted stocks were then streaked onto sterile LB plate. The plates were grown at 30ºC 3 days prior to secondary streak to purify the culture. After secondary streak, the plates were then grown for 3 days at 30 ºC. Single colony was picked using sterile loop and was used to inoculate 10ml of LB broth and was left in shaking incubator at 30 ºC, 120 rpm for 3 days. After 3 days the culture was streaked once more to ensure the stock was morphologically identical.

2.7.2 Screening for plastic degradation

Cultures were washed and subject to centrifugation at 10,000rpm using an Eppendorf Centrifuge 5810R for 10 minutes. The supernatant was carefully removed. The cell pellet was then resuspended using sterile M9 minimal media without any additional carbon. The process was repeated 3 times to ensure that most of the LB was removed. After the 3rd cycle, M9 with 0,1g/L of YE (Yeast Extract) was used to resuspend the cell pellet. Then, 1g/10ml of sterilized virgin PET or PS film was added to each falcon tube to promote degradation. As a control, 2 cultures from each strain were

inoculated with M9 with 0.1% YE without any additional plastic to compare the growth.

2.8 Isolation of Streptomyces sp. from environmental samples

2.8.1 Sample preparation

Various environmental samples were taken from geographically different location such as Coventry, Bridlington, London, Staple quarry Landfill site in Newark and samples collected near human sewage treatment plants to identify the presence of microplastic and potential microorganisms that can biodegrade plastic. The sampling was done by Dr. Edward spence and Kwon Jun Lee throughout the duration of the project. Samples were transferred into 50 mL falcon tubes using a clean wooden stick and the stick was snapped to ensure the safety of the sampler and to avoid cross contamination of the samples. For the storage of soil samples, the samples were kept at room temperature in a parafilm sealed Falcon tube until required. Prior to the isolation work, 1 g of the sample was diluted to 10^{-3} to 10^{-7} in sterile M9 media to reduce the residual carbon.

Two methods of isolation were used targeting actinomycetes selective isolation and enrichment. And for *Pseudomonas sp.*, the master stock was tested for contamination and inoculated to test the growth on different plastics as described in section 2.8.

2.82 Serial enrichment of samples

In order to isolate for potential plastic degrading microorganisms, 100 ul of diluted samples as mentioned on 2.7.1, were then inoculated into the M9 media supplemented with 1 g/L virgin plastic as the carbon source. Virgin plastics were used to minimize the potential for isolating microorganisms that may potentially degrade contaminants in the commercial plastic.

The first-generation enrichment carried on for 3 weeks at 30°C and 225 rpm. Then, the enriched samples were inspected for any signs of growth. Using the lowest dilution which showed signs of growth, the culture was subcultured into fresh enrichment media as mentioned above. The duration of second-generation growth was dependent on the sample. The initial planned 2nd generation enrichment phase was 1 month however, some samples were enriched for 5 months. After the enrichment was completed, the enriched samples were inspected for any obvious signs of fungal contamination and colonies were isolated using the steps mentioned in section 2.4.3.

2.8.3 Streptomyces isolation from samples

For selective isolation of Streptomyces, the diluted samples were inoculated onto ISP4 agar as described in 2.2.3.1. Once colonies were visible, they were examined for characteristic traits of Streptomyces, such as branching vegetative mycelium, spore production and secondary metabolite production, such as pigments change (Bennett et al., 2018). Selected colonies were restreaked on to new ISP4 agar plates to isolate single colonies and the

process was repeated to obtain pure single colonies. Isolated colonies were then plated onto different solid media such as ISP4, GYM, Oatmeal, Potato Dextrose agar as listed in **2.6** to allow for morphology and pigmentation-based categorization, reducing repetitions within the strain allowing wider variety of library to be created.

2.8.4 Morphology of the isolates

The colony morphology of the strains were evaluated based on the Washington University's handbook. The cultures were grown on LB for *Pseudomonas* sp. and for *Streptomyces* sp. ISP4 and GYM was used. The colony morphology was observed over the first 3 days of inoculation to ensure that there is no contamination. On top of the streak plating, gram staining was done to confirm that only one type of microorganism was present in the culture.

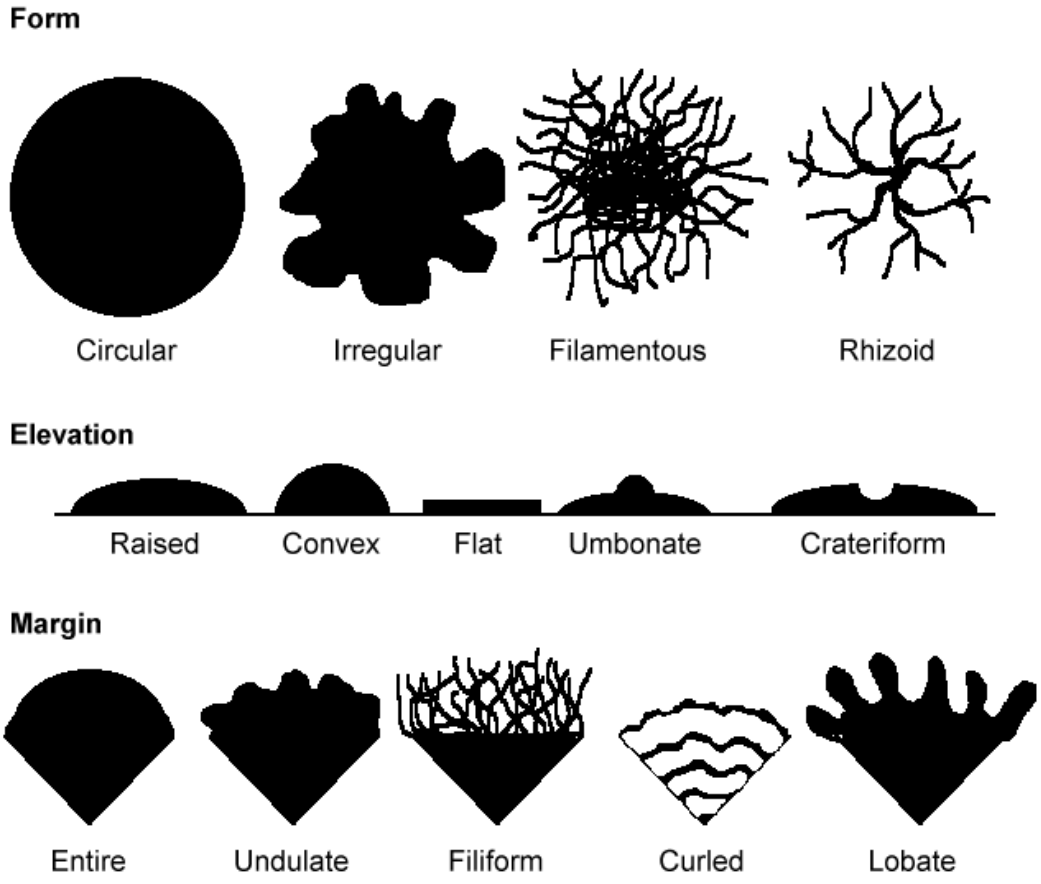


Figure 3: Diagram of Colony morphologic typing (Microbiology 101 lab book, Washington State University).

2.9 Molecular Procedures

2.9.1 Genomic DNA extraction and 16S rRNA PCR.

The genomic DNA of each strain was extracted from *Streptomyces* sp. cultures grown in 50 mL falcon tube in GYM+S media for 3 days and LB broth for *Pseudomonas* sps., according to previously published methods (Dashti et al., 2009). Briefly, 100 µL of each culture (selected based on morphological differences, as described above) were placed in sterile 2 mL tubes, centrifuged

in a mini centrifuge at maximum speed for 5 minutes, at room temperature, to pellet the cells. The supernatants (spent media and secondary metabolites) were removed, and pellets washed with sterile deionised water 2 times. After the final centrifugation step, the pellet was resuspended in 50µL of sterile deionised water and the tubes were placed in heat block set at 95 °C for 10 minutes, lysing the cells and releasing the genomic DNA. After 10 minutes, samples were centrifuged at 1000 rpm for 5 minutes and the supernatant used as template for 16S rRNA Polymerase chain reactions (PCR).

2.9.2. Amplification of 16S rRNA gene

The 16S PCR was performed in Eppendorf™ Mastercycler® nexus X2 PCR thermocycler. The primers used in the 16s PCR are shown in Error! R eference source not found.**.** The components of each PCR reaction are listed in **Table 3**: Taq polymerase used in this The thermocycler conditions used in the PCR is based on Wawrik B, et al. (2005) with a slight modification. The conditions used in this experiment are shown in

Table 4.

Table 3: Taq polymerase used in this experiment.

Components	**1x 25 µL reaction**
G2 master mix	12.5 µL
Primer Forward (10µM)	1 µL
Primer Reverse (10µM)	1 µL

Template DNA	1-2 µl (~100 ng)
Water	Up to 25 µl
Total	25 µL

Table 4: 16s PCR thermocycler conditions used in this experiment. The 2 sets of cycles were used for specificity of the product. This protocol was used as it was a laboratory standard for 16S rRNA gene of unidentified isolates.

Steps		Temp. °C	Duration
Initial		95	2 minutes
Denaturation			
30 cycles	Denaturation	95	1 minutes
	Annealing	55	45 sec.
	Extension	72	90 sec.
35 cycles	Denaturation	95	1 minutes
	Annealing	60	45 sec.
	Extension	72	90 sec.
Final extension		72	10 minutes
Hold		4	∞

2.9.3 PCR product analysis via agarose gel electrophoresis

25 µL of PCR product was loaded onto 1% agarose (Sigma-Aldrich) in TAE buffer (40 mM Tris base; 0.1% (v/v) glacial acetic acid; 1 mM EDTA) containing 0.1 µL/mL of SYBR Safe DNA stain (Thermo-Fisher Scientific). Gels were run using a Bio-Rad gel running apparatus and a Bio-Rad

PowerPac™, set at 110V, for 45 minutes to 1 hr. Gel bands were visualized using a gel doc.

2.9.4 Gel extraction

Once the PCR was confirmed to have one distinct band in the 1.5 kb-1.6 kb range (Edwards et al., 1989), an UV transilluminator was used to assist in removing target band areas with a scalpel. DNA-containing agaroge gel fragments were placed in clean 1.5 mL eppendorf tubes and DNA fragments were extracted from the agarose gel using QiAquick® Gel Extraction Kit. The protocol used for purification of PCR products was modified inorder to increase the concentration and to minimize the contaminants. The modifications to the protocol is as follows. The manufacturer's steps were followed until the washing step with buffer PE. An additional wash step was added to decrease the contaminates as one wash resulted in poor 260/230 ratio. After the second wash, the column was allowed to dry for 10 minutes to remove trace amounts of ethanol added to buffer PE. Also, instead of using 50 µL of room temperature buffer EB to elute the PCR products, the EB buffer was warmed to 55°C and only 30 µL was used with an elution time of 5 minutes where the column was left on the bench. The purified and eluted PCR products was then quantified using µLITE spectrophotometer (BioDrop).

2.9.5 Restriction fragment length polymorphism (RFLP)

Purified 16S rRNA PCR products were digested using Eco32I and Bsp1407I (BsrGI) FastDigest® enzymes from Thermo Scientific™ (typical digestion components shown in **Table 5**). All digestions were performed in 0.2

mL PCR tubes and were incubated at 37 °C for 30 minutes, to ensure complete digestion of target fragments. The digested 16S rRNA fragments were loaded onto 5% agarose gels, prepared with TAE Buffer, and containing 0.1 µL/mL SYBR safe DNA stain. Gels were run at 110V for 40 minutes, as described above, to separate the different sized bands resulting from enzymatic digestion.

Table 5:Composition of RFLP reaction

Components	Digestion
10x FastDigest® Buffer	17 µL
Digestion Enzyme	1 µL
Digestion enzyme 2	1 µL
DNA	~3 µg
Nuclease-Free water	Up to 30 µL

2.9.6 The selection of isolates for sequencing

The selection for *Streptomyces* sp. isolates for sequencing was done using 3 steps detailed above. After the phenotypical grouping was done according to the morphology, spore colour, and colour of the agar, 16S PCR and restriction fragment length polymorphism was done to differentiate between different groups of *Streptomyces* to widen the scope of isolates. After gel electrophoresis, the strains with differences in the band size were selected.

2.9.7 PCR product sequencing

The 16S PCR products from selected strains were extracted from the agarose gel and extracted using Qiagen™ QIAquick® Gel Extraction Kit. The manufacturer's protocols were followed in 2.9.4. The extracted 16S products were diluted to according to the requirements set by Source Bioscience and was sent for sequencing.

2.9.8 Whole genomic DNA extraction for sequencing of Selected isolate

To ensure good quality genomic DNA and minimal degradation, the genomic DNA was extracted from freshly grown culture prior to the shipment.

The additional two different genomic DNA extraction methods were used for sequencing of genomic DNA. The initial method was using bead beating and the second method was using the phenol-chloroform extraction method. Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA) was used. After the extraction, the quality of the DNA was checked on 0.5% Agarose gel and sent to Novogene for sequencing.

2.9.8.1. Quick-DNA Fungal/Bacterial miniprep Kit

Two modifications were made to the manufacturer's protocol. Prior to bead beating, the cells were incubated with 100mg/mL of lysozyme for 1 hour at 37 °C to weaken the cell membrane. After, the bead bashing was done for 30 seconds instead of five minutes to minimize the shearing of genomic DNA. The rest was done according to the manufacturer protocol.

2.9.8.2 Phenol-chloroform extraction

Phenol-chloroform extraction was performed as described by Moor et al (2004) with modifications made for the isolate HST. Different modifications were required to lyse the cells as the protocol did not work effectively due to the morphology of the isolated strain HST. Tris HCl-EDTA (TE) (pH8) was used as a buffer. The modified method for the isolated strain HST is as follows. 10 mL of the culture grown in 2YT medium was centrifuged and washed with 5 mL of sterile TE buffer twice. 0.5 g of wet cell pellet was placed in a clean 15 mL falcon tube. 2.5 mL of TE buffer was added and resuspended, and 0.1mg of lysozyme from eggshells (Sigma-Aldrich) was added to the suspension. The lysozyme was mixed by inverting the tube until the lysozyme was dissolved. Using a pipette, the cells stuck to the wall were dropped into the solution. The solution was incubated at 37°C for 60 minutes. 30 µl Proteinase K (10 mg/mL), and 100 µl SDS (10–20%) was added to the incubated lysate solution. The solution was mixed by slowly inverting the tube 10 times. Using liquid nitrogen (-196°C) and a water bath set at 55°C the cells were frozen and thawed 10 times for effective lysis of cells. The freeze and thaw cycle were 5 minutes in liquid nitrogen and 3 minutes in water bath. Once the cells were lysed, the solution was incubated at 37°C for 10 minutes for protease K to denature the DNase. 500 µl of 5 M NaCl was added and mixed by inverting the tube. The suspension was incubated at 65°C for further 2 minutes. 400 µl of TE+ NaCl (TE Buffer+100mM NaCl) was added to the suspension and mixed by inverting. The solution was separated into 6 individual tubes by pouring (do not use tips as it may shear DNA) into equal volumes. After shaking 700 µl of the phenol/ chloroform/ isoamyl alcohol (25:24:1) solution (Invitrogen™) was added to

each tube. The tubes were thoroughly mixed by inverting them 5 times. The phases were separated by centrifugation at 10,000 x g for 5 minutes. The top layer (aqueous) containing nucleic acids was transferred to one new tube using cut tips to minimize the shearing of DNA. The supernatant was extracted again following the step above from addition of phenol/chloroform/isoamyl alcohol solution until the interphase becomes clear. Once the interphase became clear, the top layer was moved to a clean tube and 0.7 volumes of 100% isopropanol was added to precipitate nucleic acids. The solution was mixed gently by inverting and was placed in -20°C for 30 minutes. The tube was centrifuged at 12,000 x G for 30 minutes at 4°C. The DNA pellet was washed twice using 1 mL of ice-cold 100% ethanol and centrifuged again prior to removing the ethanol. The pellets were washed twice again using ice cold 70% ethanol. The pellet was air dried for 1 hour in the fume hood. The pellet was resuspended using 100 µl of TE buffer and placed at 37°C for 30 minutes. RNA was removed by adding RNase at a concentration of 100 µg/mL and placed at 37°C for 2 hours. The RNase treated sample was extracted again using the steps mentioned above. The DNA pellet was dissolved in 50 µl of 50°C nuclease free H_2O. Quality of the DNA and purity was checked by gel electrophoresis and Biodrop.

2.9.9 Phylogenetic analysis

Phylogenetic Neighbour-joining tree was made by utilizing 16sRNA sequence obtained by above mentioned methods. The 16sRNA was first sequenced and analysed using BLASTN. Once the Blast have identified similar sequences in the library. These sequences were selected for analysis

in MegaX software. Following alignment a neighbour-joining tree was constructed (Kumar Sudhir et al., 2018).

2.10 Screening of Degradation of Plastic by Adaptation on plastic

2.10.1 Selective serial enrichment of *Streptomyces* sp on various plastics.

The isolated pure colonies were used for the adaptation to media containing plastic as a sole carbon source (plastic degradation). The methodology using 24 deep-well plates is shown in **Table 6**. The strains were subsequently incubated in 10 mL of M9 + 0.1% YE in 50 mL falcon tubes, at 225 rpm and 30°C until OD of the inoculum reached ~ 1.0. This pre-inoculation in M9 + 0.1% YE increased the biomass and aimed to reduce nutrient shock. This culture was used in the first selective enrichment. Four mL of M9 + 0.1% YE media were placed in each well, along with the ethanol sterilized plastics (about 50 mg/well). Inoculated plates were sealed using a breathable membrane (Model, Supplier) and placed in a shaking incubator at 150 rpm and 30°C, for 3 weeks. After the first-generation selective serial enrichment, the plates were prepared with the steps detailed before but, without the YE to screen for Plastic degrading organisms. 100 ul of the 1st generation selective enrichment wells were used as an inoculum. The initial selective enrichment was designed to be completed in 1 month and carried in 30°C with aeriation by placing the culture in shaking incubator. Due to COVID-19 restrictions,

selective enrichment experiments were conducted for 5 months. The wells were supplemented with sterile H_2O every 2 weeks to mitigate the water loss due to evaporation.

Table 6: 24 well plate selective enrichment setup

PP	PP	PP	nylon	nylon	nylon
PLA	PLA	PLA	PE	PE	PE
PVC	PVC	PVC	PS	PS	PS
PET	PET	PET	CTRL	CTRL	CTRL

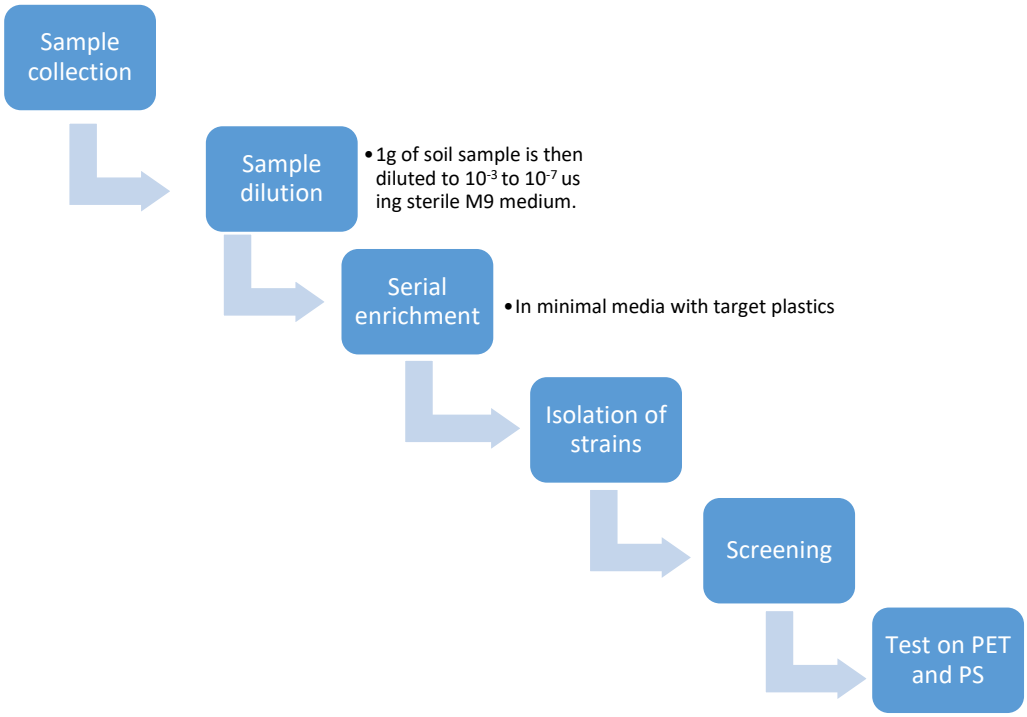


Figure 5: Process flow of the confirmation and isolation of plastic degrading microorganisms.

2.10.2 Plate based assay.

Selection and assay plates were used to quickly identify presence and expression of exported enzymes such as lipase, esterase, chitinase and cellulase as part of a characterization process to identify the isolate. The plate-based assay was performed by inoculating 100µL of GYM culture on the centre of the Chitinase and cellulase assay plate. The inoculated plate was grown in 30°C for 7 days. After the growth, the plate was flooded with 5 mL of Gram's iodine solution for 10 minutes and washed with clean H_2O. The dyed plate was left for 30 minutes before inspection for zone of clearing. For PCL degradation assay, the mixed culture was streaked onto PCL assay plate. The

plate was incubated in 30°C for 7-14 days until the zone of clearing was detected.

2.11 Biodegradation of plastic

2.11.1 *Preparation of Plastics*

Virgin plastic film and powder were purchased from Goodfellow. The plastics were weighed and washed 3 times with 100% ethanol, following the standard laboratory procedure to remove any residual contaminates such as debris and fungal/bacterial spores. The plastics were stored in ethanol for a minimum of 48 hours in 50 mL falcon tubes after being filtered and before being used. Using sterile pipette, most of the ethanol was pipetted out with care to not disturb the plastic. The ethanol-soaked plastic powders were then resuspended in 30 mL of sterile M9 medium. Plastic powder resuspended in 30 ml of M9 minimal medium was then left in 55°C with the lid lightly unscrewed to allow residual ethanol to evaporate. Resuspended plastics were then poured into the rest of the medium using aseptic methods. The pieces of plastic film were dried in 55°C prior being weighed and soaked in ethanol 48 hours. Soaked plastic pieces were then placed in sterile flask with sterile forceps. The flask with plastic pieces were then placed in 55°C for 48 hrs before adding M9 minimal media to remove residual ethanol.

2.11.2 Growth condition

The growth condition of plastic degradation was done in 250 mL baffled flasks with M9 supplemented with BG11 trace metals and modified ISP4 (starch removed) supplemented with BG11 Trace metals at 30°C,100-120 rpm for 4 weeks with supplement of glucose or TPA.

2.11.3 Colony Forming Units (CFU^{-1})

Colony forming units (CFU) was used as a method to track bio-assimilation of plastic. This was possible for *Pseudomonas* strains, however, was not possible for some isolates as the morphology did not allow accurate measurements. In order to assess the biodegradation and assimilation for those strains, final biomass was taken to be compared against glucose or TPA control.

2.11.4Scanning Electron Microscopy

The PET films were washed using water to remove the media and rinsed in absolute ethanol 3 times to remove the biofilm produced on the film to analyse the surface degradation. The films were then dried for 3 days in 55°C drying rack before being sent to be imaged.

PET film images were acquired using a JEOL 7100F FEG-SEM microscope, located at the Nanoscale and Microscale Research Centre (NMRC, University of Nottingham)

2.12 Biodegradation of TPA analysis

2.12.1 Growth conditions

The biodegradation of TPA was tested by inoculating the isolated strains in different concentrations of TPA in M9 minimal medium. Growth experiments were performed in 50 mL of M9 media in 250 mL baffled flasks with breathable membrane screw caps (0.2µM PTFE membrane, Duran). The growth conditions were 30°C at 100 rpm to minimize the culture being adherent to the side of the flask. The inoculation was done by adding 100 µL of 7-day culture from M9 + 5mM TPA medium.

500 µL of samples are taken every 24 hours to track the consumption rate of TPA. The samples were centrifuged at 14,000 x g for 10 minutes and pellet and supernatant were kept separate and stored in -80°C.

2.12.2 Hight Performance Liquid Chromatography

The concentration of TPA was measured by using C-18 reverse phased column (Agilent) on Agilent 1260 Infinity II HPLC. For the 5mM concentration, the samples were diluted to 10^{-1} and for 10 mM the samples were diluted to 10^{-2}. The samples were further precipitated by adding equal volume of 100% ice cold HPLC grade methanol to the sample and centrifuging for 30 minutes at 14,000 rpm and then the supernatant was transferred to HPLC vial. The analysis was carried out with constant 10% 0.01N formic acid and gradual increment of the concentration of Methanol ranging from 5% initially to 10% in 8 min, to 50% at 10 minutes and 90% at 13 minutes followed by post run for 7

minutes at 5% again. The flow was set at 0.85 mL min^{-1} and the column was kept at 40°C throughout the run. The injection volume was set to 10µL and the photodiode array was set to 240nM (Gamerith et al., 2017). The standard for TPA was prepared in M9 minimal medium in range of 0.001-10mM and was treated the same as the samples.

2.12.3 Liquid chromatography and mass spectroscopy

LCMS analysis was done with collaboration of Dr. Dong Hyun Kim, and Dr.Laudina Safo (School of Pharmacy, University of Nottingham). The samples were prepared by culturing the isolate in M9 supplemented with 20 mM of the carbon source and harvesting it at mid-exponential phase based on the consumption rate. The 2 mL culture was harvested and quenched in 2 mL of HPLC grade methanol pre-chilled to -80°C in 15 mL falcon tube. 800 µL of spent broth was also quenched with 800 µL of prechilled methanol in 2 mL Eppendorf tube. The quenched spent broth was snap frozen in liquid nitrogen and stored in -80°C. Quenched culture went through 8 freeze and thaw cycle, 3 minutes in liquid nitrogen and 5 minutes in ice bucket. After the extraction of intracellular metabolites via freeze-thaw, the 2 mL of the sample was aliquoted to 2 mL Eppendorf tube. All the samples in 2 mL Eppendorf tube (quenched broth and extracted metabolites) were centrifuged for 30 minutes at 14,000 rpm at 4°C. the supernatant was transferred to LC-MS vials. QC was prepared by 10 µL combining 10 µL of all the samples into one vial. The analysis was done by using the quenched broth as a blank for extracted metabolite sample to identify extracted metabolites. All of the samples were stored in -20°C until all samples were ready to be analysed.

The LCMS analysis was performed using Thermo Fisher Scientific™ Dionex™ UltiMate 3000- QExactive LC-HRMS/MS with ZICpHILIC, 4.6x150mm column with 5µm particle size. Each run was done for 15 minutes at 45°C using the mobile phase gradient mix of 20mM ammonium carbonate in water (A) and acetonitrile (B). The injection volume was 5 µL with flowrate of 300 µL/min. The gradient of mobile phase started with 20% of A and increased up to 95%(A) over the course of 8 minutes. The ratio of the mobile phase was returned to 20%(A) in 2 minutes. The column was left to re-equilibrate for 4 minutes at 400µL/min once the equilibrium was achieved, the flow rate was returned to 300 µL/min in 1 minute. Orbital trap mass spectrometer (QExactive-Orbitrap™, Thermo Fisher Scientific™, Hemel Hempstead, UK) was used for full LCMS profiling and to generate accurate mass spectra for identification. The isolated compounds were identified using Compound Discoverer 3.1 SP1 (Thermo Fisher Scientific™). The multivariate analysis was performed using Simca® P +13.

2.13 Optimal growth condition analysis and Characterization of Isolated strain HST

Streptomyces sp. are characterized utilizing several well documented techniques such as pH and carbon utilization (Abony et al., 2018),

2.13.1 Optimal pH

The optimal pH was measured by incubating the isolate in 10 mL of TSB medium with various pH from pH 1-14. The pH was adjusted using HCl

and NaOH. The pH adjusted medium was filter sterilized and aliquoted in 10 mL in four 50 mL falcon tubes. The falcon tubes were inoculated with 10µl of overnight culture in TSB pH 7. The culture was grown for 4 days at 30°C at 200 rpm. The cells were disrupted by 21-gauge syringe until the cells were dispersed. After the dispersal, they were centrifuged for 30 minutes at 12,000 x g. the pellet was washed with H_2O three times to remove residual medium. The cells were dried for 48 hours at 55°C. the dried cell was weighed.

2.13.2 Carbon utilization of HST

As a form of characterisation, metabolite utilization was checked by using 24 deep-well-plate and M9 the culture was grown for 7 days in 4 mL of medium at 30°C with 120rpm. After 7 days, the culture was sub-cultured onto fresh medium to ensure the growth was not from medium carryover or from carbon reserves within the cells. The final growth was continued for another 14 days at same conditions as mentioned above. The growth was observed by visual inspection and microscopy.

2.13.2.1 Growth of HST on different non-aromatic hydrocarbons

Eight non-aromatic hydrocarbons L-arabinose, D-Fructose, D-glucose, sucrose, D-Lactose, L-Rhamnose, and D-Mannitol were tested. 1M in H_2O stock solution was made and filter sterilized prior to usage. The growth concentration was 25 mM of hydrocarbon in M9 minimal medium. The culture was inoculated as detailed in section 2.12.2.

2.13.2.2 Growth of HST on different aromatic hydrocarbons

The utilization of benzoic compounds was performed as above. 1M in methanol stock solution for Vanillic acid, 4-hydroxy-benzoic acid, benzoic acid, and toluic acid were made. Stock solution of aromatic carbon was added to M9 minimal medium to make 5mM. The culture was inoculated as detailed in section 2.12.2.

CHAPTER THREE

Biodegradation of Plastic by *Pseudomonas sp.*

CHAPTER 3 BIODEGRADATION OF PLASTIC BY *PSEUDOMONAS SP.*

3.1 Introduction

The genus *Pseudomonas* are gram negative microorganism found commonly in the environment such as soil and water(Kim et al., 2020). The wide range of catabolic pathways and enzymes allows the genus *Pseudomonas* to be the champion by many researchers for bioremediation and degradation of synthetic compounds (Govinda Badahit 2018). The genus *Pseudomonas* was heavily cited for plastic biodegradation for their capability to produce biofilm on hydrophobic surfaces of plastic and the ability to breakdown the oligomers and monomers of plastic. Genus *Pseudomonas* have been tested and used for bioremediation of crude oil, hydrocarbons, naphthalene, toluene, and other hydrophobic polymer which is present in many plastics (Tribedi et al., 2012, Dash et al., 2013, Wierckx et al., 2015). Biodegradation of polyethylene, PET and polystyrene by *Pseudomonas* has been explored by Tribedi and Sil (2013), Badahit et al (2018), Morgan et al. (2019), and Mohan et al (2016) in corresponding order. Also, commercial grade polyurethane, polyvinyl chloride, and PE was shown to be utilized as sole carbon source for various species of *Pseudomonas.* Currently most of the degradation analysis is done using commercial plastic such as strip of polyethylene bag, PET water bottles, Styrofoam (polystyrene), and foam beads. However, commercially available plastics contain impurities such as dye, plasticizers, oligomers of the plastic, and unreacted monomers which all can be broken down naturally. These impurities can cause an inaccurate

understanding of plastic degradation as physical degradation and biomass increase may happen due to the breakdown of these compounds. Commercial plastics that can be purchase easily contains plasticizers and other chemicals such as unreacted monomers, dyes, and other contaminates. In order to confirm that the bacteria are degrading the plastic polymer, virgin plasticizer, dye free plastic was used. Continuing from my BSc final year project, 2 pseudomonas strains and 1 unidentified isolate were screened for potential biodegradation of commercial PS including further 8 other isolates provided by Dr. Nagamani Bora. All of the isolates were sampled from wastewater streams. The objective of this chapter is to revaluate and identify if there are any plastic degrading microorganisms. As my previous work was based on commercial PS, the screening step was repeated by using virgin PS. And for the assessment for potential biodegradation activity against other plastics, virgin PET, PP, PVC, PLA, PE, and nylon for potential growth on the same.

3.2 Identification of *Pseudomonas* sp.

Following the protocol listed in Chapter 2.4.1, the pseudomonas strains were revived. From the 10 strains provided, 8 strains were recovered from -80°C cryostock. Eight revived strains were then plated onto LB agar plate and inoculated in 10 mL of sterile LB in 50 mL falcon tube. The inoculation was checked for contamination on LB plate. Once the culture was tested for contamination, 16S PCR was performed and sequenced following the protocol listed in Chapter 2.9. Unfortunately, 16S results showed that 3 strains were not in *Pseudomonas* genus and was excluded from this work leaving C1, C2,

B18, B19, and B22 to be assessed in this work. The neighbour-joining phylogenetic tree was made by using MegaX software and the produced tree is seen on **Figure 4**.

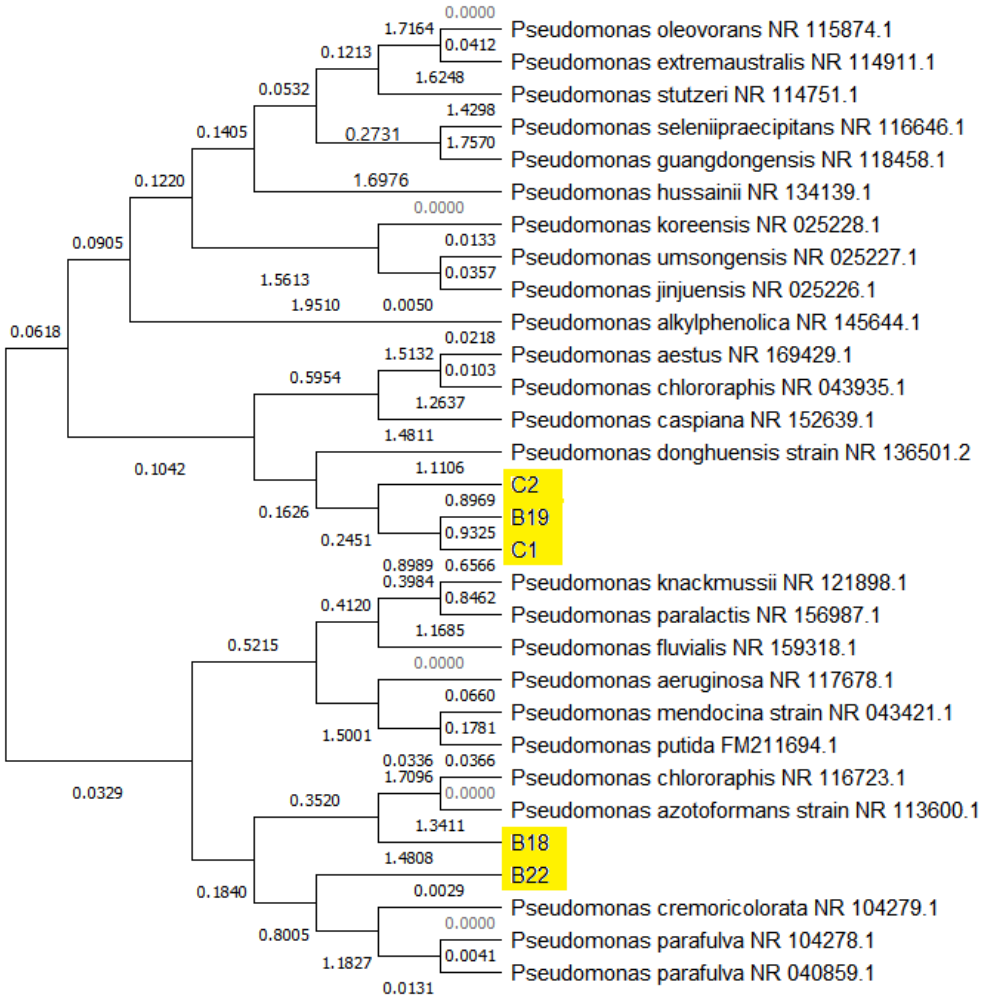


Figure 4: Neighbour-Joining phylogenetic tree of 5 *Pseudomonas* sp. with known *Pseudomonas* sp. with potential for plastic degradation.

3.3 Phenotypic Characterization

The colony morphology of the isolates was performed on LB plate. The culture was streaked onto a fresh LB plate and was incubated for 2 days. The colony morphology was evaluated and documented on **Table** 7 These characterizations are common characteristics of *Pseudomonas* sp.

Table 7: Colony Morphology of *Pseudomonas* sp. on LB agar

	C1	C2	B18	B19	B22
Color	raised	raised	brown	Yellow	yellow
Form	circular	irregular	Circular	Circular	Circular
Elevation	raised	raised	raised	raised	raised
Margin	Entire	Undulated	Entire	Entire	entire
texture	sticky	sticky	Semi-firm	sticky	sticky

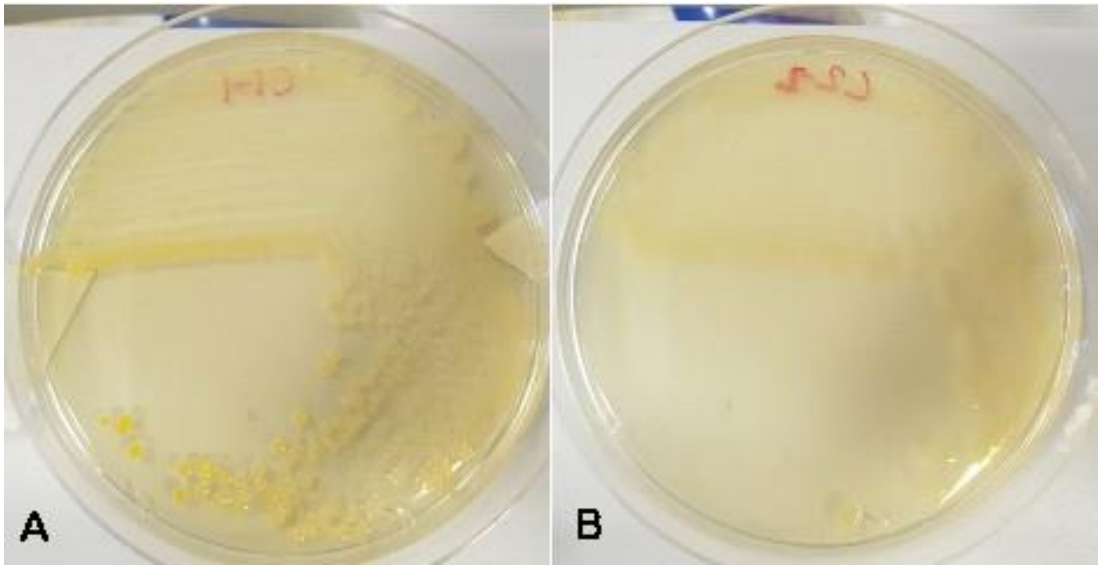


Figure 5: Photograph of *Pseudomonas* sp. on LB plate. A) *Pseudomonas* sp. (C1) on LB Plate. B) *Pseudomonas* sp. (C2) on LB Plate

3.4 Assessment of biodegradation of plastic

3.4.1 Assessment of biodegradation of Polystyrene

To assess the degradation of polystyrene, after the 1st generation adaptation process as mentioned in Chapter 2.10. The CFU was measured after 48 hours to assess the growth of the culture and the potential bio-assimilation capability. The **Table 8** below shows the CFU change of 2nd generation on the strains.

Table 8: CFU of 5 *Pseudomonas* sp. From previous study after 2nd generation selective enrichment with presence of virgin, plasticiser free polystyrene.

Strain Name	T0(10^{-5})	T48 (10^{-5})
C1	152	132
C2	140	170
B18	200	121
B19	146	111
B22ML	144	85

Based on the changes in the CFU, C2 was then selected to be proceeded to 3rd generation culture as it was the only strain where there was increase in the CFU after 2nd generation in minimal media (M9) with virgin, plasticiser free polystyrene (without any other carbon source). C2 was then inoculated in 50 mL of M9+BG11 trace metal with 1 g/L^{-1} of PS film in 250 mL baffled flask.

A third-generation culture was grown for 3 weeks in triplicate. CFU samples were taken every 48 hours. As seen on **Figure 6** the viable cell counts started to drop after day 10 for C2 in PS, which strongly indicates that intracellular carbon reserve and the carryover from previous medium was still present. The decrease in CFU is caused by depletion of residual carbon and C2 was not able to metabolize virgin polystyrene as sole carbon source. **Figure 6** shows the decrease in CFU over 26 days of *Pseudomonas* sp. (C2) growth in minimal media with virgin, plasticizer free polystyrene as the sole carbon source.

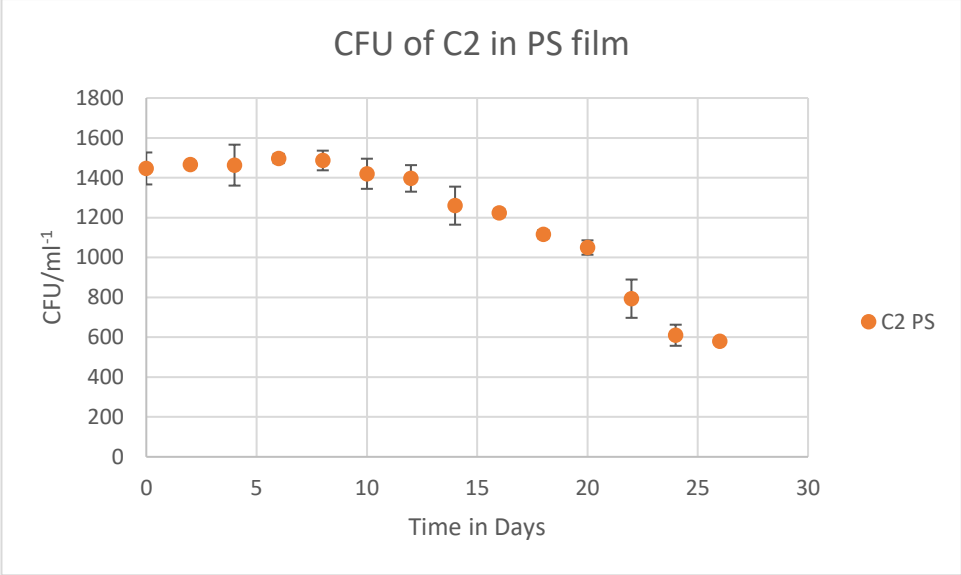


Figure 6: CFU of *Pseudomonas* sp. (C2) on virgin, plasticiser free polystyrene in minimal media (M9) over time. Error bars represent the standard deviation of triplicate cultures.

3.4.2 Assessment of Biodegradation of PET by *Pseudomonas* sp.

As five *Pseudomonas* strains did not show direct evidence towards biodegradation of polystyrene, the strains were further examined for potential PET biodegradation.

3.4.2.1 Screening of potential PET degrading *Pseudomonas* sp.

The screening method for potential PET degrader was done by plating the *Pseudomonas* sp. onto a PCL plate. PCL plate is widely used as a screening plate based assay for activity of polymerases such as esterase, hydrolase, or lipases (Molitor et al., 2020). The isolates were inoculated in fresh LB broth for 3 days before being streaked onto PCL plates. Out of 5 *Pseudomonas* strains used in this research, only one strain (C2) showed signs of clearing as shown in **Figure 7**. The zone of clearing indicates that there are

exported polymerase such as esterase, cutinase, and lipases which led to further assessment of PET degradation.

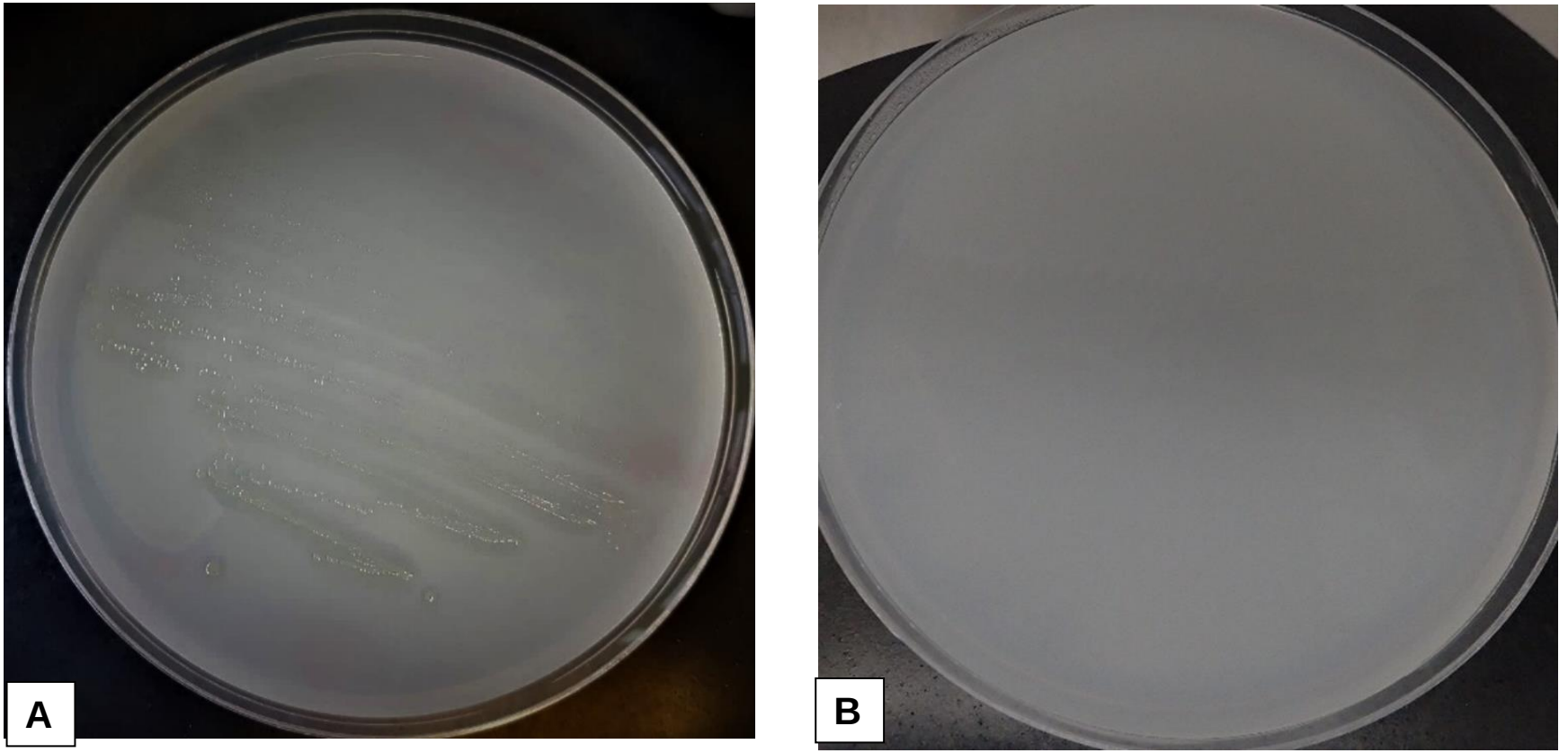


Figure 7: Polycaprolactone degradation assay. A is *Pseudomonas* sp. (C2) exhibiting zone of clearing around the colony. B is a PCL plate with *Pseudomonas* sp. (C1) without zone of clearing. This zone of clearing indicates that *Pseudomonas* sp. is capable of degrading PCL.

3.4.2.2 Growth of *Pseudomonas* sp. (C2) on PET

Upon inspection of zone of clearing on the PCL plate, C2 then went through the media adaptation process as described on Chapter 2.7.2. After the second generation, the culture was then inoculated into fresh M9 medium with 1 g/L of PET powder. The growth was continued for 26 days with CFU measurements taken every 12-24 hours. The **Figure 8** below shows the CFU counts of the C2 grown in M9 with PET powder as sole carbon source.

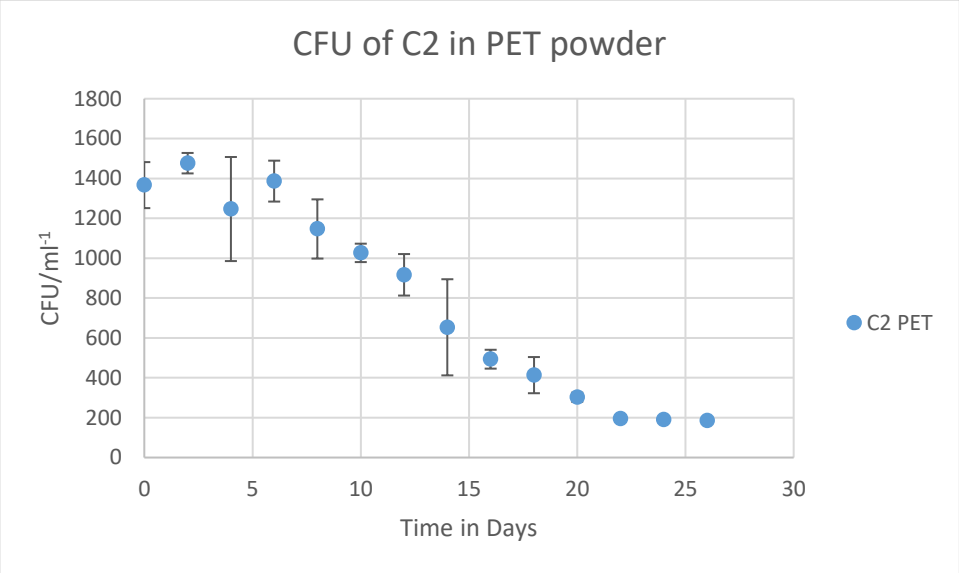


Figure 8: CFU of C2 (*Pseudomonas* sp.) in presence of PET

Similar to the growth of C2 in PS as seen on 3.4.1 Assessment of biodegradation of Polystyrene, C2 on PET also showed a slight increase in CFU then continued to decrease after day 6. Which indicates that the *Pseudomonas* sp. (C2) was not able to bioassimilate PET as sole carbon source.

3.4.3 Screening of *Pseudomonas* sp. on different Plastics.

The 5 *Pseudomonas* sp. were then screened for degradation of other plastics such as PE, PP, PVC, PLA, and Nylon. The degradation was done by long term enrichment. The enrichment became longer than expected due to Coronavirus-2019. The culture was grown in 24 well plates for 5 months. Where only H_2O was supplemented. After the 5 months of growth, no signs of growth were present which indicated that there was no growth of *Pseudomonas* sp. on any of the plastics listed above.

3.5 Discussion

Although the *Pseudomonas* sp. given by Dr. Nagamani Bora had shown potential biodegradation capabilities with commercial polystyrene and PET, upon further investigation, it turned out that they could not bioassimilate on virgin plasticizer free plastic. This result indicates that previous growth observed in my BSc. final year project could have been from the impurities that were present in the commercial polystyrene such as ethylbenzene, benzene, toluene, styrene, dye, and many more (Ahmad and Bajahlan, 2007, Thaysen et al., 2018). These impurities commonly found in commercial polystyrene such as ethylbenzene, benzene, toluene, and styrene in widely degraded by the genus *Pseudomonas* (Corkery et al., 1994, Otenio et al., 2005, Utkin et al., 1991). This becomes a common source of misinterpretation when it comes to isolation and identification of plastic degrading microorganisms. This unfortunately was the case for my BSc. final year project as the 2 strains

retested in this experiment were believed to be able to bioassimilate polystyrene when grown on commercial grade polystyrene.

The *Pseudomonas* sp. provided by Dr. Nagamani Bora was also subject to growth on PET. A zone of clearing was observed on the PCL plate. Although PCL is commonly used as screening method to identify exported esterase (Danso et al., 2018, Nyyssola et al., 2013, Almeida et al., 2019), after further investigation, the degradation of PCL does not indicate that the strain will degrade PET or any other synthetic plastics.

Further research into the degradation of the monomers of the synthetic plastic have been discussed however, due to wide number of *Pseudomonas* sp. being known to be able to degrade styrene, EG, and TPA (Muckschel et al., 2012, Oelschlagel et al., 2018, Zhi-Jiang et al., 2011), the decision was made to attempt to isolate novel strain from the environment.

CHAPTER FOUR

Isolation of plastic degrading microorganisms from Urban Environment

CHAPTER 4 ISOLATION OF PLASTIC DEGRADING MICROORGANISMS FROM URBAN ENVIRONMENT

4.1 Introduction

The genus *Streptomyces* is a wide branch of Actinobacteria with over 900 identified species defined in DSMZ to date. The *Streptomyces* are gram positive-aerobic members of order *Actinomycetales (Anderson and Wellington, 2001).* The genus *Streptomyces* DNA have a high G+C content of 69-78%.(Korn-Wendisch and Kutzner). *Streptomyces sp*. produce extensive branching substrate and atrial mycelium which guides in spreading and obtaining nutrients as seen on **Figure 9** (Anderson and Wellington, 2001). The genus is also well known for its extensive production of highly valued bioactive compounds such as antibiotics and structurally diverse compounds with pharmaceutical applications (Gopalakrishnan et al., 2020). Their wide range of secreted extracellular enzymes such as cellulase, xylanase, lipase, and many more allow the genus to metabolize on many complex natural polymers (Sevillano et al., 2016). The wide range of lyases became the reason why many researchers investigated isolation of plastic degrading *Streptomyces sp.* For instance, PETase like cutinase was found in marine *Streptomyces spp.* which showed activity towards PCL and potential docking of BHET but has not shown degradation of PET(Almeida et al., 2019). Yet, despite the presence of synthetic polymer degrading *Streptomyces,* a strain capable of PET degradation has yet to be proven. And to do so, in this chapter, will be attempting to isolate, identify and potential plastic degrading *Streptomyces*

from urban environment as well as plastic contaminated areas such as landfills and human sewage waste treatment plant.

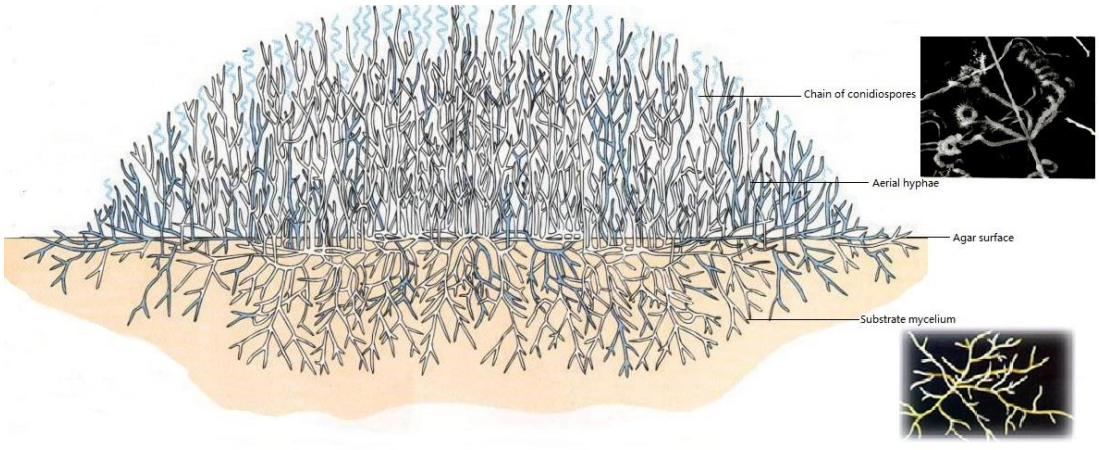


Figure 9: Morphology of Streptomyces. A) arial hyphae, B) Chain of spores, C) substrate mycelium(Li et al., 2016)

4.2 Isolation

4.2.1 Isolation locations

The sampling sites included urban environment, landfill, and human sewage waste treatment plant. To maximize the diversity of microorganisms, 4 geographically different urban locations were selected with a lot of human interaction and direct and indirect exposures to plastic. These areas included London, Coventry, and Bridlington. These increased interactions with synthetic plastic would have provided natural adaptative push towards degrading plastic. Samples from staple quarry landfill site was also tested as

it is believed that plastic contaminated areas would also have selective pressure on plastic degrading microorganisms such as sediment, soil, wastewater, and sludge (Tanasupawat et al., 2016). As increased in the concentration of plastic would have given a higher opportunity for plastic degrading microorganisms to obtain necessary carbon while inactive microorganisms would have to compete for depleting source of carbon that can be easily metabolised. The environmental samples were diluted and inoculated in M9 minimal media with PET powder to promote selective pressure on the microorganisms capable of consuming plastic as sole carbon source. However, even with the dilution of 8 folds (10^{-8}), it was evident that the residual carbon present in the sample was enough to sustain the consortium of microorganisms growing for 3 weeks. Second generation enrichment was required to decrease the diversity of microorganisms present in the sample. After the second generation, the diversity of culture dropped significantly as seen on **Figure 10**

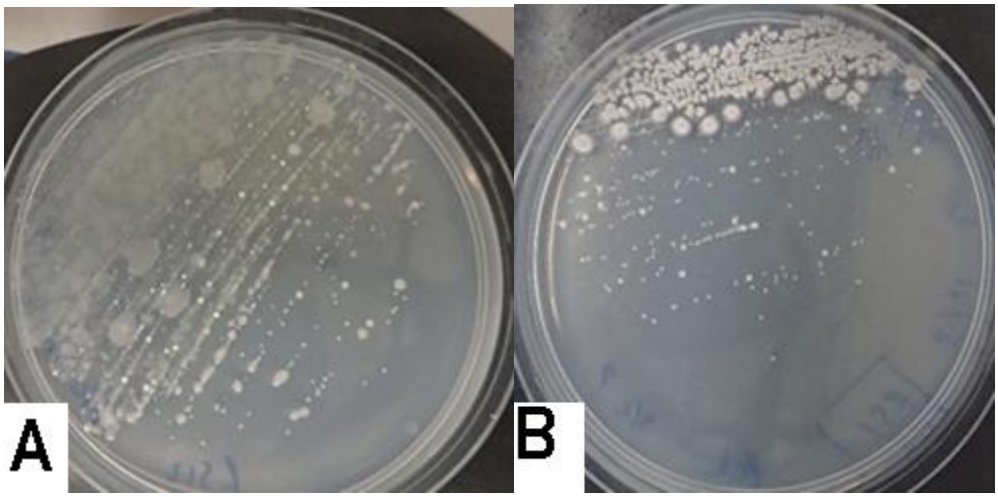


Figure 10*: (A) First generation and (B)Second-generation Enriched samples*

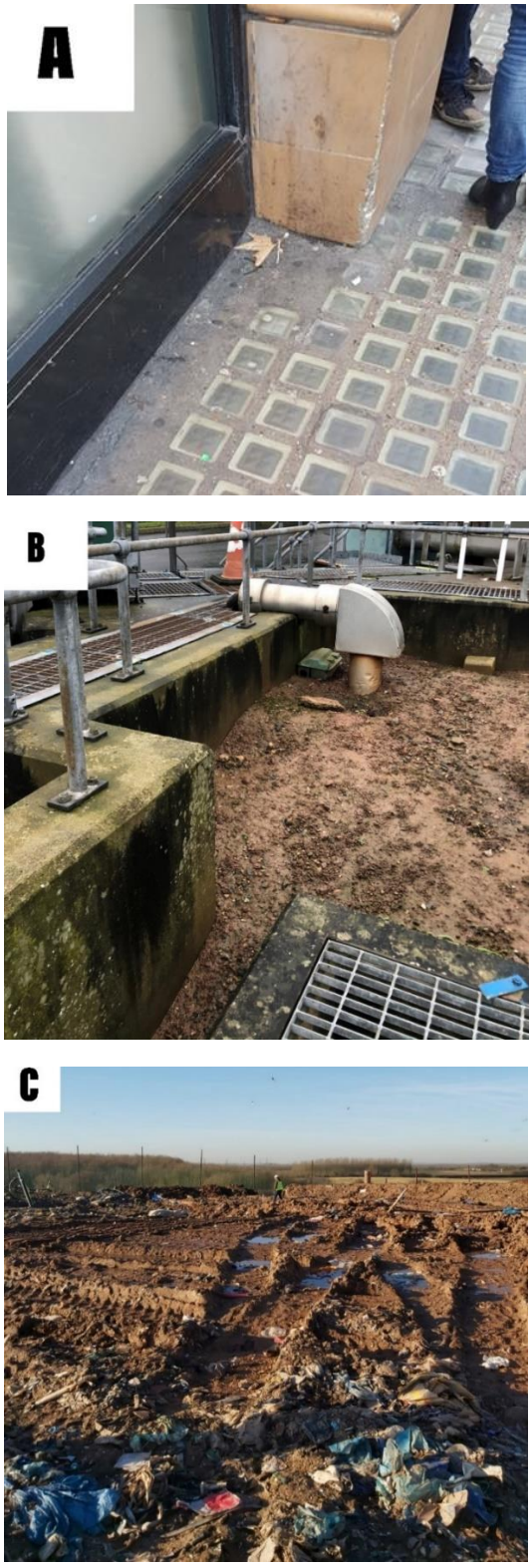


Figure 11: Examples of the locations of the sampling. **(A)** Side of the road in London. **(B)** Side of the human sewage treatment plant. **(C)** Staples Quarry Landfill site, Newark.

4.2.2 Identification

From 29 individual soil samples, 14 samples from urban soil, 1 dilution (10^{-5}) from post enrichment (5 months) for human sewage treatment plate site samples, and 2 landfill site samples exhibited signs of growth. Within the samples that showed growth, the majority of the observed growth correlated to Actinomycetes following morphological identification. As seen in **Figure 12,** spore forming colonies were visibly increased compared to the initial sample before the serial enrichment on plastic. From the 14 different samples, 31 strains were isolated which were categorized into 23 morphological groups based on the spore colours, the colour of the colony, and the change in substrate colour on 5 different plates including ISP4, Oatmeal agar, PDA, 2YT, and TSB agar. This is a common procedure utilised to characterise Actinomycetes (ref). From each morphological group, 2 isolates were selected at random giving 46 individual isolates from 16 different groupings. 16S PCR was performed on 36 individual isolates for genotypic separation. Because there was only one spore forming colony from the streak, the isolate from the human sewage treatment plant (*Streptomyces* sp. HST) was considered an individual isolate **Figure 12**. Two spore forming colonies were found in the landfill site sample from Staple quarry landfill at depth of 4.5M and 21.5M. These two isolates were treated as individual strains as they exhibited different morphologies.

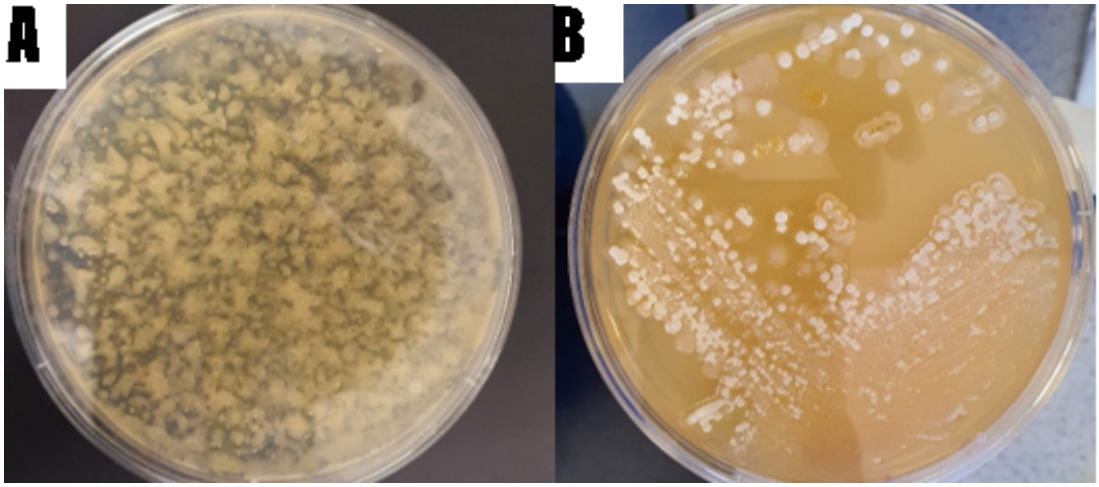


Figure 12: Enriched samples plated on AIM plate. **(A)** Undiluted Enrichment of Human sewage treatment samples **(B)** Diluted enrichment of human sewage treatment samples exhibiting spore forming colony from soil samples.

To reduce the number of similar *Streptomyces* sp., further isolation methods were done. The 3 steps taken to reduce the replicates were phenotypic separation, 16S RFLP, followed by 16S sequencing. The additional steps taken to reduce the number of replicates were crucial to avoid the same strain being characterised multiple times and to increase diversity.

4.2.2.1. Restriction fragment length polymorphism (RFLP)

RFLP was performed on 16Ss PCR products to differentiate within the group in case the phenotypic grouping was not enough to separate between species. After performing RFLP on 2 isolates from each individual group, 13 genetically different *Streptomyces* sp. were isolated and purified.

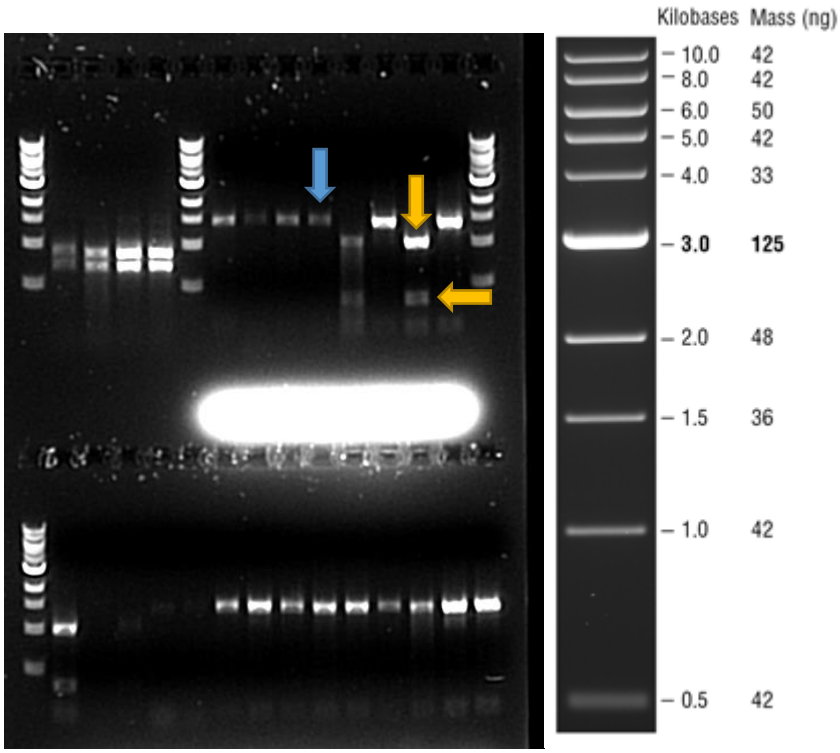


Figure 13: RFLP digestion of 16s PCR product. The digestion results in range of sizes as seen. The ladder used is NEB 1kB DNA ladder (New England Biolabs, Hitchin, UK) The arrows indicate different length of the digestion products.

4.2.2.2. 16S Phylogenetic Tree

16S PCR products from 13 selected and different isolates were sequenced and the phylogenetic tree was constructed using the neighbour-joining method (Saitou and Nei, 1987, Kumar Sudhir et al., 2018).Upon sequencing, the 13 isolates were confirmed to be individual species. From this, these 13 isolates were then selected to proceed to selective serial enrichment on PET to select for plastic degrading strains.

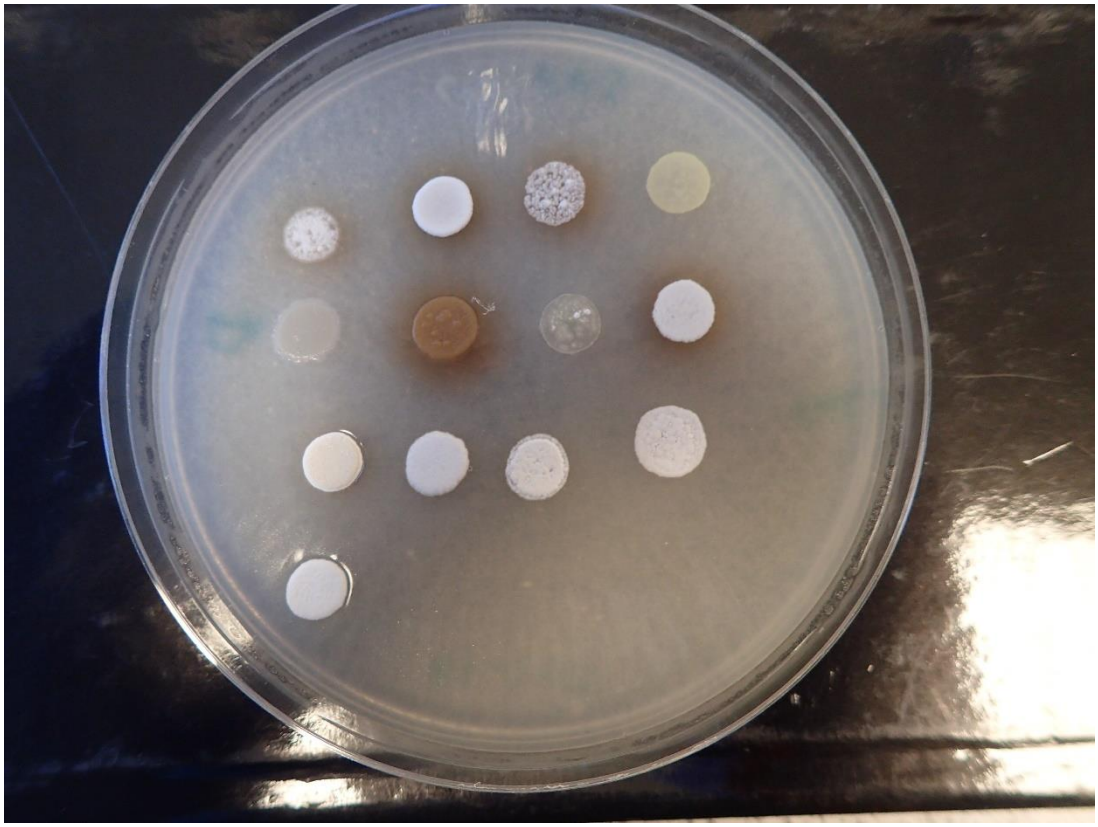


Figure 14:Drop plating of the 13 isolates from urban soil samples.

4.3 Selective serial enrichment

4.3.1 Selective serial enrichment results

The selective serial enrichment was performed to further screen for PET degrading microorganisms. The 13 isolates from urban soil sample and 2 landfill isolates went through extreme selective serial enrichment with only water being added every 2 weeks. After the 5 months of selective serial enrichment, Strains CV 2,3,4,5,11,13, and 16 showed potential signs of growth indicated by the colour change. 100µL of the samples from the wells containing PET was used to inoculate fresh media with clean PET powder. 50µL of PET

selective serial enrichment well from each colony was used to inoculate onto PCL plate for hydrolase activity assay.

4.3.2 PCL plates

The 13 isolates from urban soil samples were also plated onto PCL plates supplemented with 0.1% Yeast extract. After 14 days, isolate number 1,3,5,11,13,16, HST, from landfill site 4.5M and 21.5M showed zone of clearing which indicates esterase, lipase, or hydrolase activity. Especially 5,13, 16, and 21.5M exhibited very distinct clearing. HST and isolate from landfill site 4.5M also exhibited faint zone of clearing. However, the clearing was only visible to the naked eye.

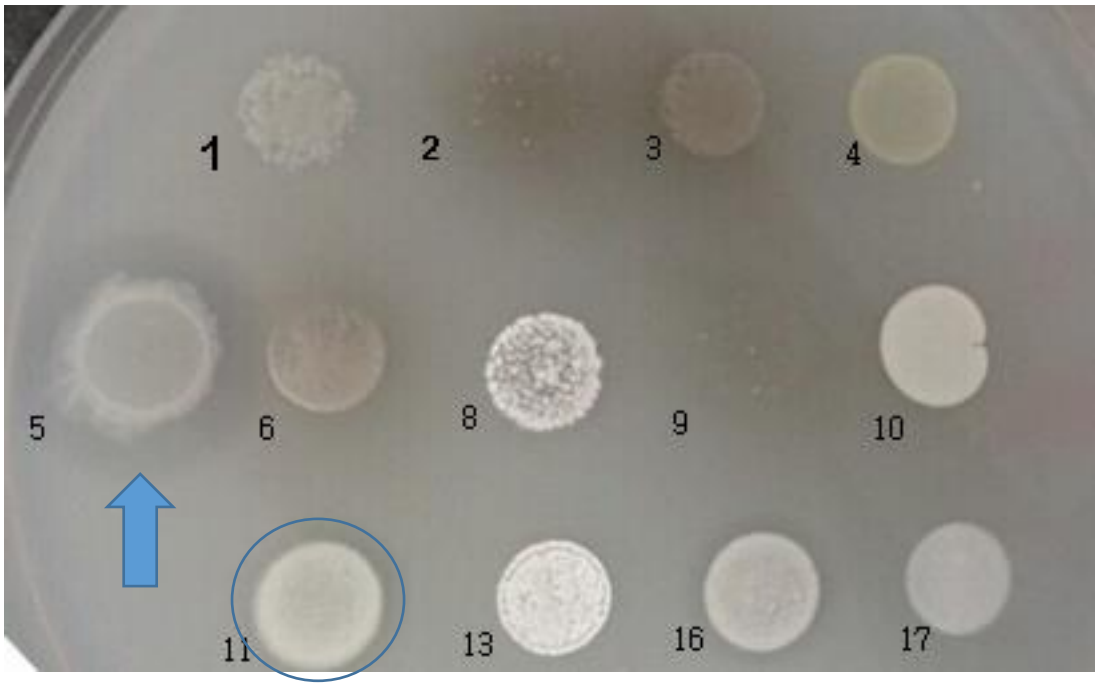


Figure 15: 13 isolates after selective serial enrichment plated on PCL assay plate for screening for polymerase activity. Isolate number CV1, CV3, CV5, CV11, and CV16 exhibited evident zone of clearing with 5 having to spread

out while 16 being local yet the most clearing. Blue arrow and circle indicate zone of clearing.

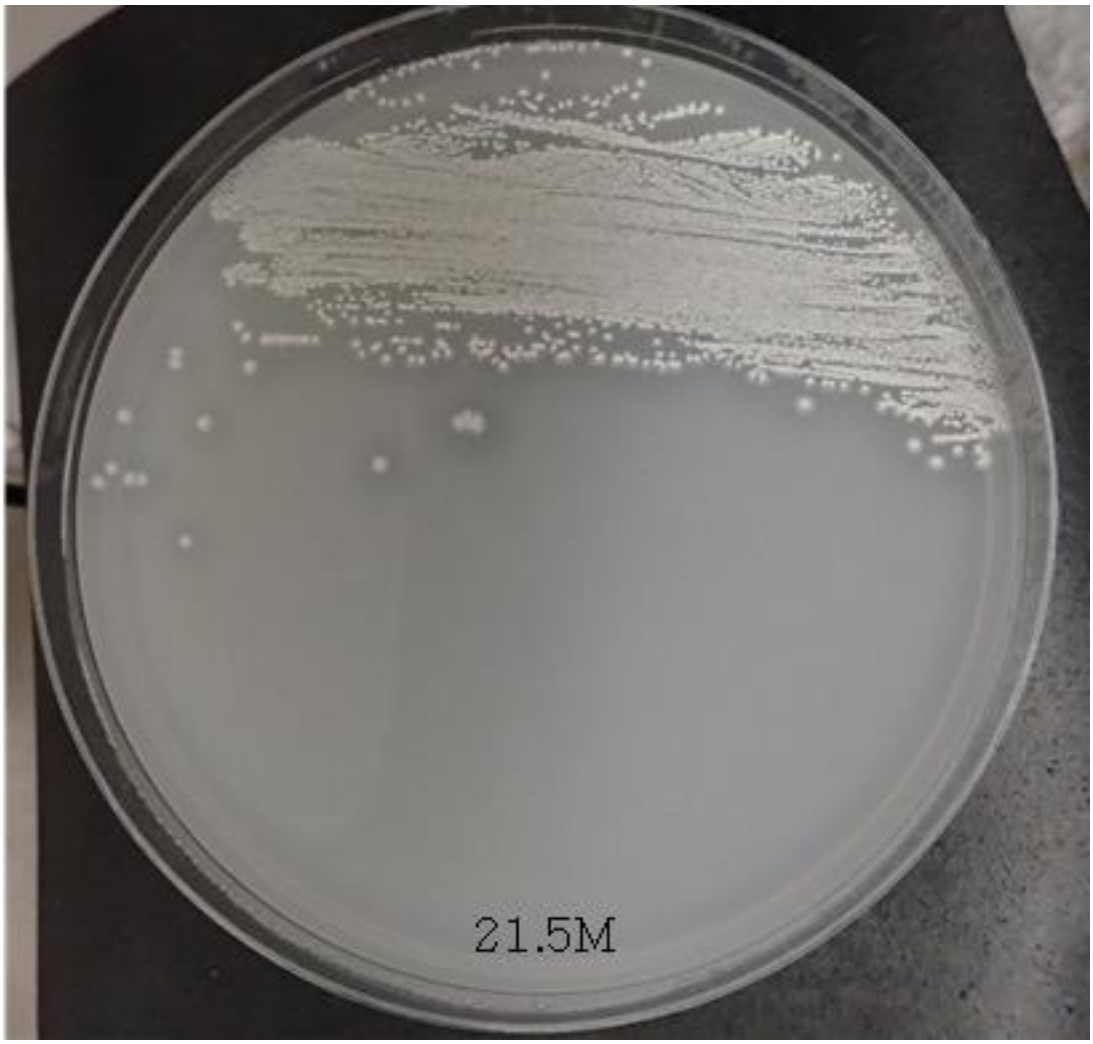


Figure 16: Isolate 21.5 was isolated from Staples Quarry landfill site. The zone of clearing is visible on PCL which indicates that there is polymerase activity after the selective enrichment.

4.4 Biodegradation of Plastic

After the PCL zone of clearing assay was complete, 6 isolates that showed signs of degradation were tested for their ability to degrade PET

plastic. The assessment of PET degradation was based on changes in biomass and HPLC analysis (Furukawa et al., 2019). Also, degradation of terephthalate was also assessed using the same methods.

4.4.1 PET degradation

The 6 isolates were then inoculated in M9 medium with 1 g/L of PET film and 0.1g/L of glucose as carbon source. The control did not have PET film to be able to compare the biomass with or without the plastic. Growth was assessed by measuring the increase in the dried cell mass of the 2 weeks old culture. To remove as much biofilm as possible, the culture was vortexed for 30 minutes prior to pipetting and centrifuging. Also, to the plastic was also weighed after drying to remove the absorbed water and to measure the loss of mass. As seen in **Figure 17** the dried cell mass did not show significant increase when PET was present Instead of having an increase in the cell mass, there was decrease in biomass which could indicate the inhibitory effect of PET in isolate 5 and in HST. The supernatant samples were also taken for HPLC analysis for presence of TPA given PET breaks down into the monomers TPA and ethylene glycol, TPA can be detected using a C18 reverse-phase column (Furukawa et al., 2019).

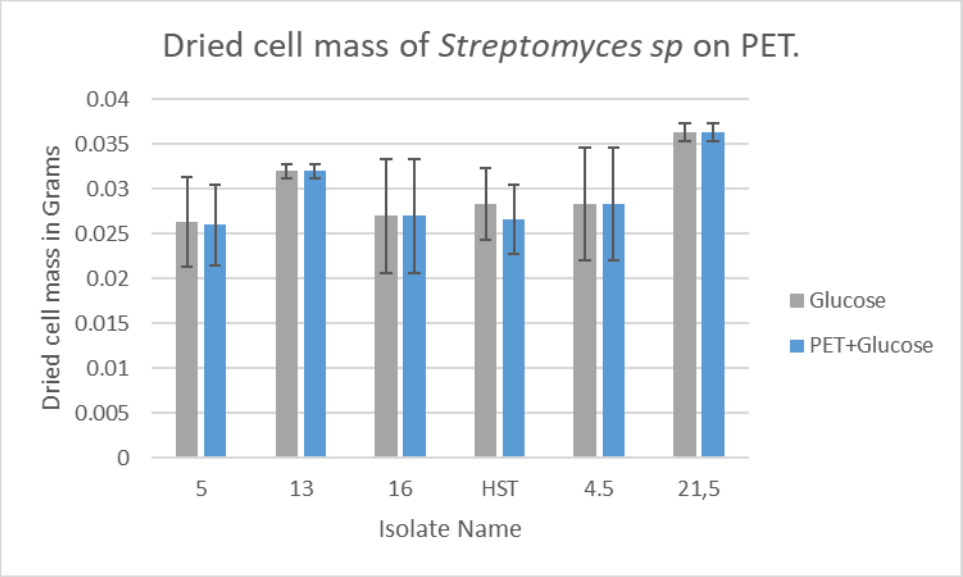


Figure 17: Dried Cell mass of Isolated *Streptomyces* sp. grown with or without PET film. Error bars represent the standard deviation of triplicate cultures. There wasn't significant increase in the biomass to indicate growth on virgin PET

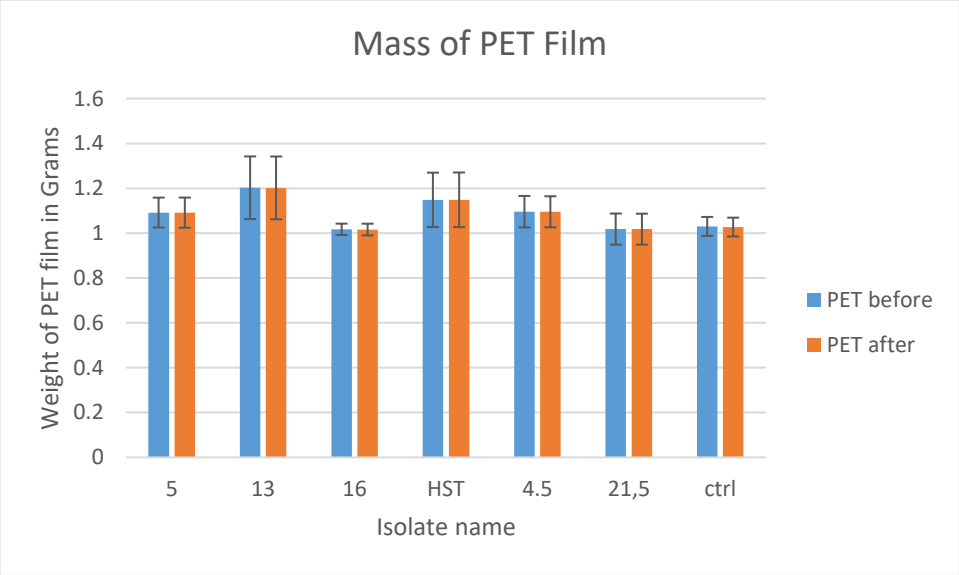


Figure 18: Mass of Dried PET film. Error bars represent the standard deviation of triplicate cultures. There wasn't significant decrease in the mass of PET to indicate degradation of PET

4.4.2 HPLC analysis

HPLC analysis showed no presence of TPA, BHET which may be an indication that either the PET was either not being degraded or the release of TPA was slower than the rate of consumption by the isolated strains in the given conditions.

4.4.3 TPA degradation

To investigate if the strains could consume TPA, 6 strains were inoculated onto TPA+M9 plate and M9 plate without any carbon source as a control. After 3 days, the colony became visible as seen in **Figure 191**, the isolates were able to grow on M9 plate without any other carbon source. This may have been caused by either utilization of agar as carbon source, impurities present in the agar or both. However, the difference between TPA supplemented plates is visibly different as seen in **Figure 202**. The visible difference in growth of HST in TPA+M9 plate, HST was inoculated in 1mM,2.5mM, and 5mM TPA in M9. The metabolism of TPA was assessed by biomass at the end of the growth. After 5 days, only HST was able to grow on TPA as seen in **Figure 202** when compared to **Figure 191**. To further ensure that HST was growing on TPA and not potential impurities in the M9 minimal media,HST was also grown in M9 liquid media. . 170mg/L of TPA was added to M9 minimal media and was inoculated for 3 days at 120rpm at 28°C. as seen on **Figure 213**, the tube without TPA showed no growth while tubes with TPA suppliment showed signs of growth. Upon this finding, HST was explored more in depth.

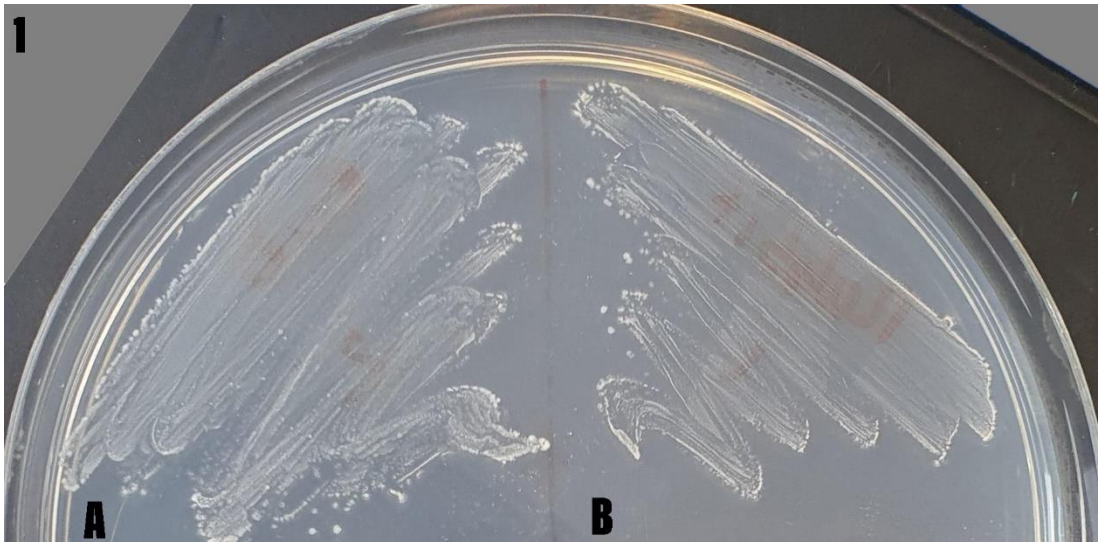


Figure 19: M9 plate without TPA. A is isolate 21.5M. B is isolate HST both showing minimal growth on M9 plate without any additional carbon.

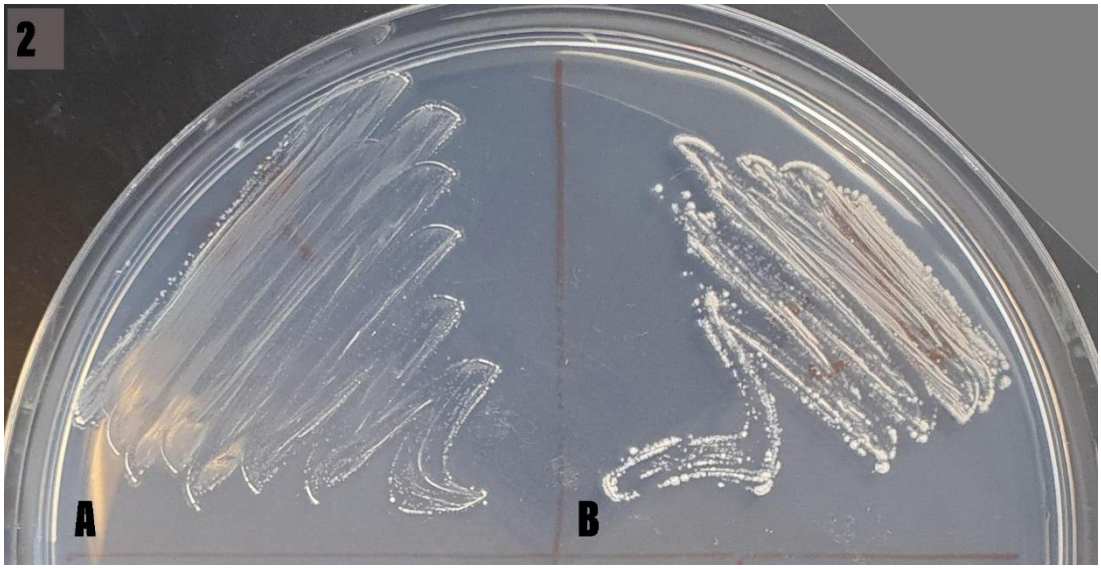


Figure 20: M9 plate with 1g/L TPA added. A is Isolate 21.5M also showing very similar growth to **Figure 19**. B is isolate HST showing significantly more growth than growth seen on **Figure 19** B.

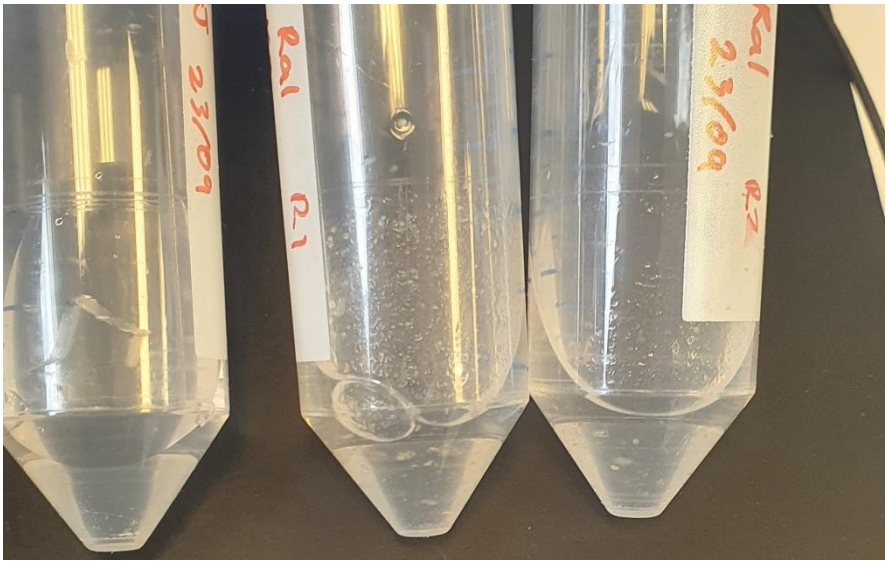


Figure 21: Growth of HST on M9 media supplemented with and without TPA. 1. No TPA added. 2&3. 170mg/L TPA added. The first tube shows no growth indicating that HST does not grow on M9 minimal media and second and third tube shows signs of growth indicating that addition of TPA allows growth of HST.

4.5 Degradation of PET by HST

With a clear increase in growth of HST on TPA, the PET film from the growth was sent for SEM analysis. However, upon inspection, the PET film did not show any signs of degradation as seen on **Figure 22** (A) and (B) having no visual signs of degradation such as holes or changes in the surface. Lack of increase in biomass, no signs of degrease in weight of PET film, and lack of biofilm formation with no visual degradation, indicates that the HST was not able to degrade the PET film in this experiment.

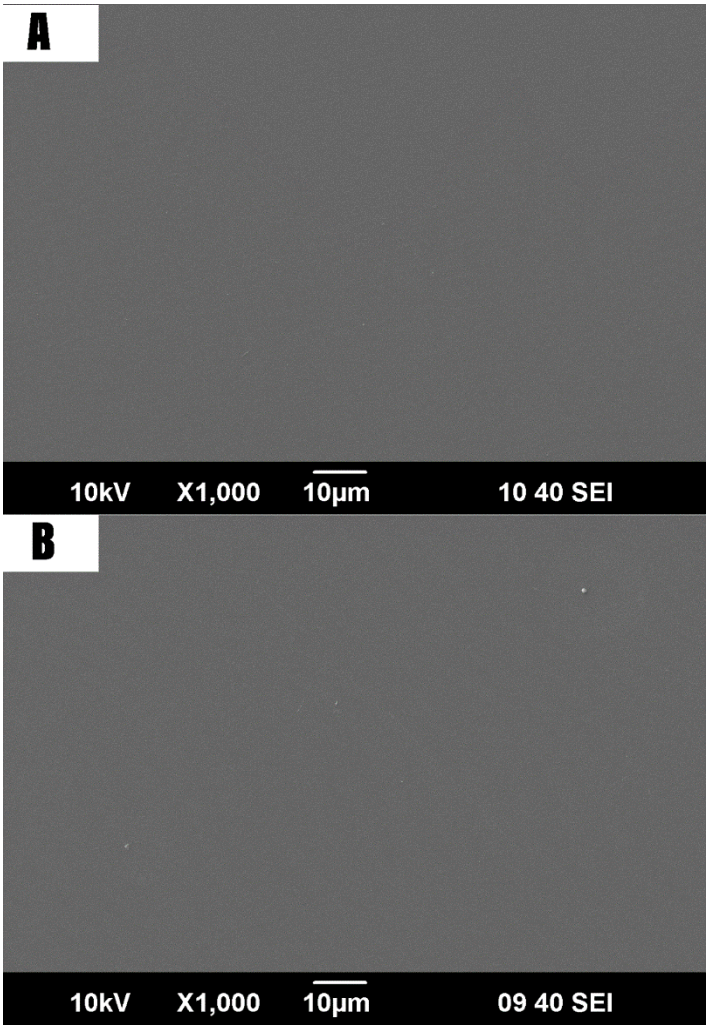


Figure 22: SEM image of PET films. **(A)** PET film of control (no cells). **(B)** PET film after 28 days of incubation with HST. As seen, there is no biofilm formation on the PET indicating that there was no residual growth on the plastic.

4.5 Discussion

The *Streptomyces* sp. is a gram positive microbe with a wide range of metabolic capabilities. The robust metabolism allows the *Streptomyces* sp. to be able to adapt to different envrionments. The nutrient stress can cause the evolution of altered substrate affinity and enzyme efficiency during the duplication event. The event causes two duplicate copies that allows cellular

process to produce specialized metabolites during the times of perturbation such as during nutrient depleation or adaptation phase (Schniete et al., 2018). These natural adaptations towards changes in nutrient availability guided the project to focus further into isolation of a novel plastic degrading *Streptomyces* sp. from wide range of environments including urban environment such as London and coventry as well as plastic exposed areas such as the human sewage waste treatment plants and landfills. From the samples, 6 isolates with potential PET degrading capabilities were screened using PCL plate. However, despite the positive results from PCL degradation, there was no release of TPA upon inoculation of the isolates in minimal media with presence of PET a strong indication that the biodegradation of PET by these isolates was not possible. Although TPA is not widely degraded by microorganisms, it was crucial aspect to inspect those isolates for potential degradation capabilities. The degradation of TPA by *Streptomyces* isolate HST was confirmed and deemed worthy to be carried forward in this research.

The inability for HST to be able to grow while not being able to metabolise PET may have been due to the fact that the growth previeously observed in the selective enrichment may have originated from bacterial community working together as a symbiotic relationship (Delacuvellerie et al., 2019, Jacquin et al., 2019). This relationship between HST and unknown microbes in the sample allowed HST to be able to breakdown TPA while the other microbes were metabolising ethlyene glycol or other byproducts HST may have released back in to the culture allowing the growth to occure.

CHAPTER FIVE

Characterization of HST

CHAPTER 5 CHARACTERIZATION OF HST

5.1 Introduction

Following the observation of potential TPA degrading *Streptomyces sp*. isolated from soil sample found near human sewage treatment plant, the strain was selected to be explored furthermore in depth. This decision was done because biodegradation of terephthalic acid by genus *Streptomyces* is a novel finding and it has not been recorded in the past. There have only been 6 genera found to metabolise TPA naturally and they are *Rhodococcus, Comamonas, Pseudomonas, Bacillus, Arthrobacter,* and *Dietzia* (Sasoh et al., 2006, Shigematsu et al., 2003, Hara et al., 2007, Chain et al., 2006, Knott et al., 2020). The degradation of TPA by the above-mentioned strains results in the generation of PCA before being taken up into the central metabolism as seen on **Figure 23.** The genus *Streptomyces* metabolising TPA is a fascinating discovery as TPA is currently considered an industrial waste in industries such as PET manufacturing, textile, and pharmaceutical. The degradation of TPA by *Streptomyces* is even more interesting as TPA degradation by other microorganisms have been observed however bioconversion of TPA and EG into higher valued products has not been explored in depth. However, with identification of *Streptomyces* capable of utilising and converting TPA into potentially higher valued compounds via secondary metabolism by *Streptomyces* sp. may indicate potential chassis for *I. sakaiensis* PETase for complete bioconversion of PET into higher valued

compounds. This chapter will characterize TPA degrading *Streptomyces* sp. isolated from soil samples near human sewage treatment plant.

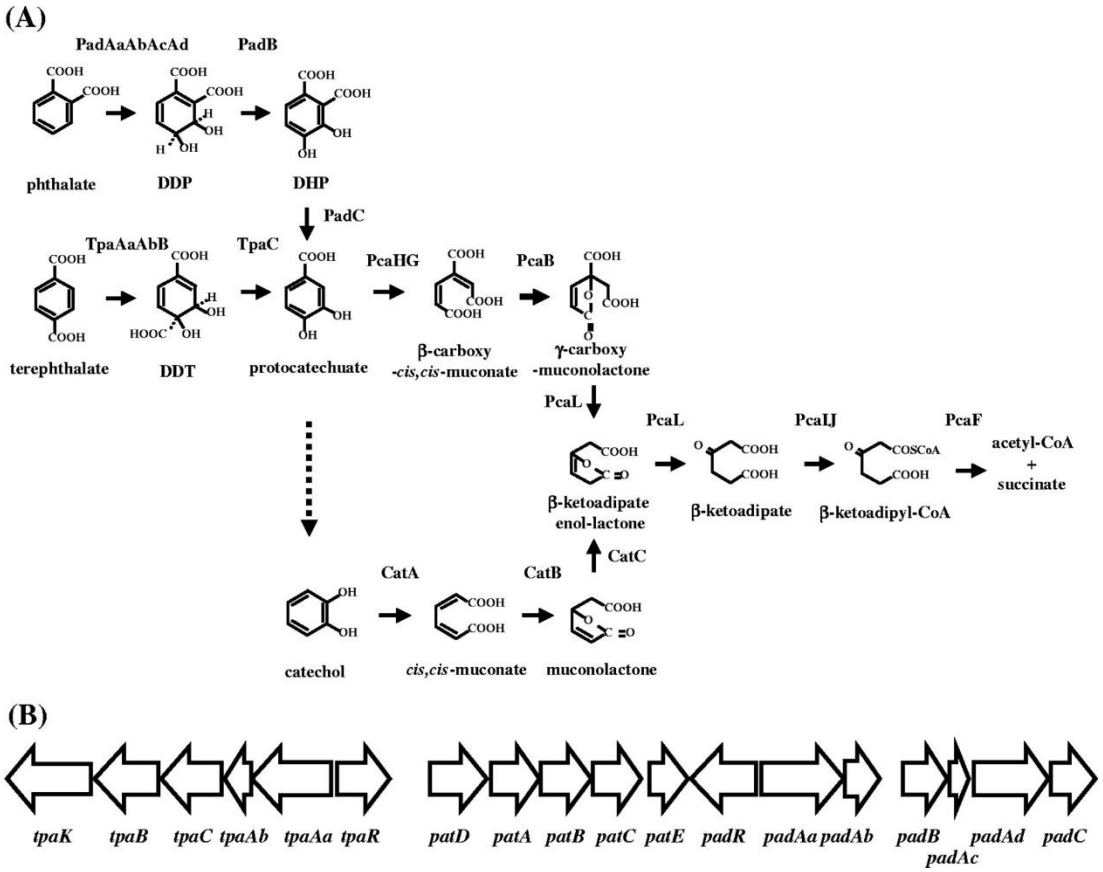


Figure 23: currently proposed process of TPA and PCA degradation by *Rhodococcus* sp.(Hara et al., 2007). Similar process is also observed in *Comamonas* sp.(Sasoh et al., 2006)

5.2 Morphology

Colony morphological characteristics of the TPA degrading *Streptomyces* sp. (HST) was evaluated on 4 different media. On all 4 media, the substrate changed colors due to spreading of secondary metabolites as seen on in **Table 9**

. The morphology of HST matches the description of genus *Streptomyces* as described by Anderson and Wellington, 2001. Exhibiting the arial hyphae and spore formation *Streptomyces* genus is well known for. The colony morphology on different agar plates is described in **Table 9**

Figure 24: Colony morphology of HST on Oatmeal agar.

Table 9: Chart of morphology and visual characteristics of HST on different Streptomyces selection agar

Medium	Growth	aerial mycelium	substate mycelium	Form	Elevation	margin
Oatmeal agar	good	white, brown	brown	Circular	Umbonate	entire
PDA	good	white	Yellow	Irregular	Raised	Entire
SFM	good	white and grey	yellow	Circular	Raised	Entire
ISP4	moderate	grey	yellowish	irregular	umbonate	Entire

.

5.3 Growth conditions

5.3.1 pH range of HST

To further characterize the newly Isolated strain HST, optimal pH was measured, a commonly utilised technique to validate Streptomyces morphology (Abony et al., 2018, Wei et al., 2020). HST was able to show growth in various pH's ranging from pH 5 to 9. The optimal growth was shown at pH 7 by measuring the dried cell mass after 3 days of growth. The pH range of growth indicates that HST is a neutrophile.

Table 10: pH range of HST growth in pH adjusted Tryptic-Soya broth

pH	HST
5	+
6	+
7	+
8	+
9	+
10	-

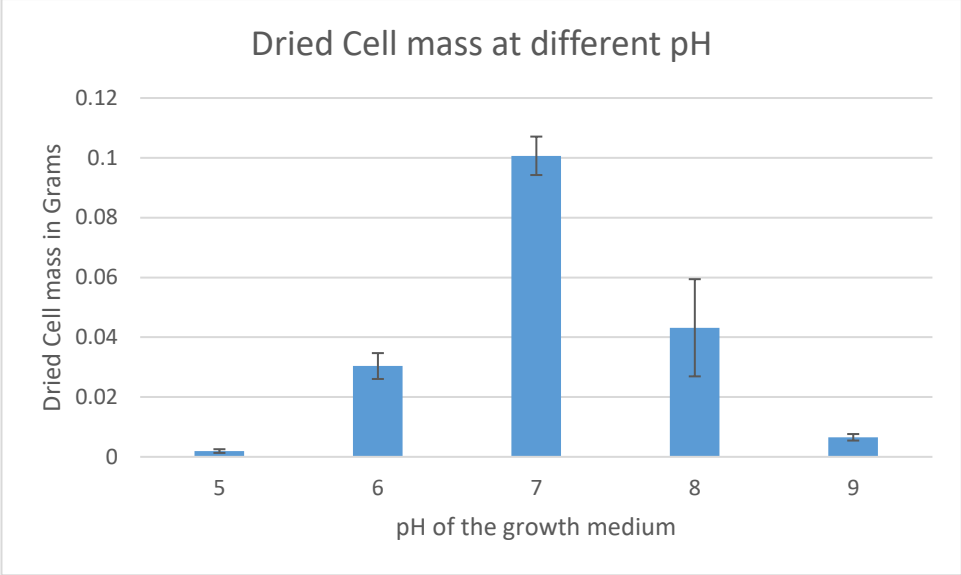


Figure 25: Dried cell mass of HST at different pH adjust Tryptic soya broth incubated in 50 mL falcon tube for 3 days at 30ºC at 200rpm. Error bars represent the standard deviation of triplicate cultures.

5.4 Metabolic capability of HST

In order to further characterize HST, the consumption of different carbon sources was evaluated (Shirling and Gottlieb, 1966, Law et al., 2019). The types of hydrocarbons tested were non-aromatic and aromatic hydrocarbons. The non-aromatic hydrocarbon consumption was very effective as HST was able to utilize various sugars provided. The non-aromatic hydrocarbons utilized by HST are listed in

Table 11. The utilization was confirmed by subculturing multiple times to visually inspect the growth. The activity of exported enzymes was tested by plate-based assay. The substrate tested were cellulose and chitin. The plates were inoculated for 1 week before being stained for zone of clearing.

Newly isolated HST showed activity towards both complex hydrocarbons. The formation of a zone of clearing around the colony confirms the excretion of extracellular enzymes capable of degrading chitinase seen in **Figure 26** and extracellular enzymes capable of degrading cellulose **Figure 27** (Gupta et al., 2012). Given TPA is converted to PCA, HST was grown on PCA and catechol and the growth was compared to TPA. The growth on PCA and on catechol was unusual, Table 12. At 1mM and 10mM of PCA, the growth was visible, however, despite how long the culture was left, the growth in 10 mM did not increase more than the growth observed in 1mM. Meanwhile in 1mM of catechol the growth was now visible while at 10mM there was growth, however, the growth was very slow and too small to be measured. This was an interesting finding as the current known degradation pathway of TPA involves conversion of TPA into PCA to be broken down into β-carboxy-*cis, cis*-muconate or converted into catechol, which is degraded to *cis, cis*-muconate(Hara et al., 2007, Sasoh et al., 2006). The lack of growth observed with vanillic acid and 4-hydroxybenzoic acid also supported the observation that HST struggles to metabolize PCA.

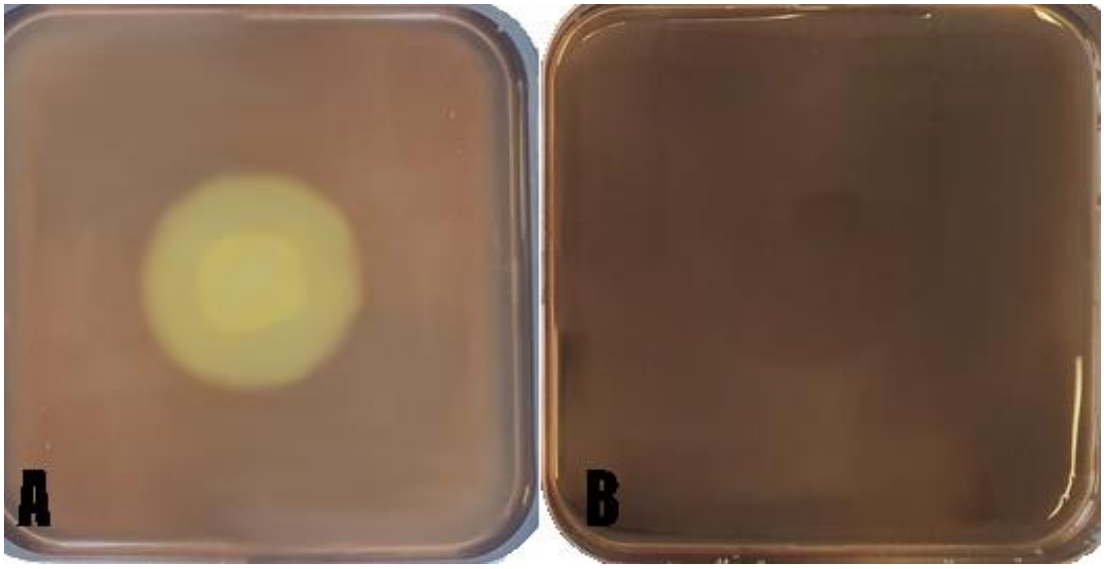


Figure 26: M9 based colloidal chitin plate. (A) Zone of clearing created via excretion of chitinase by HST. (B) Control plate. The yellow halo indicated degradation of colloidal chitin as Iodine does not dye the halo as the chitin have been degraded,

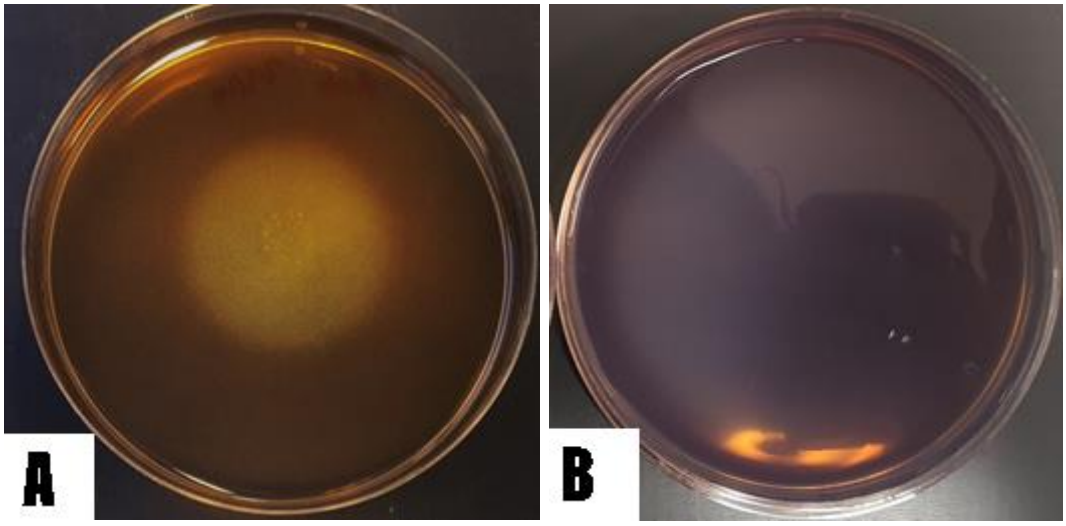


Figure 27: M9 based cellulose plate. (A) Zone of clearing created via excretion of cellulase by HST. (B) Control plate. The yellow halo indicated degradation of cellulose as Iodine does not dye the halo as the cellulose have been degraded,

Table 11: Consumption of Different types of sugars by HST. + indicates visual growth when compared with control and – indicates no visual growth difference to control.

Characteristics	HST
Glucose	+
Sucrose	+
Lactose	+
Rhamnose	+
Mannitol	+
Xylose	+
Arabinose	+
fructose	+

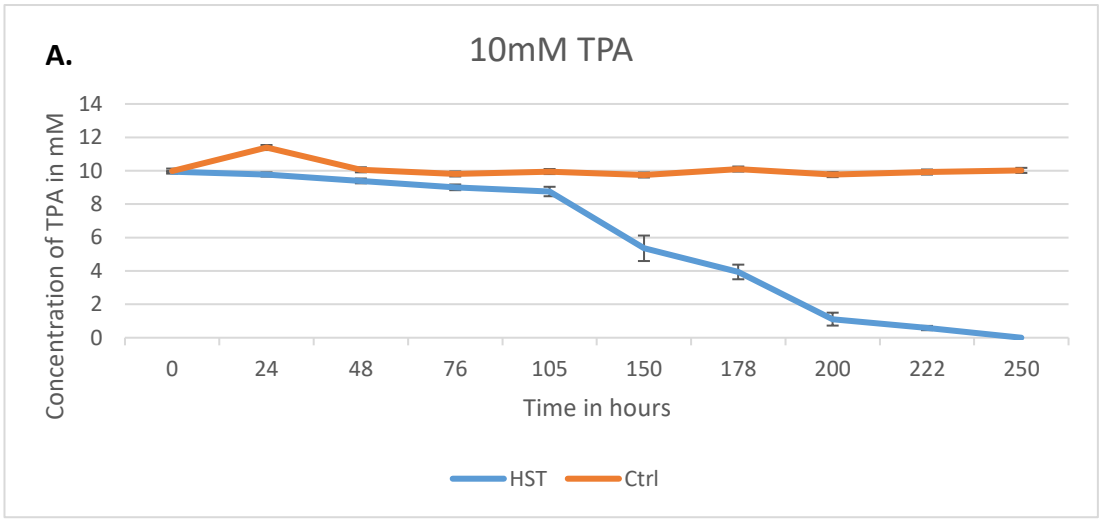
.

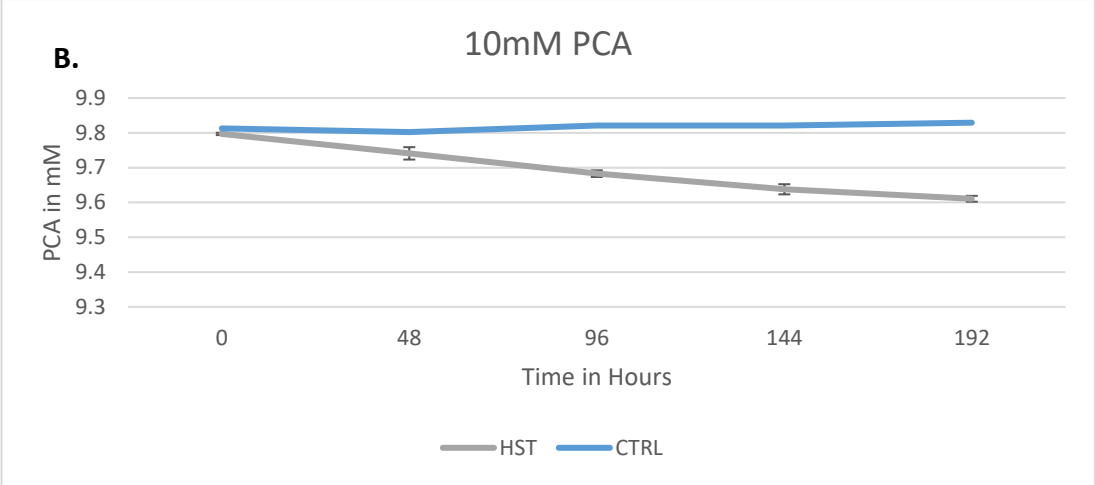
Table 12: List of Benzene ring containing compounds tested for degradation via HST. + indicates visual growth when compared with control and – indicates no visual growth difference to control.

Benzoic Carbon	HST
4-HBA	-
PCA	+ Very small growth
Catechol	+ at higher concentrations
Vanillic Acid	-
Terephthalic Acid	+
Benzoic Acid	+ very slow growth
Toluic acid	+ very slow growth

5.5 Degradation of TPA

In order to confirm that the HST was capable of degrading TPA while not being able to utilize PCA as a sold carbon source, the HPLC was used to compare the concentration of TPA and PCA with the control over time. The degradation of TPA and PCA was done by dissolving 10 mM of TPA or PCA in M9 minimal medium with BG11 trace metals added. The cultures were grown in triplicates with the negative control done with one flask. The supernatant was prepared for HPLC and was stored in -80°C for maximum 3 days before being processed. The concentration of TPA and PCA was estimated using a standard curve with known concentration ranging from 0.1mM to 20mM. The standards were processed by the same method as the supernatants and were analyzed with the samples to minimize the deviation caused by the equipment. The degradation of TPA goes into exponential rate at about 105 hours (**Figure 28A**) while the PCA degradation is minimal and only achieving about 0.2 mM degradation in 192 hours (**Figure 28B**). The TPA degradation starts with a very long lag phase of 105 hours. Once the lag phase was ended, the degradation rate of TPA increased significantly indicating the metabolic pathway for TPA degradation was activated. About 48 hours prior to complete depletion of TPA in the culture, the secondary metabolite production seems to be triggered indicated with colour changes to the culture media.


A.
10mM TPA
Concentration of TPA in mM
14
12
10
8
6
4
2
0
0
24
48
76
105
150
178
200
222
250
Time in hours
HST
Ctrl



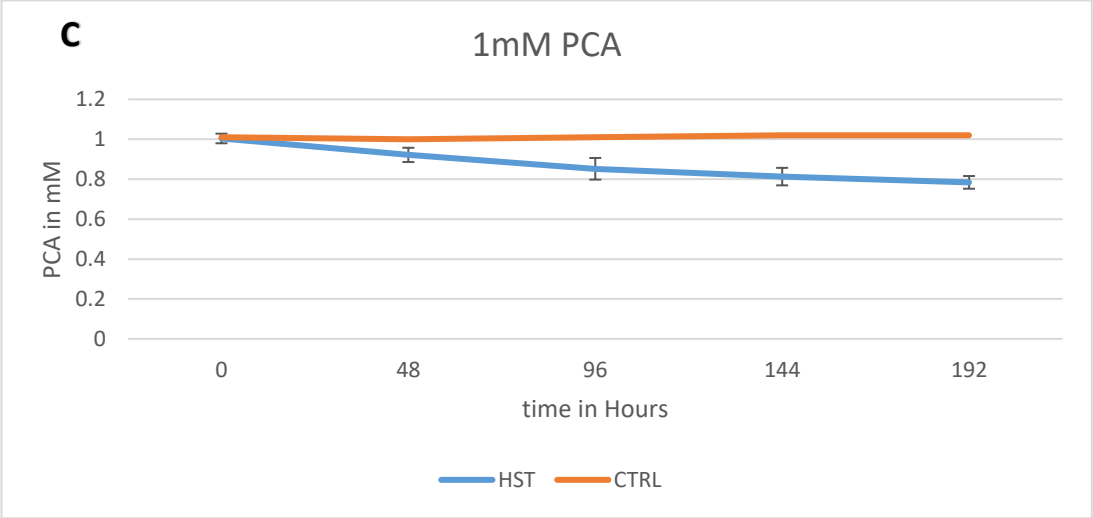


Figure 28: Degradation of TPA and PCA over time by HST. **(A)** Degradation of 10mM TPA by HST over time. **(B)** Degradation of 10mM PCA by HST over time. **(C)** Degradation of 1mM PCA by HST over time Rapid degradation of TPA is observed after 105 hours, however in PCA despite the concentration of PCA and inoculation volume being identical, the degradation of PCA is heavily delayed and HST was only able to degrade very small amount (about 0.2mM for 1mM and 0.4 mM for 10 mM concentration). Error bars represent the standard deviation of triplicate cultures.

5.6 Identification of potential metabolic pathway utilized for bio-assimilation of TPA by HST.

After the confirmation of the lack of degradation of PCA compared to TPA by HPLC analysis of the spent broth, the hypothesis that TPA degradation pathway in HST is not linked to the PCA degradation pathway became more plausible. To confirm that the two-degradation pathways are not linked, HST was cultured in M9 with BG11 trace metal supplemented with 20mM TPA, 20mM PCA, or 20mM Glucose in 6 biological replicates to maximize the statistical accuracy. Seed culture used was grown with 1mM of each corresponding carbon source. Because glucose consumption was much faster than TPA or PCA, glucose growth culture was grown for 2 days while TPA and PCA culture was grown for 6 days.

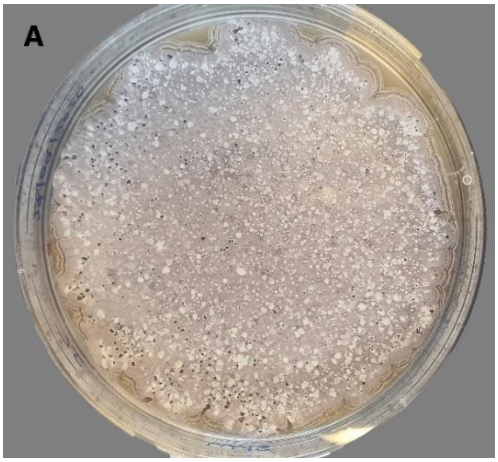
Once the experiment was completed, the cell mass was collected and pipetted into sterile Eppendorf tubes.

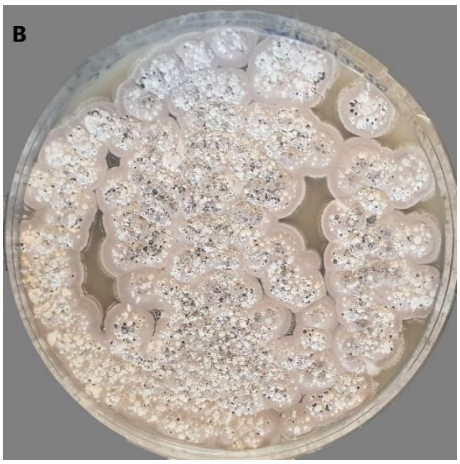
Initially, the colony morphology was indistinguishable from one another despite having been grown in either rich media, TPA, or PCA. However, the morphological mutations were visible once the incubated plates began sporulating (**Figure** 29). The changes in morphology indicates that there has been changes in developmental switch caused by disruption in the signal cascade. Similar morphological changes have been observed in *S. coelicolor* when the culture was supplemented with glucose causing change in the tricarboxylic acid and/or glyoxylate cycle (Viollier et al., 2001). Upon this inspection, the prepared samples were then sent to Dr. Laudina Safo for LCMS analysis of intracellular metabolite profiling.

LCMA metabolite analysis was done using untargeted metabolomics to give wide range of metabolite profile as we did not know what pathway was expressed by HST. The intracellular metabolites were used to understand how TPA is being taken into the cells and how it is being metabolized. To maximize the precision of the data, the samples were cultured in 6 biological replicates.

The principal component analysis was performed to give unbiased overview for any possible trends and grouping within the sample datasets collected from the LCMS runs. Combined with the principal component analysis, partial least squares – discriminant analysis (PLS-DA) was also performed in order to model the differences between the sample sets as well.

The PLS-DA performed from data collected from LCMS analysis showed significantly different metabolic profiles between glucose, TPA, and PCA as seen on **Figure 30.** The clear separation in the grouping of metabolic profiles indicate that different metabolites were present in the 3 different conditions. This result was also confirmed by pathway analysis comparison done to evaluate the difference between metabolite profiles between PCA and TPA supplemented cultures. The pathway analysis shown in **Figure 31** indicates that there was a significant increase in sulphur metabolism, alanine, aspartate, and glutamate metabolism and pyruvate metabolism in the culture with TPA in comparison to culture with PCA. This data is qualitative and requires further validation with standards.





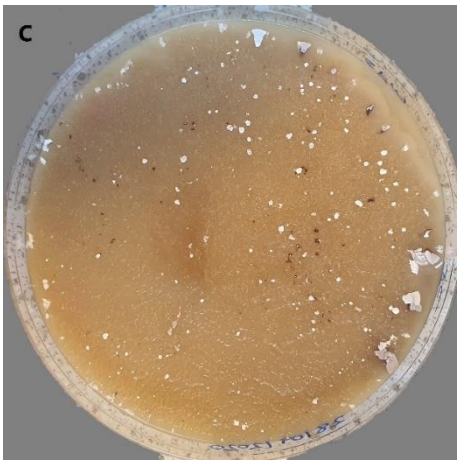


Figure 29: sporulation of HST on SFM plate after growing on various carbon source. (A) Sporulation after growing on Glucose. (B) Sporulation after growing on

TPA. (C) bald mutation after growing on PCA. The plates were incubated in 30°C for 7 days.

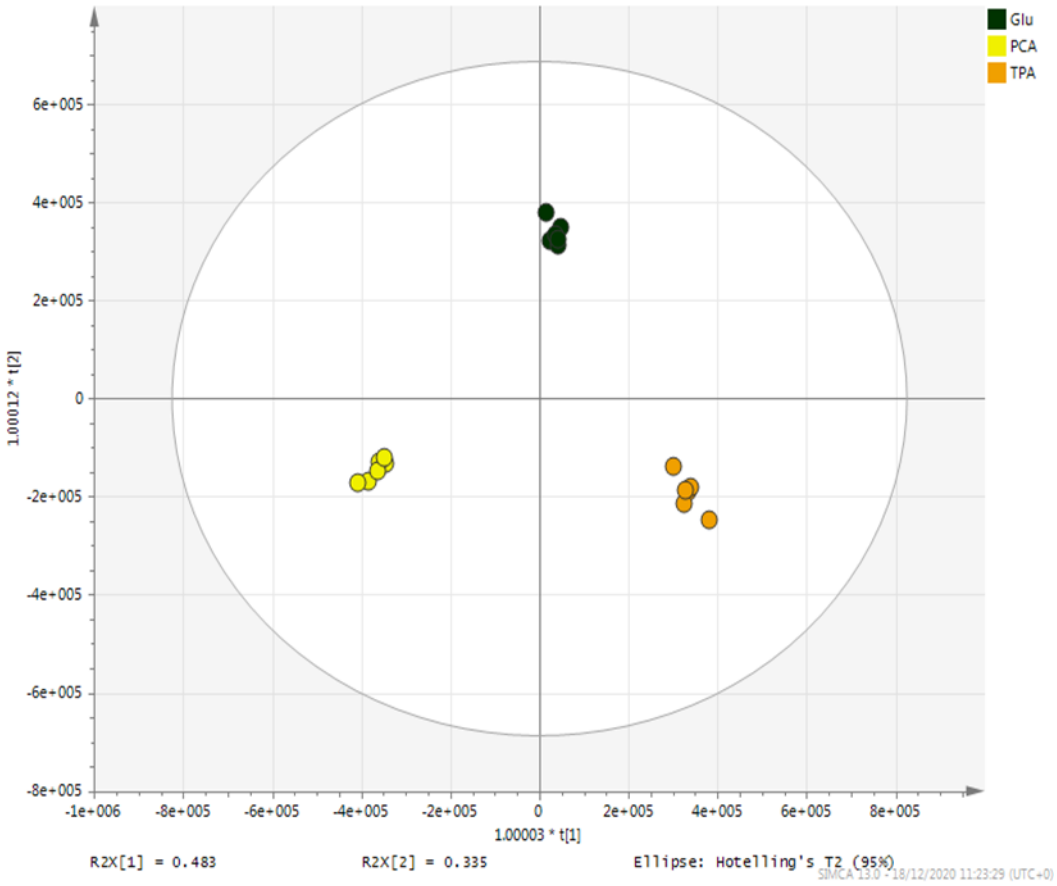


Figure 30: PLS-DA of Extracted metabolites. Green is extracted metabolites from culture grown in glucose, Yellow is extracted metabolite from culture grown in PCA. Orange is extracted metabolites from cultures growin in TPA.

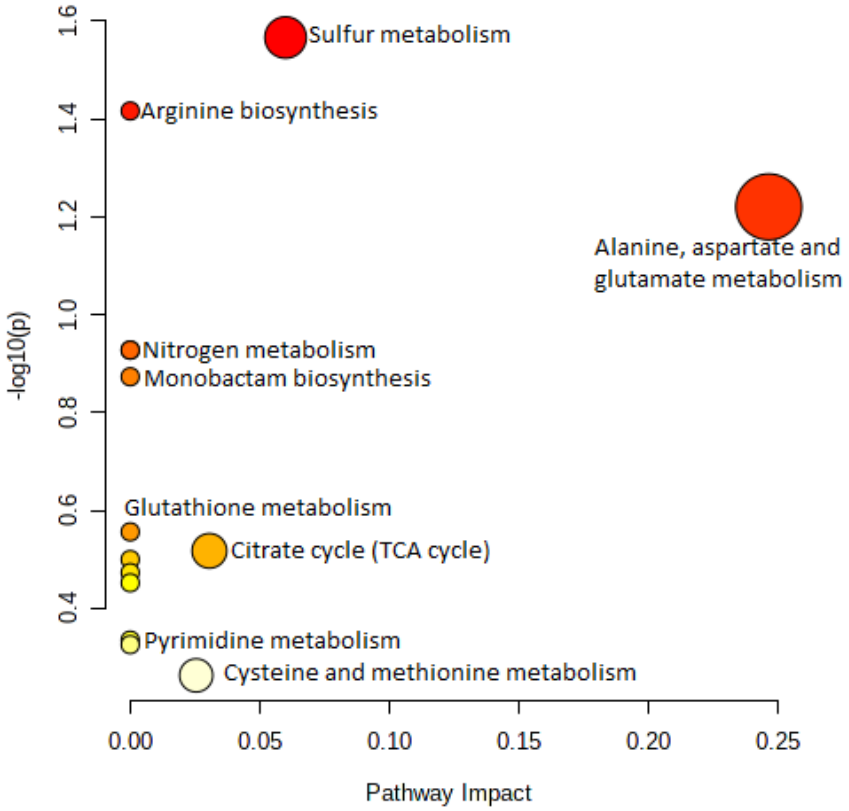


Figure 31: Metabolic pathway analysis of significantly altered metabolites in the intracellular and extracellular extracts from degradation product of PCA and TPA by HST

5.7 Genomic analysis of HST.

The whole genome of HST was sequenced. The complete genome was 7,353,031 bp long with a GC content of 75% Figure 32. Within the genome, the genes for the Beta ketoadipate pathway were present in the genome, *pcaA, pcaB, pcaF, pcaG, pcaH, pcaI, pcaJ*, and *pcaR*, demonstrating that genetically HST can utilise PCA. The TPA gene cluster was also present tpaA*, tpaB, tpaC*, and *tpaK*. Despite the presence of the *pca* genes, it is not immediately obvious as to why they are not expressed.

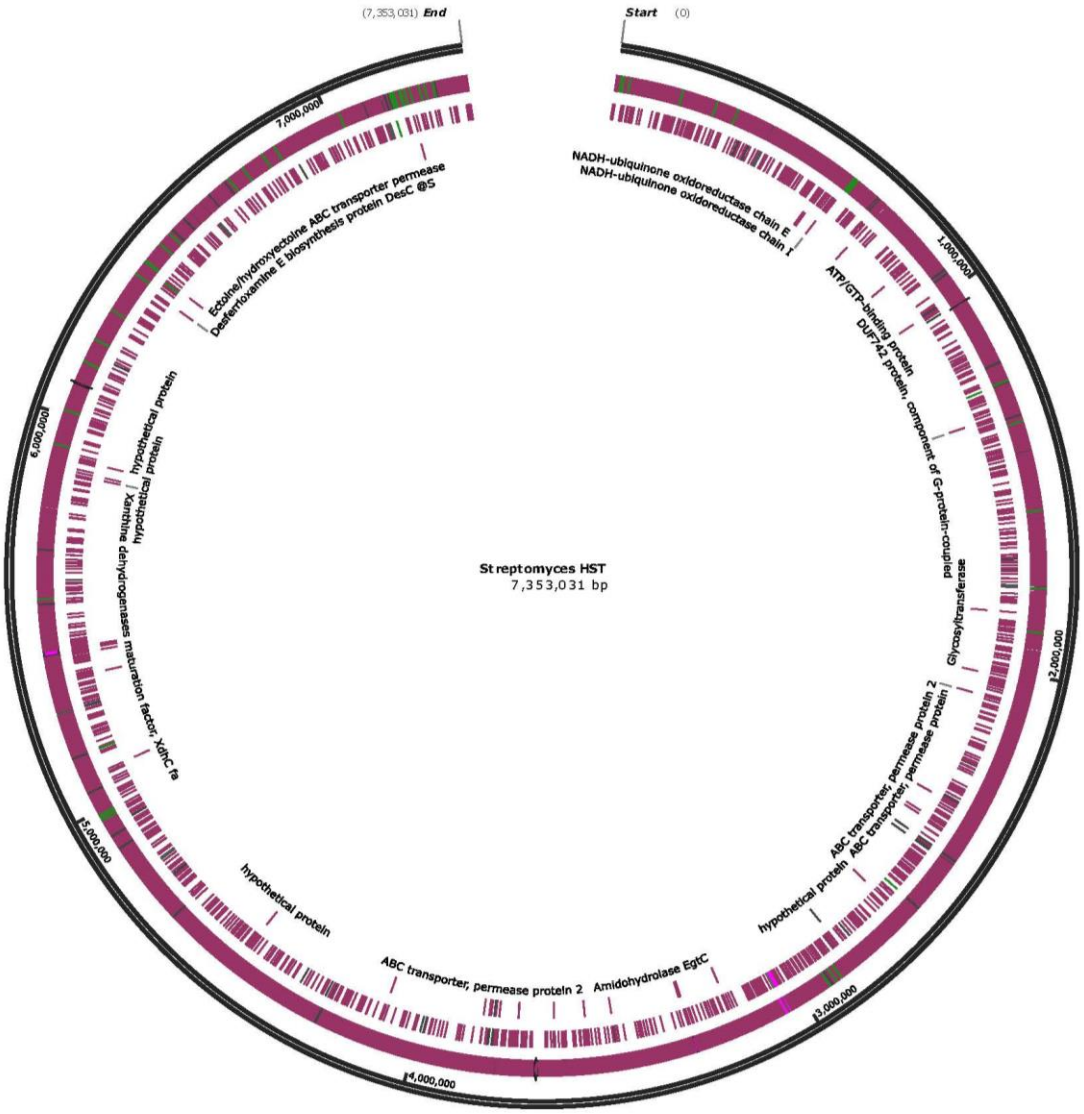


Figure 32: Whole genome of newly isolated *Streptomyces* sp.

5.8 Discussions

The bio-assimilation of TPA by genus *Streptomyces* has not been recorded in the past. This finding is effectively an opening of the potential for commercialization of the strain to utilize the waste TPA as a substrate and produce higher valued products such as antibiotics, antifungal (Jakubiec-

Krzesniak et al., 2018). The isolated strain HST exhibited ability to degrade TPA however failed to show effective degradation of PCA which may be an indication that TPA is degraded into different intermediates rather than PCA as observed in other microorganisms such as *Rhodococcus, Comamonas, Bacillus, Arthrobacter,* and *Dietzia* (Sasoh et al., 2006, Shigematsu et al., 2003, Hara et al., 2007, Chain et al., 2006, Knott et al., 2020). Although the potential for novel pathway is possible, the inability to bioassimilate PCA in large quantities may also indicate the membrane uptake of PCA is inefficient compared to TPA uptake. Leaving a potential for TPA degradation pathway to have PCA as intermediate and follow the already proposed pathway as seen in Error! Reference source not found.**3**. Even though it is uncertain how the T PA is being assimilated into the cell but, it is evident that higher concentration of PCA is toxic to the cell meanwhile same concentration of TPA is not. In order to fully understand the metabolic pathway involved in TPA degradation by HST, obtaining the genomic information or transcriptomics will be required.

CHAPTER SIX

General Conclusions

6.1 Summary of the research

In this work, *Streptomyces* sp. (HST), an environmental isolate capable of the biodegradation and bio-assimilation of terephthalic acid, has been isolated and characterized. This is the first documentation of *Streptomyces* sp. being able to bioassimilate terephthalic acid.

6.1.1 Re-evaluation of *Pseudomonas* sp.

The need for identification of plastic degrading microorganisms is in high demand to expand the knowledge of plastic biodegradation in the environment. The first part of this work involved re-evaluation of *Pseudomonas* sp. previously showed growth on commercial plastic in previous research done in my BSc. However, the *Pseudomonas* sp. provided did not show signs of degradation on virgin plastic indicating that the growth observed in the past may have occurred from impurities in the commercial plastic such as plasticizer, dye, and unreacted monomers that may have been left behind. This is a crucial aspect in plastic degradation as by screening plastic degrading microorganisms on commercial plastics may result in isolation of microorganisms that may grow on various contaminants in the plastic instead of the polymer. As the result of this, further isolation work was done to isolate plastic degrading microorganisms. Although this experiment showed no signs of successful degradation of PET and PS by *Pseudomonas* sp. mentioned, it

may indicate that the given growth conditions, including, but not limited to; temperature, oxygen availability, growth media, etc. may have caused silencing or suppression in certain metabolic pathways that may be responsible for the degradation. In order to properly test the degradation capabilities, it would be interesting to attempt the experiment using a wide range of conditions and growth media to maximize the chance for the expression of potential pathway for degradation of plastic as seen in previous work. It would also have been beneficial to perform PCR to screen for potential gene sequences such as serine hydrolase, hydroquinone peroxidase, and other potential enzyme capable of breaking the carbon-to-carbon covalent bonds (Kim et al., 2020, Nakamiya K. et al., 1997). Another key aspect of this work that I could not achieve was the utilization of styrene gas for screening of polystyrene degrading capability of provided *Pseudomonas* sp. and degradation of ethylene glycol and TPA was excluded. Styrene gas was not available given the health and safety implications. Given the large amount of data already published, on the consumption of both TPA and EG by *Pseudomomas* sp. this was not deemed a worthwhile effort, as it would not have represented a novel piece of work. (Haines and Alexander, 1975, Muckschel et al., 2012, Zhi Jiang et al., 2011). The confirmation of metabolism of styrene by strains would have provided more rapid method of analyzing the potential for polystyrene degradation.

6.1.2 Isolation of novel plastic degrading microorganisms.

Microorganisms capable of biodegradation of plastic have been isolated in areas with high plastic pollution such as plastic recycling centers, landfills, and wastewater (Hiraga et al., 2019). In this work, soil samples from wide range of areas with potential exposure to plastic such as urban city centers, Landfill, and human sewage treatment center. These soil samples were subjected to enrichment followed by selective enrichment using virgin plastic with no additives such as plasticizers, dye, and minimal unreacted monomers. Once the selective enrichment was performed, the samples were screened for plastic degrading microorganisms. After the selective enrichment, eight *Streptomyces* sp. showed potential for degradation of polycaprolactone, commonly used polymer for screening of PET degradation. Eight polycaprolactone degrading *Streptomyces* sps. Were then inoculated into media with virgin PET plastic. Upon further growth of these strains in PET, they did not show any release of TPA after 14 days on virgin PET. This was a crucial aspect of PET degradation as the release of TPA is believed to be one of the main indicators for degradation of PET by microorganisms, as natural biodegradation of TPA is uncommon. As a negative control to confirm that there was no degradation of TPA by these isolates, the eight strains were grown on TPA supplemented minimal medium. Even though the growth on TPA was done as a negative control, the work indicated that isolate HST had the capability to biodegrade and assimilate TPA. Which was then processed for characterization of the strain.

Due to the limitation of time and resources, only few types of plastics were screened for degradation. Also, the selective enrichment being carried on for 5 months instead of 2 weeks as planned which was caused by the current Covid-19 situations. It would have been appropriate to be able to screen the samples for various types of plastic commercial and virgin plastic.

6.1.3 Characterization of Isolate HST

With this isolation of TPA degrading *Streptomyces* sp. (HST), the work was shifted more towards the characterization of the strain and to understand the biodegradation pathway of TPA via LCMS analysis. The characterization of HST involved capability to metabolize various carbon source, different pH range, and finally the metabolite profiles of TPA and PCA supplemented cultures. The LCMS data showed significant difference in the metabolism of TPA in comparison to glucose and PCA. The significant difference in the metabolic profiles of TPA and PCA may indicate that the TPA is not being degraded via proposed TPA degradation pathway by Hara et al, 2007. Proposing a potentially novel degradation pathway have not seen before. However, as LCMS data relies on the predicted structure based on the mass and by compound discoverer, the lack of standards for metabolites makes it impossible to be able to accurately predict the metabolic pathway. Despite the lack of secondary confirmation of potential degradation pathway, it is still important that TPA degradation by the genus *Streptomyces* have not yet been reported in the past making the isolate HST the first *Streptomyces* sp. to degrade TPA. More work is required to understand how the TPA uptake and

degradation is possible while PCA is not being metabolized as two compounds are very similar in structure and PCA is considered to be more easily metabolized by various microorganisms. Proteomics and transcriptomics studies will not only be able to understand the metabolic pathway, but it will also allow deeper understanding of how presence of TPA does not affects the cell while PCA seems to be toxic to the cells.

The isolation of plastic degrading microorganisms, the objective of this work was partially successful by isolation of HST, an isolate capable of degrading part of the PET that was deemed toxic to the environment. Although more work is required to fully comprehend the capability of the isolate HST however, it is a novel finding and potentially economically, and environmentally beneficial finding in reducing the plastic pollution.

CHAPTER SEVEN

Future Work

7.1 Future Work

Although this work was able to isolate first recorded case of terephthalic acid degradation by the genus *Streptomyces*, Due to the current circumstances of Coronavirus-2019, many things were not achieved.

First, regards to work done with *Pseudomonas* sp. the degradation capabilities were not fully explored. To fully explore any potential degradation capabilities, wide range of media composition and different conditions including the monomers should have been tested to confirm that inhibition or silencing did not happen due to the growth conditions. On top of the limitation of time, genetic sequencing analysis would have also given in-depth understanding the genomic background to the degradation capabilities.

Second, the isolation of plastic degrading microorganisms would have been also interesting to see potential degradation caused by bacterial community and explore how each type of bacteria takes a role in the degradation of the plastic (Morohoshi et al., 2018, Lear et al., 2021). As in this work, the carbon and other nutrients were heavily limited due to sudden suspension in the serial enrichment, it would have been an interesting to see how the community would change as each generation is carried onto the next. Furthermore, deeper exploration of types and location of the samples would also be interesting to attempt

in the future, attempting to find environments with long term exposure to plastic and sampling the plastic particles found in those areas for signs of biodegradations. Microbial community may be isolated and characterized from the samples that had long term environmental exposures proving the importance of community in biodegradation and the existence plastisphere (Wen et al., 2020). Furthermore, more rapid methods of isolation will have to be developed in the future to screen though the samples to save time, consumables and to be able to automize the isolation step.

Finally, the characterization of first recorded case of terephthalic acid degradation of streptomyces could have been research more in-depth to understand the full process. More specifically, proteomics will be able to identify which protein is responsible for transportation and consumption of TPA giving us the full picture of the pathway. It will also be beneficial to assay the crude cell extract for activity against wide range of benzoic carbon to identify potential intracellular proteins that may affect the degradation. Further research such as metabolism of radiolabeled TPA will also confirm how the metabolites are being assimilated and converted into other compounds. These cellular understanding of HST will allow utilization of the strain as the chassis to create PET degrading microorganism by insertion of genes responsible for PETase and/or MHETase. By doing this, HST would be able to fully metabolize PET reducing the pollution and successfully biodegrading and bio converting PET into more expensive compounds such as antibacterial, antiviral, and other high value compounds.

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