Biochemical characterisation of *Ideonella sakaiensis* and the influence of culture conditions on plastic degradation

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Abstract

It has become apparent that the global reliance on plastic is not only due to the increased commodification of modern society, but also due to practical applications which have become too inextricably intwined in every industry. The global Covid-19 pandemic in 2020 has particularly illustrated this. The solution to the plastic problem lies in a systematic overhaul of international sustainability initiatives, waste management strategies, and prioritising recovery of materials trapped in end-state plastic, as well as developing comprehensive complete recycling systems.

With the discovery of microorganisms that are able to degrade plastic, an avenue of sustainable recycling has opened for closing a loop on a circular economy system. One such bacteria is *Ideonella sakaiensis* which produces a hydrolase capable of depolymerising PET, aptly dubbed a PETase. In addition, with an MHETase which aids in converting dimers for complete recovery of monomers, *I. sakaiensis* is able to efficiently degrade PET of varying crystallinity, with a preference of low crystallinity PET.

In this work, the objective was to characterise growth conditions which would produce reliable and reproducible set of methods to obtain the biggest biofilm growth, while investigating how it affected the degradation of PET. The project did not fulfil the goals set out, however, it concluded that the research is valuable even without the use of molecular tools, in order to identify the base characteristics of the species, for comparative purposes as well as better insight into the direction future research may go towards.

Acknowledgements

I would like to take this opportunity to acknowledge all of the people responsible for this thesis coming to fruition. Firstly, I would like to thank my supervisor, Cristiano Varrone, for the incredible amount of patience and willingness to provide guidance with anything I needed with the project. Our research was cut short, but in the time, we had, I have learned a lot and gained many valuable practical skills.

I would also like to thank our laboratory technician who was always forthcoming in listening to my questions. The student assistant, Andrew Rennison, who was working on a project parallel to mine and spent a considerable amount of time at the start sharing work with me and sharing his extensive molecular biology knowledge. Without him the start would have been filled with more uncertainty than it already was. I thank him from the bottom of my heart. All of the Masters students who were in the laboratory with me, doing their own work, but always lending support; we might have been doing completely different projects but we always had words of wisdom and encouragement for each other. The exchange of information not only between us as students but with other supervisors as well, has proven to be invaluable.

And finally, I would like to thank my family and friends who trusted in me and my dream to study about a more sustainable world. As well as supporting me the entire duration of the thesis programme.

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List of Abbreviations

NAFTA North American Free Trade Agreement

PE Polyethylene

PET Polyethylene terephthalate

PVC Polyvinyl chloride

PP Polypropylene

PUR Polyurethane

PA Polyamide

PS Polystyrene

PHA Polyhydroxyalkanoates

PLA Polylactic acid

MHET Mono(2-hydroxyethyl) terephthalate

BHET Bis(2-hydroxyethyl) terephthalate

TPA Terephthalic acid

EG Ethylene glycol

TCA Tricarboxylic acid

BSL 1 Biological safety level 1

YSV Yeast-sodium-vitamins

MC MacConkey

1 Introduction

1.1 Reliance on plastic

Modern society brings with itself a wide catalogue of conveniences as well as luxuries not before experienced in human history. As more countries across the globe become more industrialised, the necessity for plastic polymers rises. This does not only follow the convenience aspect but improvement in sanitation, industry and all aspects of society. An immediate association with plastic falls to the conveniences that surround us. Mainly these are single use plastics, often linked to the food industry. Packaged foods and disposable cutlery and beverage containers. However, plastic comes in the form of electronic devices heavily in use today, toiletries, furniture, textiles, transportation devices and other miscellaneous objects. Plastic permeates todays existence, partly due to being cheap to produce and the variety of polymers mean that the use of it is widespread. The first synthetic polymer ever produced was Bakelite in 1907 by Leo Baekeland(Powers, 1993). It was hailed in its time as a new and exciting look into the future of humanity. It could be used to produce so many things, and incorporate in already existing products to make them more resilient and more appealing. After that initial discovery, it did not take long for other types of synthetic polymers to begin widespread production. While it may be hailed a luxury, the way society has become reliant on plastics, and unwilling to part with its many conveniences, an alternative would not include removing plastic from all the aspects it is imbedded in. Finding more sustainable counterparts, and the change of perception what the unmonitored use of it would mean for the environment as a whole are more pertinent issues to address. With the raise in sanitation standards and sterilisation technique in both food production as well as the health sector, these pose large areas where single use plastic became almost an irreplaceable necessity. Nitrile and rubber gloves which are single use and prevent any cross contamination, while generating a large amount of waste. In the health sector, single use medical equipment brought with it lowered infection rates, due to the ease of disposing of used equipment. However, this means the medical system in conjunction with research laboratories produce an unprecedented amounts of plastic waste, daily averaging 25% of all plastic waste produced in the USA(Wagner, 2016).

1.1.1 Types of plastic polymers and their uses

Plastics are defined as polymers of either natural or synthetic origin which, when soft, can be moulded, and when cooled retain that shape. Based on which monomers the polymer is constructed will determine its unique properties(*Science of Plastics | Science History Institute*, 2019). The raw polymer before pouring is called a resin. The top industrial resins of importance produced are: polyethylene (PE), polyethylene terephthalate (PET), polyvinylchloride (PVC), polypropylene (PP), polyurethane (PUR), polyamide (PA) and polystyrene (PS)(Danso et al., 2019).

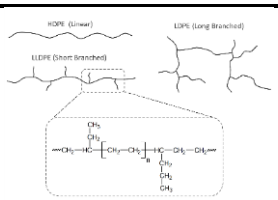
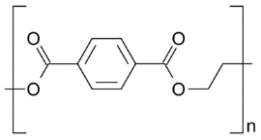
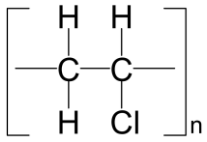
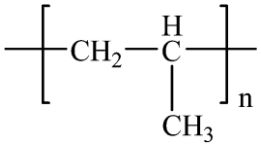
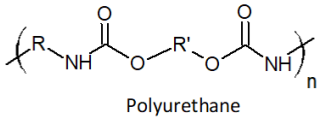
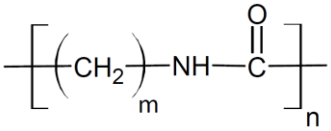
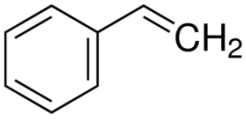
Polymer	Monomer(s)	Structure	Uses	Reference
PE (LDPE, HDPE)	Ethylene		Packaging, thin films	(Polymer Database, 2015d)
PET	Ethylene glycol, terephthalic acid		Textile fibres, packaging	(Polymer Database, 2015c)
PVC	Vinyl chloride		Construction industry	(Polymer Database, 2015a)
PP	Propylene		Packaging; the same market as PE	(Polymer Database, 2015d)
PUR	Organic diisocyanate, diol compound	 Polyurethane	Foams, sealants, elastomers, adhesives etc.	(Polymer Database, 2017)
PA	Diacid, diamine		Automotive, engineering industry	(Polymer Database, 2015b)
PS	Styrene		Food industry consumer goods	(Polymer Database, 2015e)

Table 1. Most common commercial plastics, their monomers and uses

PE represents the most commonly used polymer on the market. Due to a large extent of the market being taken by the packaging industry, and PE being the most versatile in terms of the types of stable films able to be produced from it, it is highly valued. It most commonly is produced in 3 formats: low-density polyethylene (LDPE), linear low-density polyethylene (LLDPE) and high-density polyethylene (HDPE). They vary in their crystallinity, HDPE being the more crystalline and the highest value PE. The higher crystallinity means it is more durable and less likely to be affected by abiotic and biotic degradation(Polymer Database, 2015d).

PET is most commonly used in the production of bottles and the textile industries. Many polyester labelled fibres are PET. It is a durable textile fibre with good moisture resistance and low wear over time. It can be used in industry where it is reinforced with fiberglass, graphite etc(Polymer Database, 2015c).

PVC comes in various forms. It is the third highest selling plastic in the world. It has poor resistance to photo and thermal degradation. Therefore, additives are added to stabilise it. The largest use of PVC is in the construction industry. PVC pipes, window parts, machine extruded parts, and added to plasticisers to create plastisol which can then be moulded at room temperature(Polymer Database, 2015a).

PP is the second most popular plastic polymer in industry use. It shares many similarities with PE and its properties fall between those of LDPE and HDPE. It fulfils the gap for packaging needed with the resistance and properties where PE cannot fulfil. It can withstand much higher temperatures than PE and therefore is used to secure items that will experience more thermal stress(Polymer Database, 2015d).

PUR is a polymer with a wide range of capabilities, based on which compounds are chosen to create the backbone. Based on the combination of aromatic or aliphatic isocyanates; low molecular weight or polymeric diols, the physical properties of the foams will be different. Applications of PUR are for foams both industrial and household ones, adhesives, coatings, sealants etc(Polymer Database, 2017).

PA are a group of polymers more readily used in the engineering industry. They are more resistant to alkaline hydrolysis and have better resistance to organic solvents than PET. They can withstand continuous use at below freezing temperatures and are heavily used in the automotive industry. A popular PA, nylon, has been used in the textile industry for decades

before losing some market share to PET fibres as those are less wrinkle prone(Polymer Database, 2015b).

PS is one of the largely used commodity plastics as it is cheap and easy to produce. The food industry uses large amounts of PS for packaging, disposable cutlery, plastic cups etc. It is a less readily recyclable plastic, and in its expendable polystyrene form, it takes up a lot of space on landfills. It is in most cases produced as a single use plastic(Polymer Database, 2015e).

1.1.2 The cost of convenience

The various applications of plastic have led to a boom in production. As it is cheap and versatile, the use of plastic is dominated globally. Due to the lack of marine conservation research at the turn of the 21st century, there was a lack of data, how the waste produced from the use of plastic would affect the worlds marine systems(Derraik, 2002). In 2006, the global plastic production was 225 million Mt per year. Due to the global spread of factories and different sorting and collection capabilities of plastic waste, the recovery of the waste produced was varied(Barnes et al., 2009). The composition of waste generated globally is around 10% in favour of plastic, while the marine litter and waste washing up on the beaches is a disproportionate 50-80% of plastic(Derraik, 2002). This is prescribed to several factors. The plastic produced for packaging is light-weight, and readily carried by the wind if not properly secured. The lightweight nature of it also ensures it is buoyant and readily distributes in water systems(Barnes et al., 2009).

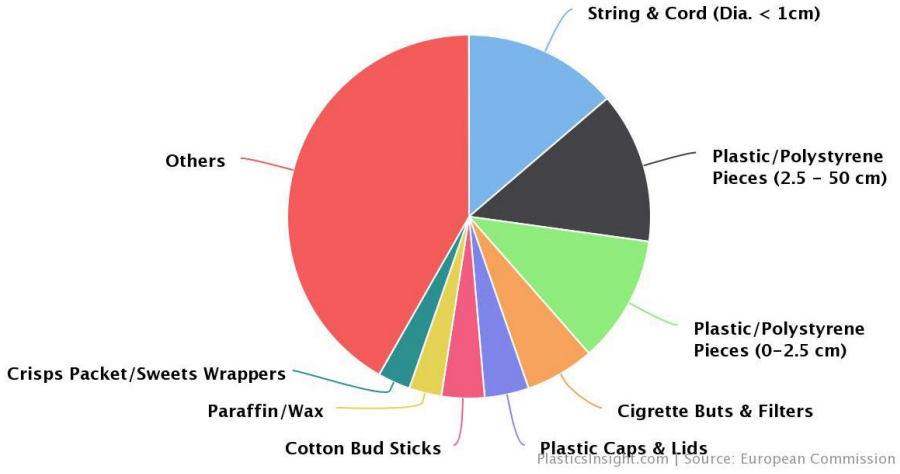


Figure 1. Top marine litter on European beaches, 2016(Editorial team, 2018)

This figure showcases the distribution of various litter on European beaches, 31.64% being broadly plastic items(Editorial team, 2018). In 2012, the global resin production had risen to 288 million Mt per year, an increase of 620% from 1975. The largest sector to experience this was packaging. The use and production of plastic keep increasing each year. The resulting waste from this is proving to a large challenge. Mismanaged waste that occurs within 50km of the coastline is the biggest problem. The highest concentration of global human population lives in this area. 16 of the top 20 mismanaged waste producing countries are middle income countries, which were rapidly industrialised, but with a slowly developing waste management infrastructure(Jambeck et al., 2015). Global consumption puts the NAFTA area at 139kg/person, Western Europe 136kg/person and Asia(excluding Japan) 36kg/person(& A. C. Horodytska, 2019). Consumption includes domestically produced as well as foreign imported objects(Suranovic, 2005). This is important to highlight as the top global importer of PVC, PP, PE, PA and PP resins are India and China. China holds 50% of the worlds PET resin market. And while they produce the largest amounts, they also have the highest consumption. The prediction models for helping lowering the mismanaged waste in the countries that have the biggest problems are not accurate as there are too many variables that cannot be predicted as economic states, political decisions and cultural circumstances(Jambeck et al., 2015).

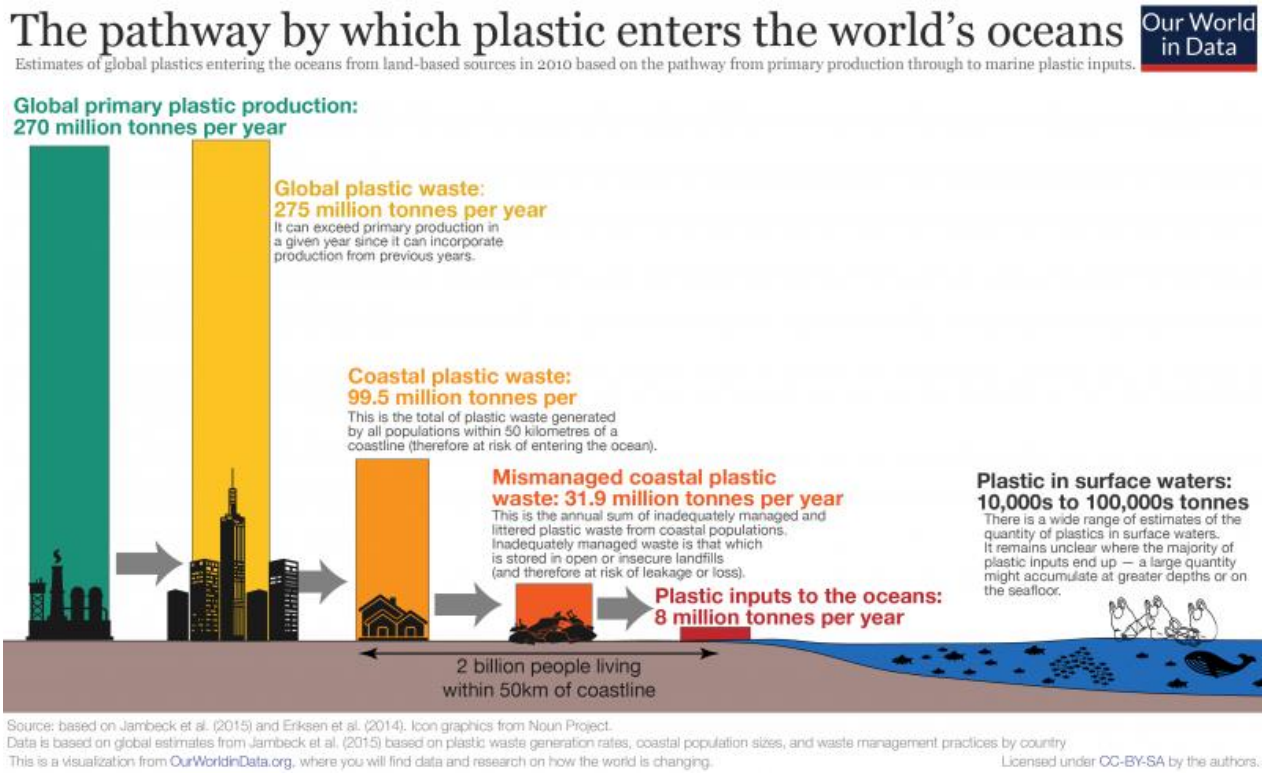


Figure 2. Path of mismanaged waste into the ocean(Ritchie & Roser, 2020).

The mismanaged waste leeching into the water systems and settling in plastic islands floating in the open oceans is broadly categorised in macro(>25mm) and microplastics. Macroplastic is more regularly included in cleaning efforts as it is easily spotted and retrieved. It also poses a larger danger for sea life as seabirds, cetaceans and fish ingest them whole. Microplastics are primary and secondary. Primary microplastic are ones that were already produced in a microscopic size. Scrubbers in cosmetics, some drug vectors and industrial air-blasting media. And while modern water treatment removes 99% of these, the amount left in the effluent is still significant due to the large amount present in the original stream. Secondary microplastic is formed as abiotic and biotic factors fragment larger waste(W. C. Li et al., 2016).

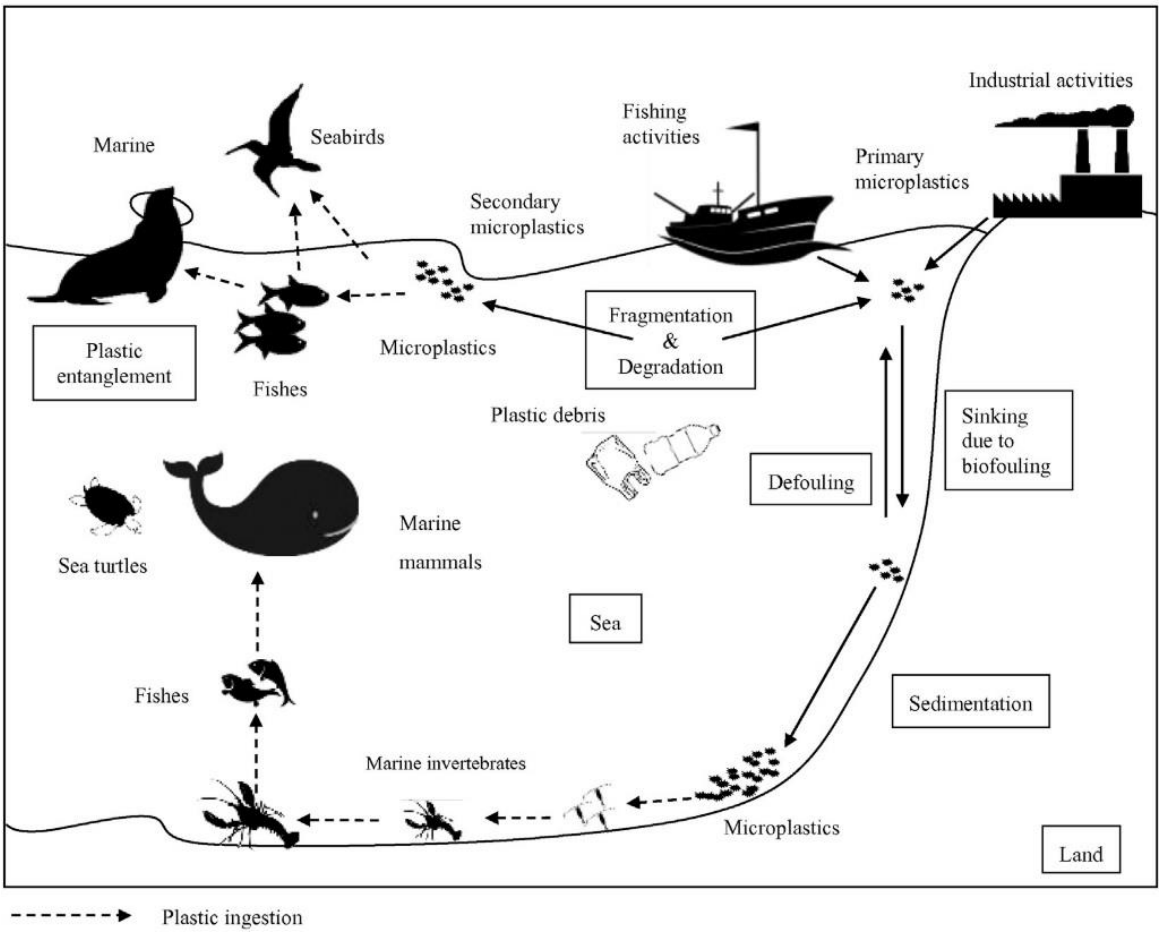


Figure 3. Potential pathways of plastic in the environment(W. C. Li et al., 2016)

1.1.3 Modern waste treatment

As plastic production continues increasing, by 2050 it is predicted the accumulated waste globally will reach 33 billion tons(Joo et al., 2018). Looking at PE degradation rates in a landfill, it was calculated at about 0.5% of its mass per year. PE is the highest utilised plastic

in the world, and globally it is faced with a similar fate of ending up in a landfill(Palanisamy et al., 2011).

An international standard was created by the American Society for Testing and Materials (ASTM) for identification of resins in industry. This was based on the markers created by the Society of the Plastic Industry (SPI) in 1988 to clearly identify resins used to produce a product, and have since been printed on plastic products to denote their composition(ASTM D7611 / D7611M-20, 2020).

Polymer	SPI marker
PET	
HDPE	
PVC	
LDPE	
PP	
PS	
PU, polyurea, polycarbonate, nylon, polyethersulfone, epoxies, biopolymers	

Table 2. SPI markers of plastic polymers for identification(Rahimi & Garciá, 2017)

Waste management is divided into: collection, sorting and treatment. In the developed world, the mindset of recycling is well established. In Europe it varies in terms of the rate of recovery of waste; with Germany on the higher end, and Spain on the lower end. The EU monitors the rates at which collection increases or reduces. Germany had a 40% growth in terms of collection, while Spain had a 7% decline in 2016. The collection at a municipal level

involves sorting general waste in each household, the availability of different waste bins for disposing of household waste, buy-back schemes for plastic bottles, and drop-off sites for larger waste and waste that cannot be sorted in the common categories. Sorting is done at material recovery facilities. The technologies used at these sites are: bag splitters, ballistic separators, air separators and optical detection systems. Once there and sorted, based on the type plastic, different recycling processes are applied. Thermoplastics such as polyesters are ground or pulverised to then be extruded into pellets which can be reused. Others are combusted. There are two methods of classifying the recycled plastic: the open and closed loop. The closed loop method implies scrap from production is re-extruded and reused, creating a higher-grade recycled product. In an open loop, the plastic had been used by a consumer and has accumulated impurities, either intentionally or unintentionally introduced. This plastic needs to be washed, filtered, or degassed first. The quality of this plastic decreases with each processing step, and it cannot be recycled ad infinitum. Usually this recycled plastic is later used for trash bags, pipes etc. As for PET, which is recycled at the highest rate, as it is inert and recycles very well, similar products to its starting point may be produced from it. Flexible plastics and mono-, and multi-layered plastic are more difficult to recycle as the machinery is more optimised for hard plastic. Films are also quite often coupled with aluminium and inks, and flexible plastics with inks and adhesives which require more processing to prepare for pulverising it(O. Horodytska et al., 2019).

Based on the collection strategy of a given country, the sorting and processing process will be more or less complex. If there are schemes for separate collections of drinking bottles, away from other plastic waste, the processing will be more straightforward for the drinking bottle waste. As it is known that the plastic in this waste is predominantly PET and PP, which are recognised by IR sensors and sorted. For mixed waste, the detection of which plastic is present is more complicated. If there is an automated system with an IR sensor, it may be limited to reading the type of plastic that will be turned towards it at that moment and disregard other plastic present in the waste. If the factory has human sorters, it can be more precise however, it is more costly and time consuming. There is a distinct difference in mechanical recycling, between what can be modelled and what is practically possible. Multiple sensor systems exist for sorting through the different polymers: tribo-electric separation, froth floatation, magnetic density separation, x-ray detection. These all require different pre-treatment, and specialise for a specific polymer set, therefore might not be cost effective to implement. One of the biggest challenges of mechanical recycling, is the

degradation of the plastic, both by abiotic factors such as UV, moisture, general mechanical damage, and heat. All of these factors add to the disruption of the crystalline structure of the polymers, decreasing their molecular weight. In the case of a mixed polymer waste, the thermal processing is done at the temperature needed to process the most resistant polymer, and as such, the others undergo higher thermal stress. PET recycling is the most common "cover-all" recycling strategy, and particularly in Europe, 57% of PET was recycled in 2014. This is due to comprehensive EU directives urging the collection and compensation for PET bottles. The PET is heated between glass-transition and melting temperature in a process called solid-state post-condensation. The mass is then reinforced with additives that are chain extenders, and extruded for industrial use. The loop of product-waste-product requires a design approach from the raw material constructed polymer, and then a new product made with recycled material. This means optimising the starting product and recognising how the changed recycled material may be utilised afterwards, as discussed before, not all recycled material may be reused in the same capacity the virgin material was used for(Ragaert et al., 2017).

There is another recycling strategy which is chemical recycling. Chemical recycling is considered a tertiary method, with the closed and open loops with mechanical recycling being the primary and secondary respectively. Chemical recycling aims to recover the monomers that make up the polymer through processes such as pyrolysis. They are not considered as scalable to industry as they require significant power outputs. PET and HDPE are the polymers that are most frequently recycled, as others require sophisticated depolymerisation technique. PET can be depolymerised via pyrolysis or chemolysis which can include acid catalysts or not. The backbone is transesterified using ethylene glycol, methanol, water or hydroxide. These processes require high temperature and pressure, and are often less efficient if there is a presence of colourants in the feed. A future for chemical recycling would include discovering catalytic reactions that do not require high temperatures and pressure to be effective and applicable on an industrial scale(Rahimi & Garciá, 2017).

As previously discussed, the lack of municipal waste management infrastructure heavily contributes to mismanaged waste in developing countries. It is more common for individual members of society to organise efforts for collecting and sorting plastic waste by hand. PE and PET is mechanically recycled using similar technology as the one in Europe as overseers of these sorting operations are buying the technology for their use. Latin America is upping the municipal engagement in waste management. Historically, more than half of plastic waste

that was meant for recycling was exported primarily to China. Since 1992, China has taken cumulatively over 45% of global plastic waste produced. It is estimated that 111 million Mt of plastic waste will be misplaced as a result of China instating an import ban on non-industrial plastic waste in 2017. During a review, it became apparent that higher-income countries were exporting up to 70% of plastic waste in 2016 to lower-income countries in East Asia and the Pacific. The problem of mismanaged waste may not be the sole problem of lack of local collection strategies, but a global “change of hands” and who holds the plastic waste at the end of the chain(Brooks et al., 2018). Still however, China, with 260 million tons of uncollected plastic waste contributes to 84% of global plastic pollution. This comes from both poor enforcement of waste management strategies as well as China holding a large proportion of the global population. The government has made efforts in bringing new waste management laws however, there is a big discrepancy between the collection rate in urban and rural areas. For urban areas it is 65% of plastic waste collected through official means, while in rural areas it is 5%. In rural areas the plastic waste it put in wild landfills with no supervision or burned in uncontrolled local fire pits. Pyrolysis and combusting waste are something done in the developed world; however, these uncontrolled and unsupervised open fires pose both a threat for the public health of the people in the surroundings as well as far reaching pollution concerns. The low rates of recycling and the burning of waste are both an indicator of the current economic model where plastic is produced from finite fossil fuel

sources, used and then disposed of immediately. The recovery is too low to be sustainable(O. Horodytska et al., 2019).

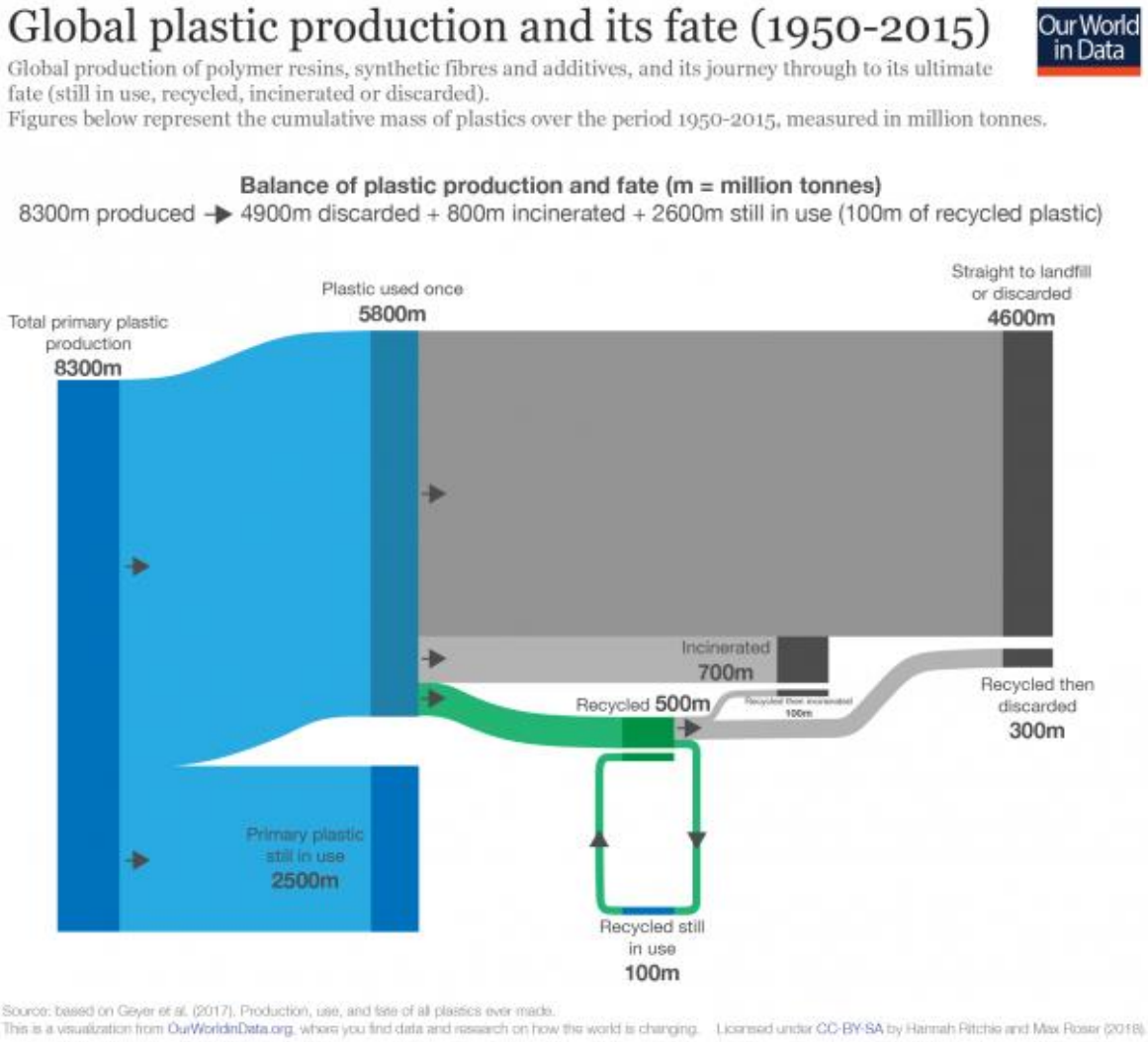


Figure 4. The flow of plastic from production to waste(Ritchie & Roser, 2020).

As can be seen in the figure above, recycling represents a very small percentage of plastic waste recovered and reintroduced into the system, with only 6% entering the recycling loop.

An example of 2 large recycling plants is the Montello S.p.A, and the MyReplast from NextChem. Montello uses mechanical processing with an input of 300 000 t/y, to produce PET, HDPE flakes in different colours, PP, and LDPE granules and solid-state fuel(Montello S.p.A, 2020). MyReplast uses a proprietary thermo-mechanical system dubbed “Upcycling”, promising a 95% efficiency and a 40 000 t/y production of recycled material at a premium quality, one that can be rivalled by plastic produced from virgin materials(NextChem, 2020).

1.2 Alternative management strategies

An alternative not only for the recycling system but the overall consumer mindset is necessary as well. The linear path of producing, using and then immediately disposing of the plastic is combining well with the widespread mismanaged waste ongoing globally, to produce a global plastic waste crisis. Therefore, an alternative in the economic structure is also an area of import for solving this problem. The new economic model would be of a circular economy, where the used products would be recovered and reintroduced back into the circulation. Recycling is trying to do this very thing, but the rates are too low for the current plastic use to be considered circular. In 2018, the EU adopted a plan for circular economy and plastics were recognised as a priority. A focus on recycling, sustainability and consumer and producer responsibility were identified as key components for the plan to come to fruition(EU (European Union), 2018). However, the EU directive from 2019 considers the responsibility of the producer is more important than the behaviour of the consumer. While the municipal waste management laws are made stricter, the directives made for industry highlight the importance of increasing the proportion of recycled plastic that products need to have. Elimination of unnecessary packaging as well as lowering the amount of fossil-based raw material and instead using more biobased options. This highlighted that the consumer does not bear sole responsibility for the pollution, but industry bears the brunt of the damage, and if guidelines are not followed, they will be appropriately sanctioned. The EU has given clear goals for 2025 and 2030 on how much recycled plastic needs to be included in the materials produced. This equals 25% and 30% respectively for PET bottles produced in that time period, as well as ensuring producers of the materials listed in the directive will bear the cost of collecting, transporting and raising awareness as well as data collection as it pertains to their products(EU (European Union), 2019).

A new system of a circular economy would include developing a new plastic economy, where all levels from sourcing, production, collection and recovery of materials would need to be redesigned. As the use of plastic is so prevalent globally, each country respectively would see economic gain due to elimination of import of virgin materials or already made plastics for their use. As discussed before the supply and production chains have become centralised to a single region of Eastern Asia, and present a potential problem in case of global emergencies. Denmark sees itself as a potential leader in this new circular economy system. A proposed 5 step system to: recycle all plastic waste, minimise or phase out plastic that cannot be recycled, alternatives for plastic currently in use, reduce use of plastic that goes directly into the waste

stream and help create policies that can be implemented in the EU and globally as the plastic problem is a global problem. The primary goals would focus on research and development of both recycling and recovery strategies(Høngaard Andersen et al., 2019).

Another alternative is in terms of the plastic production itself. Constructing new polymers that are biodegradable, use no or less fossil fuels for production but perform the same way as conventionally used plastic.

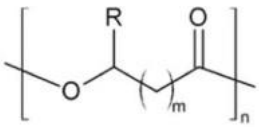
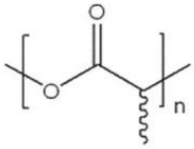
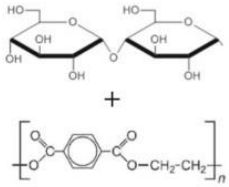
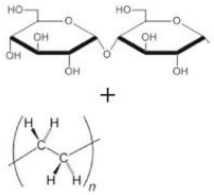
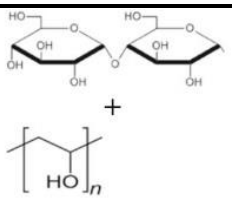
Polymer	Monomer(s)	Structure	Uses
PHA	Hydroxy fatty acids		Packaging, paints, single use medical devices
PLA	Lactic acid		Packaging, coating
Starch/polyester	Starch; dicarboxylic acid; diol		Fibres
Starch/PE	Starch; ethylene		Highly biodegradable
Starch/PVA	Starch; vinyl alcohol		Packaging, agriculture

Table 3. Biobased plastic alternatives(Ahmed et al., 2018)

However, biobased plastics are not necessarily biodegradable. The addition of starch to existing fossil based polymers makes the final product more readily biodegradable(Ahmed et al., 2018).

1.2.1 Biodegradation

Biodegradation as a waste treatment strategy has become more popular in the last decade as more knowledge is gained about the systems required for the degradation of various polymers. However, the wide array of polymers available and disposed of, in addition to the various additives to the plastic which make it resistant to microbial attacks, have made it so a very specific set of enzymes are needed to be able to attack its structure. It has only been 6 decades since the global spread of mismanaged waste leaking into the environment, giving a short time span in which, these enzymes could have evolved. Even in this short time span, with the presence of microplastics which are already partially degraded due to UV degradation, becoming more available to these enzymatic systems, and several species, both fungal and bacterial, have been identified to be able to degrade a wide array of plastic polymers. There enzymes work on a very simple principle of degrading the polymer into mono- or dimers which are then uptaken by the cell and metabolised. However, due to the resistant nature of these polymers: hydrophobicity, strong C-C bonds, high molecular weight,

anti-fungal, and anti-microbial additives; biodegradation is a very lengthy process(Danso et al., 2019).

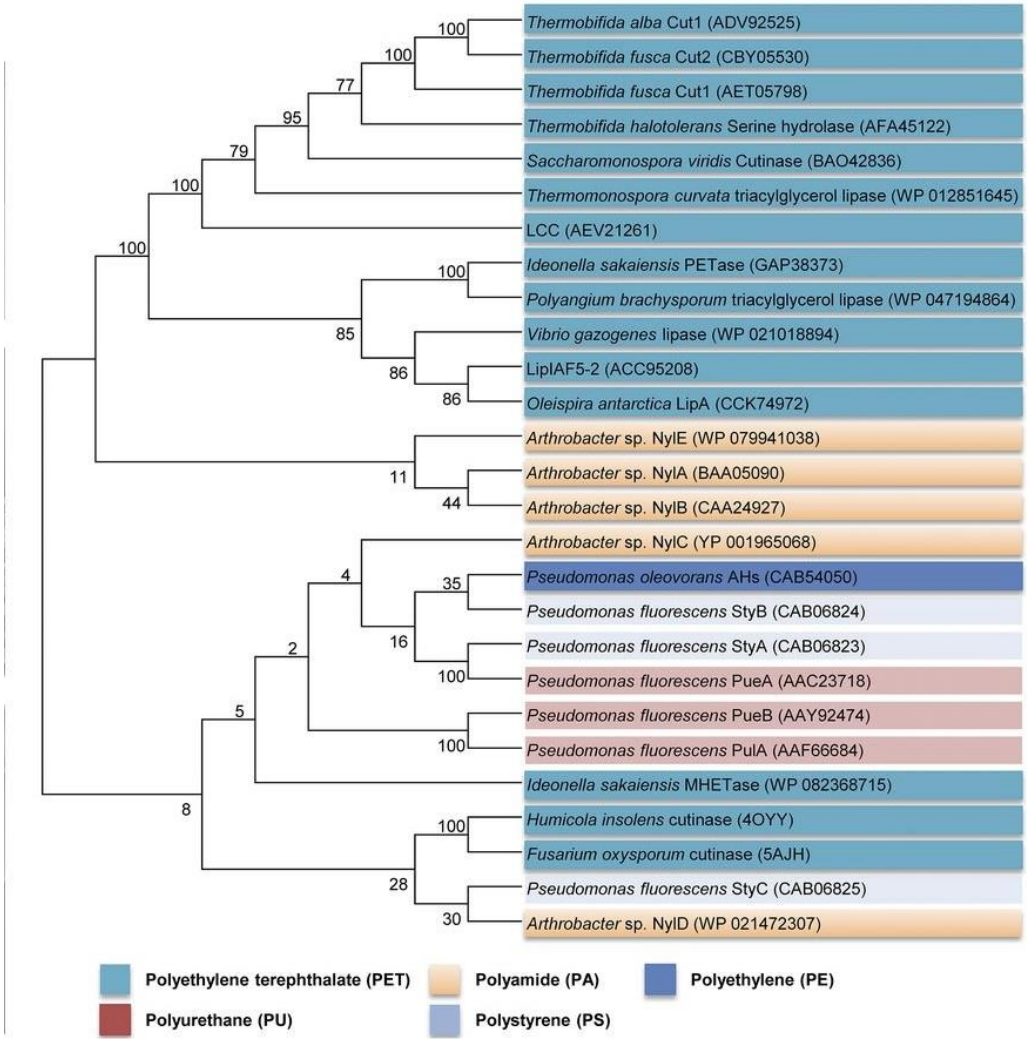


Figure 5. Neighbour-joining tree of the majority of currently known synthetic polymer degrading enzymes(Danso et al., 2019).

As shown in the figure above, the majority of currently known plastic degrading enzymes are for PET. Most of the enzymes discovered so far are highly specialised to the backbone of the polymer they degrade. And even then, the degradation rate itself is quite low. This is prescribed to the crystallinity of the plastic itself. The higher the crystallinity the lower the degradation rate. Currently, biodegradation is presented as a future perspective of what waste management will look like, as the technology for it is very limited to discovering the enzymes themselves and trying to optimise them. As well as searching for enzymes that will degrade a variety of polymers instead of using one for each type of plastic. The focus of the waste management is not only on recovering the recently used materials but the waste currently

polluting the oceans and rivers. And these are all mixed sources of plastic which over time have degraded into microplastics and would be not cost or time effective to separate.

1.2.2 Commonly used strains

As can be seen in Figure 5. there are many bacterial and fungal strains already identified as having synthetic polymer degrading enzymes. The most prominent are cutinases from *Thermobifida fusca* and *Fusarium solani*(Eberl et al., 2009). *F. solani* has previously shown the ability to hydrolise PET into its monomers using the cutinase(FsC)(Silva et al., 2005). And in the same year, a cutinase(TfH) was discovered in *T. fusca*(Müller et al., 2005). Both TfH and FsC are opportunistic in terms of attacking the amorphous segments of PET, and not the highly crystalline area. Addition of plasticisers seems to add to the degradation ability of these enzymes, as they reduce glass-transition temperature and contribute to greater chain mobility(Eberl et al., 2009). TfCut2 is thermo stable, and able to operate at 65ºC, which is near the PET glass-transition point, making it more available for degradation(Wei et al., 2016). FsC has show degradation of PUR and polycaprolactone, even seen as the dominant species residing on PUR surface(Pathak & Navneet, 2017). The cutinase from *Humicola insolens* (HiC) which has also shown to be able to degrade low crystallinity PET, and a recovery of 97% pure terephthalic acid was observed(Quartinello et al., 2017). HiC also operates at 65ºC, and has shown greater degradation of powder that has smaller particle size(Simone Weinberger et al., 2017). Leaf and branch compost cutinase (LCC) has shown particularly high PET hydrolysis activity, and consistently shows higher performance than the previously mentioned enzymes in terms of PET degradation. The native organism is not known but it shares 46% identity with TfC in *Thermobifida cellulosilytica*. It reportedly maintains 85% of function at 75ºC for 60 minutes. Due to its ability to reach temperatures where PET is at glass-transition makes it highly efficient for hydrolysis. It is able to reach ~95% weight loss of a $1cm^2$ sheet of low crystallinity PET(Shirke et al., 2018). It seems that across research, the high work temperature of enzymes, that contributes to increasing the mobile amorphous fraction of PET, and therefore increasing the hydrolysis susceptibility is one of the most important factors in PET degradation. However, the aging process of PET caused by high temperatures creates micro-structures which show resistance to enzymatic degradation. A mitigation strategy for this would include a catalyst added to the reaction which would facilitate degradation before the formation of inaccessible components(Wei et al., 2019)

1.3 *Ideonella sakaiensis*

I. sakaiensis is a gram-negative, aerobe; discovered in 2016, while screening for PET degrading organisms. It was isolated as a bacterium that solely was thriving on the degradation on PET films.

I. sakaiensis is a rod-shaped bacterium which does not form spores. The taxonomy of the species is as follows: class *Betaproteobacteria*, family *Comamonadaceae*, genus *Ideonella*. It was isolated from a soil sample taken at a plastic waste landfill. The family *Comamonadaceae* members share a place in the phylogenetic cluster of 16S rRNA phylogeny of *Betaproteobacteria.* They can be found in various natural habitats as well as those man-made and do not discriminate between polluted or clean areas such as: soil, fresh and groundwater, activated sludge and industrial processing water. Due to the wide variety of habitats, the individual features of the genera both morphological and physiological, vary greatly within the species themselves as well(Willems, 2014). Other members of the *Ideonella* genus include *I. dechloratans, I. azotijigens* and *I. paludis*(Sheu et al., 2016). The genus was first described in 1994, with a discovery of *I. dechloratans* isolated form an activated sludge sample from a municipal treatment plant in Malmö, Sweden(Malmqvist et al., 1994). With dimensions: 0.6-0.8 x 1.2-1.5Øμm. It has a polar flagellum with which in the early stages of life it moves in the substrate quite readily. As the bacterium matures into a biofilm it stops the rapid movement. When grown on solid NBRC 802 media at 30ºC, the colonies produced are raised, translucent and have an entire margin. It was tested for optimal temperature and pH which are 30-37ºC and pH 7-7.5. It is an aerobic bacterium which does not respond positively or negatively to light and does not possess any visible pigments. It is positive for maltose and

adipic acid assimilation. It is negative for denitrification, nitrate reduction, indole formation, glucose fermentation, β-galactosidase and d-glucose assimilation(Tanasupawat et al., 2016).

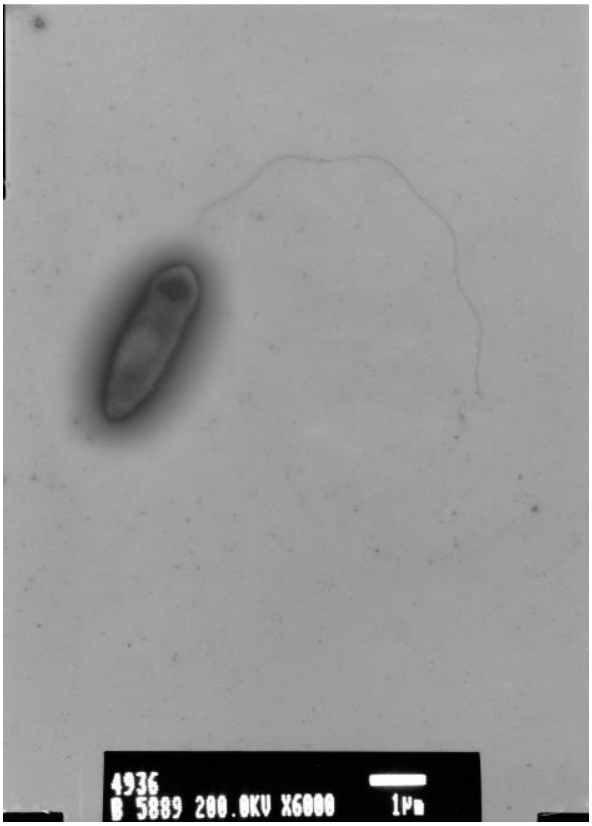


Figure 6. TEM picture of a single *I. sakaiensis* cell(Tanasupawat et al., 2016).

1.3.1 *I. sakaiensis* PETase

I. sakaiensis adheres to PET using specialised appendages and proceeds to establish a biofilm upon contact. It excretes an enzyme which seemingly degrades the plastic. No detectable mono- or dimers of the PET are present in the surrounding media where the bacteria is grown indicate that the enzymatic system completely breaks down the plastic into monomers which are taken up by the cell and completely metabolised into CO_2 which is then released into its aerobic environment. SEM pictures of the PET extracted from degradation experiments show

pitting on the plastic surface where it was degraded. The proposed mechanism of the enzymatic system is as following.

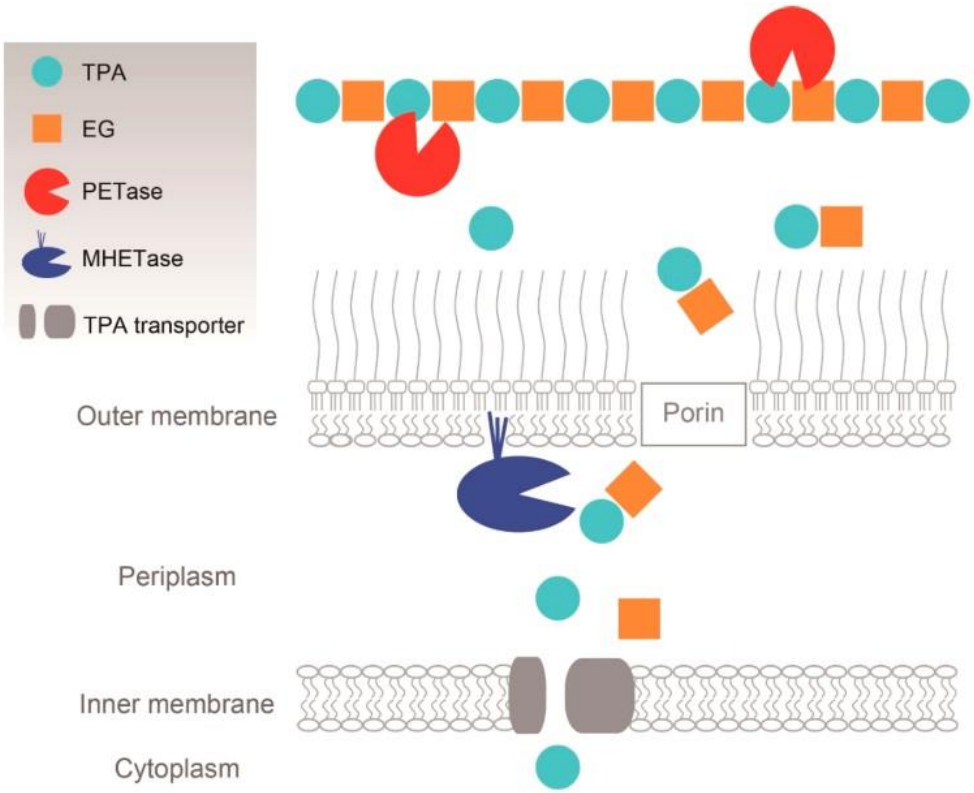


Figure 7. Proposed mechanism of PET degradation by *I. sakaiensis* PET hydrolase(Taniguchi et al., 2019).

PETase hydrolyses the PET backbone, breaking the ester bonds and producing the monomer, terephthalic acid (TPA) and a mono(2-hydroxyethyl) terephthalate (MHET) dimer. These are transported into the periplasm where the additional membrane bound enzyme MHETase further breaks down MHET into TPA and ethylene glycol (EG) which are the 2 PET monomers. The TPA is taken up through a specific TPA transporter into the cytoplasm and metabolised. TPA is uptaken as protocatechuic acid into the tricarboxylic acid(TCA) cycle, and EG as glyoxylic acid(Taniguchi et al., 2019). The hydrolase also initially produces a small amount of bis(2-hydroxyethyl) terephthalate (BHET) which is then further hydrolysed into MHET. The enzyme was tested against the TfH and FsC cutinases, which were already known enzymes capable of degrading PET, in terms of its ability to hydrolyse p-nitrophenol-linked aliphatic esters. It shows a much higher affinity for PET as opposed to the other enzymes which show more affinity for pNP aliphatic esters, leading it to be dubbed a PET

hydrolase. In this way it presents a better option for PET degradation as it shows a better affinity(Yoshida et al., 2016a).

In the scope of PET degradation by *I. sakakiensis,* the importance of MHETase cannot be lessened. Systems that use the whole organism must take into account PETase and MHETase as subsequent enzymes that work together to depolymerise PET to its monomers TPA and EG. The interaction between the two opens avenues of research into creating new bioplastic that will be more susceptible to these enzymatic systems, and produce materials that can be put into a recycling system that will be highly efficient(Palm et al., 2019).

Studies of the structure of the protein have shown that it is not completely optimised for degradation of crystalline PET. It has shown to be effective at degradation of polyethylene-2,5-furandicarboxylate (PEF) which has emerged to the market as an attractive PET substitution. This further opens venues for protein engineering of PETase as it pertains to its structure and activity of the active site(Austin et al., 2018). However, PETase is heat-labile and the direction of research currently is into changing the structure of the enzyme to make it thermo-stabile at higher temperatures. The current working temperature is between 30-37ºC(Taniguchi et al., 2019).

Newly published research compared the efficiency of the afore mentioned commonly used cutinases TfF, LCC and IsPETase. LCC proved to have the highest degradation efficiency with being reportedly 33 times more efficient. It was selected as the enzyme for modification. Improving thermal stability was one of the main factors investigated. The resulting mutant ICCG was optimised to have a 90% conversion depolymerisation after 10 hours of post-consumer coloured PET. It was also show that micronisation of the samples and making the surface area greater, contributed to the higher degradation rate. Resulting monomers could be recovered at a 99.8% purity making them suitable for use of producing virgin PET. This presents a closed loop in a circular economy system(Tournier et al., 2020).

Carbios, the company behind the ground-breaking research, is partnered with industry leaders in PET bottle manufacturing and usage: Nestlé Waters, PepsiCo and Suntory Beverage & Food Europe. With the new proprietary enzyme, and a deal with Novozymes A/S for enzyme production, it is scheduled to open a large-scale industrial recycling plant by 2024/2025(Carbios, 2019).

1.4 Biochemical characterisation

The foundation of biochemical characterisation is establishing how a species behaves similarly or dissimilarly to another one in terms of its metabolism. As methods for identification skew more towards 16S rRNA molecular biology techniques, the biochemical aspect becomes more of a feature for looking into how similar species evolved towards or away from each other in terms of their unique features. At this point in time, with numerous species discovered, based on their initial molecular identification, certain biochemical tests are singled out to be performed as they are relevant. An enteric bacterium is more likely to be tested for gelatine hydrolysis than a staphylococcus. It is a matter of previously acquired knowledge, as to which tests will be performed in addition to any tests of interest based on where the bacteria were isolated(Cowan et al., 2004). As was the case with *I. sakaiensis* where a PET hydrolysis test was of importance for it.

Commercial kits are available were specific biochemical tests were selected as being relevant to determining the specific species or generally determining certain metabolic features. These range from testing the assimilation of certain carbon sources, presence of certain enzymes, proteins and amino acids etc. These tests give very simple: positive, negative, or weakly positive or negative results. They are not only for identification of novel bacteria but also quick tests in hospital or industry settings to see if an organism of interest is present. In a hospital setting these are used to quickly give a direction as to sterilisation and treatment needed to be performed. And specialised tests for common bacteria that cause infection or are contaminating production lines in for example the food industry(Betts, 2014).

1.4.1 Testing culture conditions

While biochemical characterisation yields the data on which condition a microorganism grows in, and how it behaves in regards to its environment, to use this organism in a laboratory or industry context, it is necessary to determine the optimal conditions under which it operates. Initially tested, an organism may display a range of temperatures and pH under which it will grow, however, if it is a bacteria or fungi that will be used for production or degradation of a substance, it is imperative to establish an optimal growth media environment(Cowan et al., 2004).

Culture conditions which are tested most frequently are: type of growth media, incubation temperature and time, pH, salinity and agitation. These are first tested under a standard media

which the organism prefers for growth, from which the temperature, pH, salinity and agitation can be tested. If a bacterium is needed for a specific purpose which can only be accomplished with a very specific media, then tests can be made in parallel to see its response to multiple variable change at the same time. For example, in case of *I. sakaiensis* where the degradation of PET is tested, and PET is offered as a sole carbon source, a less nutritive media is necessary as a starting point to insure the bacteria will not opt for a more easily accessible carbon source available in a nutritive media such as NBRC 982. A high throughput method is necessary in order to be able to test many variables changed at the same time. Using a microplate reader is a faster way of testing many variables simultaneously.

An end goal of testing culture condition for any purpose would be getting a stable biomass which can reliably be replicated over and over again. A starting point for any experimental purpose, where an organism can be grown in a substantial amount in an expedient manner. After which, it has defined parameters for growth, and any future use of it can begin with these variables already recorded.

If, in the future, it is used for production, or degradation, how will the pH naturally change due to something being consumed and a product being made needs to be taken into account. This will give insight if a buffer is needed as a base for the growth media. Temperature will not only affect the organism but the products as well, so if the bacteria may be a thermophile and offer higher growth rates at hotter temperatures, there could be a risk of product degradation. These become considerations from which the optimal conditions for its growth can be used as a starting point and expanded upon.

1.5 COVID-19 impact on global plastic consumption

Following global lockdowns and complete staggering of international travel starting February 2020 due to a Covid-19 outbreak, a major industry in particular experienced an oversupply and drastic decrease in demand; the crude oil industry. On April 20th 2020, the Western Texas Intermediate (WTI) price for oil fell to -$36,98. This concerns low density crude oil-based commodity for future stock contracts. This comes after Saudi Arabia and Russia increased production to outcompete their market prices(van der Made, 2020a).

Meanwhile, personal protective equipment (PPE) became instrumental in the fight against the pandemic. Masks, gowns, facial shields made primarily of PP, PE and PET fibres are used as

single-use plastics. Italy alone is predicted to go through 1 billion face masks, and 500 million gloves each month during the lockdown. Face masks were not covered in the EU single-use plastic directive, and therefore need to be monitored with increased scrutiny so they are disposed of correctly(Montalto Monella, 2020).

Increased demand for PPE has led to the US Environmental Protection Agency (EPA) to loosen their environmental protection regulations by issuing an enforced discretion policy. The plastics industry has been exploiting the crisis both by calling for lowering of routine monitoring, and using historically low oil prices for production of virgin plastic(van der Made, 2020b). There is concern over reverting to old habits before the education of decreasing single-use plastic started becoming effective. An additional worry is that the plastic used during the crisis is inappropriate for recycling and therefore will need to be burned in sterile conditions to not risk infection. This also being coupled with many recycling operations being disrupted by the pandemic and lines being stopped. In case of recycling of PPE, the recycling plants would have to apply appropriate sanitation standards to insure no cross contamination will occur both between workers and onto the material produced(Sahni, 2020). In times before the pandemic, 85% of all medical waste was incinerated even as only 15% is considered a biohazard. The reason is identified as a lack of in-house segregation of plastic waste. Inappropriate disposal of injection devices led to 33 800 HIV, 1,7 million hepatitis B and 315 000 hepatitis C infections in 2010. A proposal for reducing the waste is education of staff in reducing plastic use where possible and awareness of disposal, as well as developing a comprehensive waste segregation(World Health Organisation (WHO), 2018).

The Covid-19 pandemic has overall lead to a decrease of the plastic additives industry profits due to lowering of industry demand for certain products. However, the industry expects a full recovery in the medium to long term future. New applications are considered for additives, with plasticizers having the most substantial value-creation impact(Research and Markets, 2020).

One of the greatest revelations of the global pandemic was how vulnerable the oil industry was, even with the global demand of PPE, it is expected that governments will start passing stricter and stricter sustainability laws, not only on national but international basis as well. The shift to renewable energy, and renewable materials, as well as research into recovery of material trapped in waste, may lead to the peak demand of oil arriving a lot sooner than predictions have been before(Sheppard, 2020).

1.6 Research questions and project aims

The research questions for this project are as follows:

- What media conditions are necessary for activation of *I. sakaiensis* overnight to reach an OD greater than 1?
- What is the most reliable and consistently reproducible method for producing PET powder in a small-scale laboratory setting?
- What are the methods for separating PET powder from biofilm, for comprehensive degradation quantification?

The aim of this project was to find an optimum media for activating *I. sakaiensis* which will produce a uniform biomass each time it was used, and the inoculum for each experiment can be reliable and easily reproducible. The media would be modified and tested in terms of its effect on plastic degradation. In the confines of this project it was PET as it is the preferred plastic for this organism and it has the enzymes necessary for degradation. A method for easy PET powder production would be established and a system for sieving the powder into different size grades. These sizes would represent an additional variable that would change in the plastic degradation experiments. A unified method for quantifying the degradation would also be established starting from measuring the biomass, the total protein content, the enzymatic activity, change in plastic weight, and finally the presence of monomers in the culture media. This way it could be comprehensively tracked how the degradation happens and at what rate.

2 Materials and Methods

The project was originally planned to focus very specifically to the development of an optimal growth media for *I. sakaiensis* and the to continue to a large variety of experiment which would test the culture conditions that would then measure how the degradation rate would behave. However, due to some complications with a contaminated cryo stock of *I. sakaiensis* which was noticed within the first week of the start of the project; the following methods pertain more to the isolation of *I. sakaiensis* from the contaminant and subsequent testing of different solid and liquid media for this purpose. As well as creating a method for producing PET powder in-house.

2.1 Microbial strains

- *Ideonella sakaiensis* 201- F6, purchased previously (cryo stock contaminated); BSL 1
- *Ideonella sakaiensis* 201- F6, newly purchased from BCRC; BSL 1

2.2 Culture activation

Both batches of *Ideonella* were activated using the recommended NBRC 982 media(BCRC, n.d.).

Hipolypeptone	10 g
Yeast extract	2 g
$MgSO_4 \cdot 7H_2O$	1 g
Agar (for solid media)	15 g
Distilled water	1 L

Table 4. NBRC 982 activation media recipe

Narrow neck, baffled, 250 mL glass Falcon flasks were capped with cellulose stoppers and autoclaved at 121ºC for 20 minutes. The media was autoclaved in the same way and 25 mL pipetted into the flask. This was done in a laminar flow hood to insure sterility. Scrapes of the cryo culture were taken with a sterile spatula and inserted into the vessel. The flasks were placed in a shaking incubator set at 30ºC, 150 rpm. These were tested as necessary.

2.3 Solid and liquid media

Previously stated, the *I. sakaiensis* arrived with a preferred activation media. The isolation and optical density experiments utilised a variety of liquid and solid media with a range of available nutrients.

2.3.1 LB media

LB media was used as a starting widely-used media with a high nutrition(*LB Broth Media Recipe*, 2013).

Bacto-tryptone	10 g
Yeast extract	5 g
NaCl	10 g
NaOH	For adjusting to pH 7
Distilled water	1 L

Table 5. LB media recipe

2.3.2 YSV media

YSV media was used as a media during the degradation experiments in (Yoshida et al., 2016a). The media is made in a 10 mM, pH7, phosphate buffer. With an addition of 0.1% vitamin mixture and 1% trace elements.

Yeast extract	0.01 %
Sodium hydrogen carbonate	0.02 %
Ammonium sulfate	0.1%
Calcium carbonate	0.01%

Table 6. Base YSV media recipe

The base YSV media is mixed and autoclaved at 120ºC for 20 min. The vitamin and trace element mixtures are filter sterilised and added to the media in a laminar flow bench to insure sterile conditions.

Thiamine- HCl	0.25%
Biotin	0.005%
Vitamin B12	0.05%

Table 7. Vitamin mixture for YSV media

$FeSO_4 \cdot 7H_2O$	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
$CuSO_4 \cdot 5H_2O$	0.01%
$MnSO_4 \cdot 5H_2O$	0.01%
$ZnSO_4 \cdot 7H_2O$	0.01%

Table 8. Trace elements for YSV

2.3.3 M9 and M9+ media

M9 is classified as a minimal media as it has a very low amount of carbon sources required for bacterial growth(*M9 Minimal Media Recipe (1000 Ml)*, 2013). In this way it is a very good media for selecting which carbon source will be selectively fed to the microorganism in question. M9+ is the original M9 recipe with an addition of the trace element and vitamin mixture from the YSV media. It provides a bridge between a low nutritive media, and a minimal nutrition media.

Na_2HPO_4-$7H_2O$	64 g
KH_2PO_4	15 g
NaCl	2.5 g
NH_4Cl	5 g
Distilled water	1 L

Table 9. M9 salts solution

The M9 salts solution is autoclaved at 120ºC for 20 min before being added into the M9 media.

M9 salts solution	200 mL
Sterile 1M $MgSO_4$	2 mL
Sterile 1M $CaCl_2$	100 µL
Sterile distilled water	Adjust to 1000 mL

Table 10. Assembling M9 media

For the M9+ media, the 1% trace element and 0.1% vitamin mixture are added after autoclaving in addition to all the other constituents of the M9 media.

In order to create solid media of all the listed above, 1.5% of agar was added to each media respectively. These served as parallel tests to see how *I. sakaiensis* responded to solid versus liquid media.

2.4 Purifying and verifying the species

With the discovery of the contaminated cryo stock of *I. sakaiensis*, several efforts were made to separate these contaminants and obtain a pure culture at the end. This was done by activating the culture on both liquid and solid NBRC media; directly from cryo stocks, and from several cultivated liquid cultures. The resulting single colonies were streaked and re-streaked every 24 hours in order to obtain single colonies for 16S rRNA sequencing. This was done to help selection of selective media and confirmation that *I. sakaiensis* was still present in the mixed culture and could ultimately be isolated. The plan for the isolation followed 3 lines of experiments: streaking from cryo directly onto agar plates, using PET film and YSV media to try and isolate *Ideonella,* and using PET powder and M9 media created in the lab to try and isolate *Ideonella.*

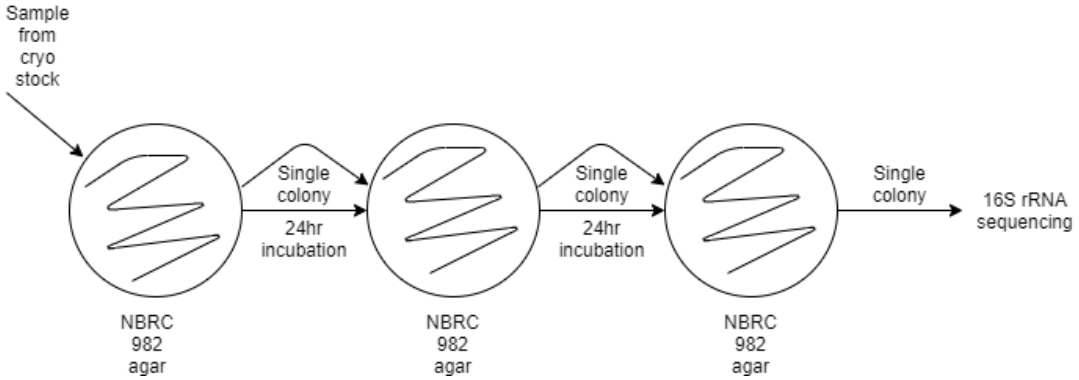


Figure 8. Experimental plan for isolating using streaking plates

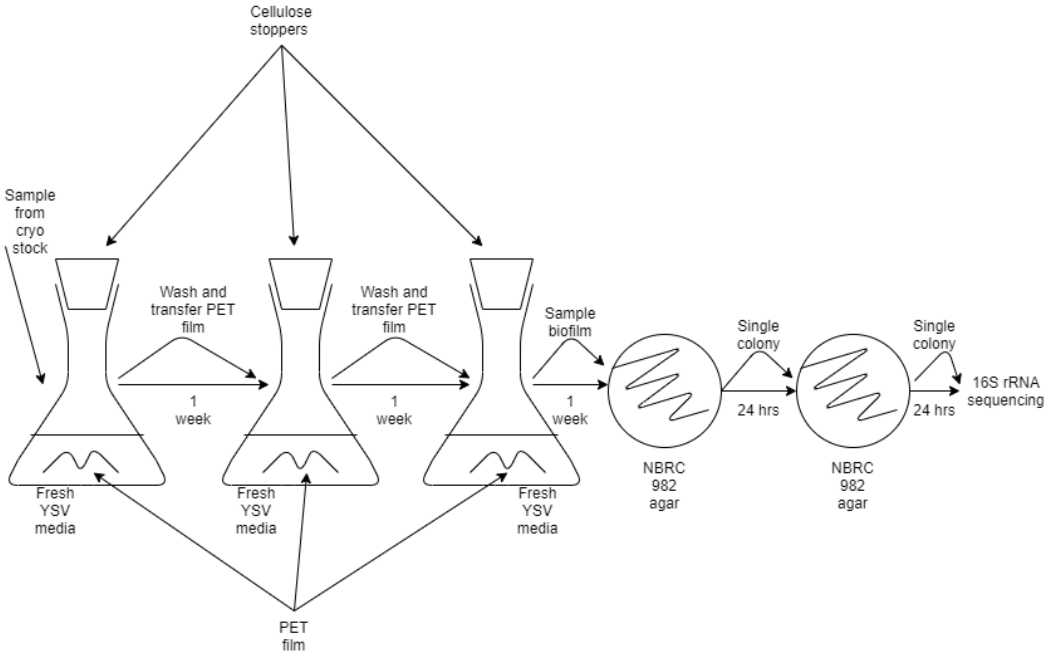


Figure 9. Experimental plan for isolating using YSV media and PET film

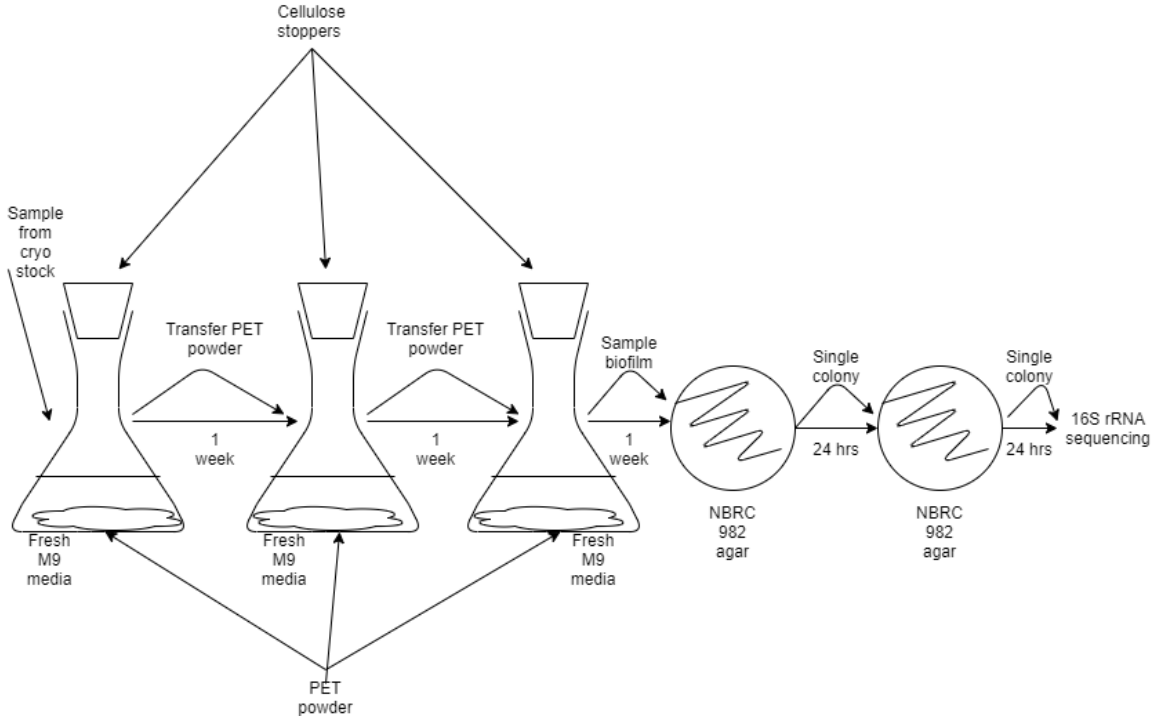


Figure 10. Experimental plan for isolating using PET powder produced

2.4.1 Solid media colony isolation

NBRC 982 solid media was created and poured in 10 mL amounts into sterile 90mmØ plastic Petri dishes. Cryo stock or liquid culture was picked up with a sterile loop and streaked onto the plate. The plates were incubated at 30ºC in a standing incubator. This was repeated 3-5 times, every 24 hours. Each time a single colony was selected to ensure the final plate would have a pure culture.

A modified MacConkey agar was used later on, in order to discourage the growth of gram-positive bacteria, as *I. sakaiensis* are a gram-negative bacterium. In the same proportion as the NBRC 982 and same incubation temperature.

Peptone	17g
Proteose peptone	3g
Bile salts	1.5g
NaCl	5g
Crystal violet	0.001g
Agar	15g
Distilled water	1L

Table 11. Modified MacConkey agar

2.4.2 Verifying the species

When consistent single colonies were obtained on the solid media, they were sampled, PCR was performed, and they were washed and send for 16S rRNA sequencing. Colony PCR is performed according to the Mix2Seq Overnight kit from Eurofins, and instructions followed according to manufacturing guidelines. The PCR product can then be run through a gel electrophoresis to check the size of fragments obtained. Then the product is washed of any salts in the reaction, quantified using a nanodrop and appropriately diluted to fit the sequencing kit specifications. The results are then checked in Benchling to then identify the specie of microorganism growing on the plate. These results further influenced media selection.

2.5 Creating PET powder

Mechanical processing of PET to produce powder does not only affect the final crystallinity but the state of PET that is reformed with the powder. Cryomilling, the process of milling the PET under cold temperatures, has shown to produce highly amorphous powder(Zhu et al., 2006).

While the isolation experiments were being conducted, a protocol for creating PET powder was created. There were several forms of PET available: highly crystalline sheets of PET (~50%), soft drink PET bottles (~14-30%), and PET pellets supplied by BioMi with an unknown crystallinity.

The PET film and bottles were cut in $1cm^2$ squares, while the pellets were around the same size as well. As PET becomes more brittle at lower temperatures, samples of each plastic were put in a freezer bag and submerged in liquid nitrogen for 10 minutes. The external plastic bag ensured the PET pieces would not develop condensation while they were transferred to the milling machine. As an increase in moisture would prevent a proper milling.

Preliminary tests were done in a high-powered food blender, and a FRITSCH® Pulverisette 6 planetary ball mill. The latter experiments were conducted in a FRITSCH® Pulverisette 14, with a 12-rib rotor, and a 0.5 mm sieve. For the film and bottle fragments at a speed of 10k rpm, and for the pellets 6-8k rpm. This is due to the films being flat and if the rotor speed is slower, the particles catch between the rotor and sieve and turn into liquid, therefore jamming the mechanism.

The collection of the particles is done with a stiff bristle brush, and it is mandatory to wear a facemask to protect from inhaling the plastic dust. These powders may then be sorted in size categories or kept as a singular multi-granule size powder. For separating it into different sizes, steel mesh of several sizes was used: 2000 mm, 1000mm, 500 μm, 250 μm and 50 μm. The separation process is tedious and long if done by hand and not with automated shaking sieves. For this project it was done by hand. The powders are then labelled and stored individually.

2.6 Measuring OD_{600}

In order to optimise the time and amount of media that can be tested at one time, OD_{600} experiments were conducting using a BioTek® Epoch 2 microplate reader.

This is an indirect way of determining cell growth and from OD an approximate cell count can be determined. For accurate reading, it is important to either measure dry cell weight of the sample, or determine the colony-forming units (CFU) from agar plates.

This was not performed due to time constraints once the pure organism was obtained, but it can be calculating by diluting OD readings to establish a correct base reading, plating the sample and count the colonies formed.

2.6.1 Preparing the microplate

96-well microplates were used for this experiment. The working volume for each well was 200 μL. Each media was pipetted as a blank in triplicates, as well as the samples with *I. sakaiensis.* The inoculum size was 10% or 20 μL. A culture was preactivated 24 hours in advance in an NBRC 982 media in a 250 mL narrow necked, baffled Falcon flask. The OD_{600} was measured in a standard 1 mL UV/VIS spectrophotometer cuvette to check the optical density and determine how many cells were contained in the media. This sample was then diluted so the starting OD_{600} inside the microplate well would be around 0.1. As the media used for experiments was lower in nutrients than the activation media, the samples of the inoculum had to be washed.

This was done by taking 500 μL of the activation broth with grown cells and washing it 3 times with each corresponding media. The activation broth was put in a sterile 1.5 mL

Eppendorf tube and centrifuged at 4000 rpm for 5 minutes. The supernatant was taken out, and the pellet was resuspended in the corresponding media. This was repeated 3 times to ensure no leftover nutrients would interfere with the reading.

Appropriate amounts of media and inoculum were then pipetted into the microplate wells and the plate was covered with a plastic film that was optically compatible with the reader.

2.6.2 Microplate reader

The microplate reader was set in the following specifications.

Light wavelength	600 nm
Temperature	30ºC
Shaking	Continuous orbital
Shaking speed	180 cpm (3mm)
Experiment duration	24 hours
Reading frequency	15 min intervals

Table 12. Microplate reader experiment settings

The data was generated after 24 hours and transferred into Excel for processing.

2.7 PET degradation experiments

Two PET degradation experiments were conducted. The experimental setup from (Yoshida et al., 2016a) was used to test several consortia for their PET film degradation ability. Strips of PET bottles were put inside a test tube with a 10 mL working volume of YSV media. These were sterilised and capped with cotton plugs and shaken in an incubator at 30ºC, 300 rpm. The PET strips were pre-cut to fit the vessels, weighed, and sterilised with 96% ethanol. The ethanol was left to evaporate and they were then washed with sterile water and left to dry before being inserted into the vessels. Samples of microorganisms were taken from biofilms and added into the test tubes. The test was conducted in triplicates. The media was added as necessary each week; this equated to 500 µL of fresh YSV added each week. The strips were taken out after 3 weeks and measured gravimetrically.

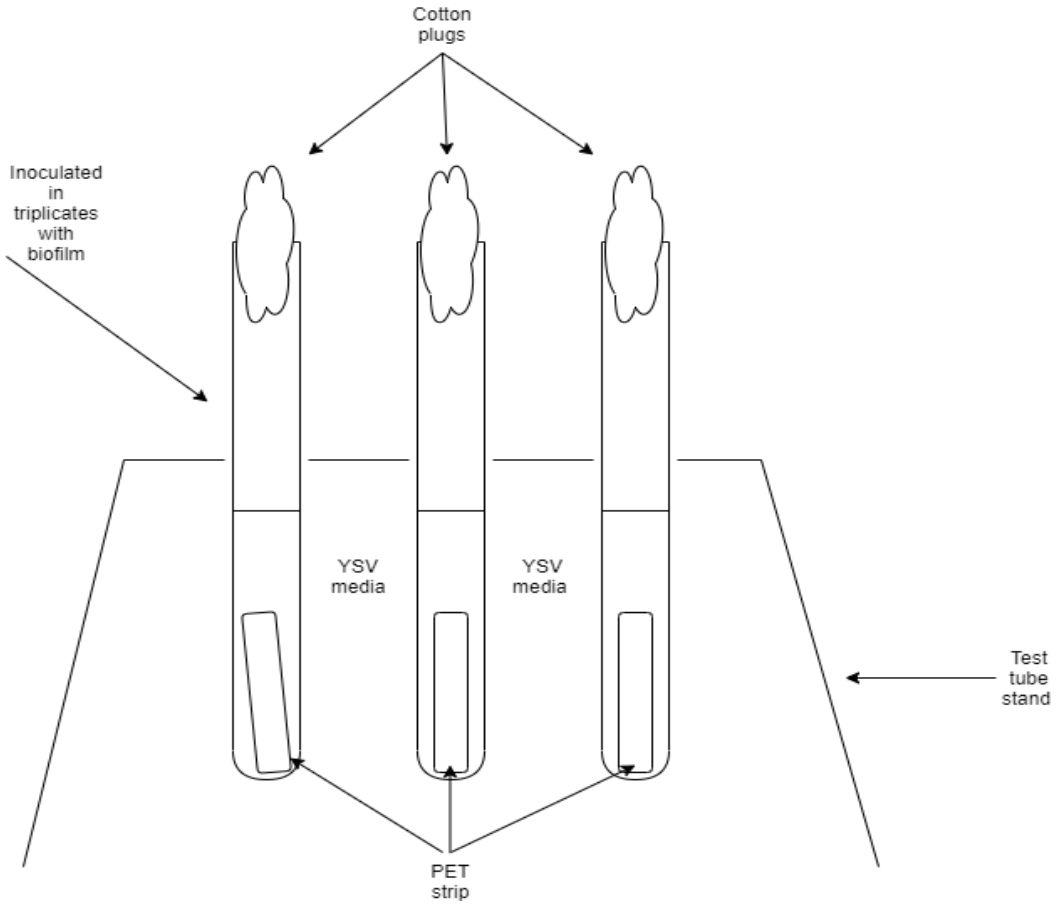


Figure 11. Experimental set up of PET degradation experiments

2.7.1 Quantifying degradation

Gravimetric quantification of the PET film and bottle strips were done straightforward with a measuring boat and an analytic scale with an error of ±0.0005g. Using PET powder presented an additional step of processing before weighing, separating it from the biomass. As well as doing protein quantification, enzymatic activity quantification and testing solvents for PET powder separation.

The protein quantification was done to compare with an enzyme activity and along with gravimetric measuring would give a full picture of the PET degradation. A Pierce™ BCA Protein Assay Kit was used to conduct the protein quantification. The protocol given by the manufacturer was used in the microplate variation to use smaller sample sizes.

For enzymatic activity assay, the preliminary tests were done using the protocol in (Salgado et al., 2019), with the following specifications.

Sodium phosphate buffer	50mM
pNPB	2.63mM
Triton X-100	4% (v/v)
pH	6.8

Table 13. Enzymatic activity assay buffer composition

The assay was conducted in microplates. The microplate was placed on an ice bed in a Styrofoam box to prevent the reaction from taking place. The volume in each well was split up as: 195µL of the assay buffer, with 5µL of the sample added. The microplate was covered and incubated for 15 minutes at 37ºC, and once the time elapsed, returned on ice and taken to the microplate reader to be read at 405nm. The pNPB starts degrading at room temperature, therefore it needed to be kept on ice. The assay buffer was only made in the quantity necessary for each experiment as it does not keep well after several days even at -20ºC. This allows the timeframe of 15 minutes for incubation to be more accurate and therefore the results more accurate. Alternatively, to be 100% certain the reaction is stopped, after the incubation 100µL of pure acetone is added to the samples. However, due to the use of plastic microplates, this resulted in the acetone reacting with the wells and making the mixture cloudy, invalidating results. Later it was decided a new method was to be used but before testing it time ran out.

2.7.2 Solvent experiments

Using PET powder in experiments with whole cells presented the issue of separating it properly. Preliminary tests with chloroform as a solvent were conducted to test how the powder could be dissolved and separated from the biomass and filtered. This would present a twofold gravimetric quantification of both the biomass and the powder. A modified method of (Bengtsson et al., 2010) was used. 50mg of PET powder were placed in glass Pyrex tubes along with 5mL of chloroform, which were capped with Teflon lined caps. These were then warmed in a glass of boiling water for 1 hour, with vortexing every 15 minutes for 10 seconds. The mixture was then vacuum filtered using a 0.45µm Teflon filters. This method was later deemed inappropriate as it did not comply with sustainability efforts of the project as it used a strong organic solvent.

3 Results and discussion

Contamination of the cryo stock of *I. sakaiensis* was noticed within the first 2 weeks of the project. This stalled furthering the plans for the project as the following experiments focused more on trying to resolve the contamination and re-obtain a pure culture which can then be propagated and stored adequately. Preliminary results that pointed to the contamination included: a much faster growth of liquid culture than is reported in literature(Yoshida et al., 2016c), the colonies on solid media did not match up to known colony morphology, and when put under the microscope, it was very clear there was a mixture of several organisms in the media. NBRC 982 is a highly nutritive media, so a degree of human error was assumed at the beginning. However, after several activation attempts where negative and positive controls proved to be in line as they were supposed to react, the contamination continued to emerge. LB and M9 media were also used to try and eliminate as many contaminants as possible but these efforts proved unsuccessful. The biggest indicator at this stage of experiments was microscopy of liquid and solid media samples. The samples had several sizes of rods which were not consistent with *I. sakaiensis* as well as unidentified cocci present in samples.

There were several parallel preliminary experiments to clear up the contamination. M9 media with PET strips was left in a 250 mL Falcon flask in a 30ºC, 150 rpm shaking incubator. Samples were taken every week and streaked onto solid NBRC 982 media to check colony consistency. After 5 weeks this did not prove successful. At the same time, PET powder was put in M9 media at the same conditions as above, and each week the powder itself was transferred into fresh media. This was done due to the fact that *I. sakaiensis* physically attaches itself onto PET and creates as biofilm. This was done with a belief that any microorganism that persistently stayed with the PET powder would be the *Ideonella*. However, this did not work either.

As these samples were being taken weekly and streaked and re-streaked every 24 hours onto solid NBRC 982 media, single colonies obtained were sent for 16S rRNA sequencing, so more selective media could be utilised to help clear up the contamination.

3.1 Selection of liquid and solid media

The flow of experiments at the start consisted of checking the activation media every 24 hours and monitoring the contamination. Some preliminary PET powder was created using a

FRITSCH® Pulverisette 6 planetary ball mill. This was to both start testing the capabilities in the laboratory to produce our own in-house PET powder, and to use it as a means of isolating *I. sakaiensis*. Some of this PET powder produced was put on a solid NBRC 982 media, and a sample of the liquid culture was streaked onto it, and incubated for 48 hours at 30ºC in a standing incubator. After this plate was taken out, localised growth around the PET powder was noticed. This was taken as a sign that the microorganism that was gravitating was preferential towards it, as the negative control both testing the streaking technique with and without powder was negative of any growth. This indicated that the bacterial growth did not start proliferating from the powder onto the media.

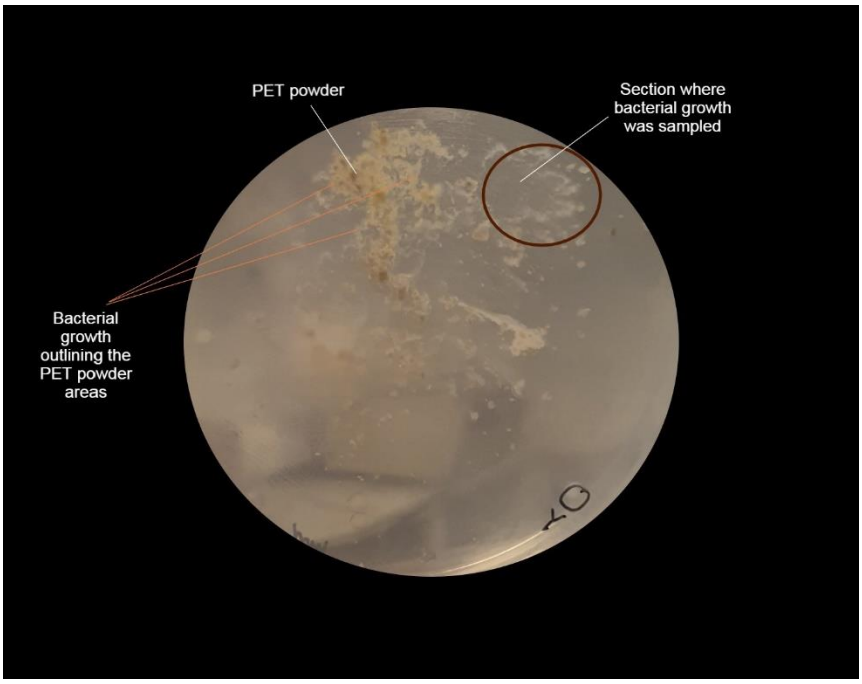


Figure 12. NBRC 982 solid media with bacterial growth localised around the PET powder

This plate was titled “Plate X” as it served as a starting point for a lot of experiments moving forward. Two lines of work were run in parallel after Plate X was established: testing this microorganism growing around the powder as a potential plastic degrader, as well as an isolation of *I. sakaiensis*. Andrew Rennison, a student assistant in the laboratory was handling the sequencing of organisms, as there were other collaborations in the laboratory happening on a similar topic. The sequencing of single colonies obtained by streaking and restreaking was an instrumental part of the isolation effort as it was necessary to know which selective media needs to be used. Several isolations confirmed that there was a presence of *I. sakaiensis* in the contaminated cryo stock, however there were several gram positive and gram negative contaminants as well. These were a gram-negative *Pseudomonas sp.*, and gram-positive

Bacillus cereus. To help removing *B. cereus*, a modified MacConkey agar was used, which contains Crystal violet which does not allow the growth of gram-positive bacteria. This was later proved successful as single colonies which were sequenced as *B. cereus*, when restreaked to the MC agar would not grow. To prevent the *Pseudomonas sp.* from growing, adipic acid was used as a carbon source as *I. sakaiensis* has shown to be able to utilise it for growth while *Pseudomonas sp.* mostly cannot(Tanasupawat et al., 2016). These efforts ultimately had proved to not yield the results that was expected. While the modified MC agar and addition of 1g/L of adipic acid as a carbon source helped with reducing contamination, *I. sakaiensis* was not growing readily on it either. In February 2020, a new batch of *I. sakaiensis* was ordered from BCRC in Taiwan.

3.2 Establishing biofilms

As mentioned, another line of experiments alongside sequencing and isolating the target bacteria, was testing the microorganisms that started growing around the PET powder on Plate X. 4 streaks were taken from the plate, and inoculated into a YSV media. Narrow necked, baffled, 250 mL glass Falcon flasks, were autoclaved capped with cotton plugs. 25 mL of YSV media was pipetted and inoculated. These were shaken at 150 rpm at 30ºC incubator. Cleaned strips of PET film were crimped to have folds and inserted into the media. These were left for 1 weeks before checking them again. A biofilm started forming in the folds of the PET film. During the first 3 weeks, the surrounding media was turning turbid, therefore it was decided that each week, the strip would be taken out and transferred into a fresh flask with fresh media. This was done by preparing the new flask and media in the same way as the original one, preparing a sterile pair of angled forceps, 70% ethanol for sterilising

between samples, and sterile water and glass container to rinse the PET film. This was done to ensure the only bacteria transferred would be the ones already forming biofilm on the plastic.

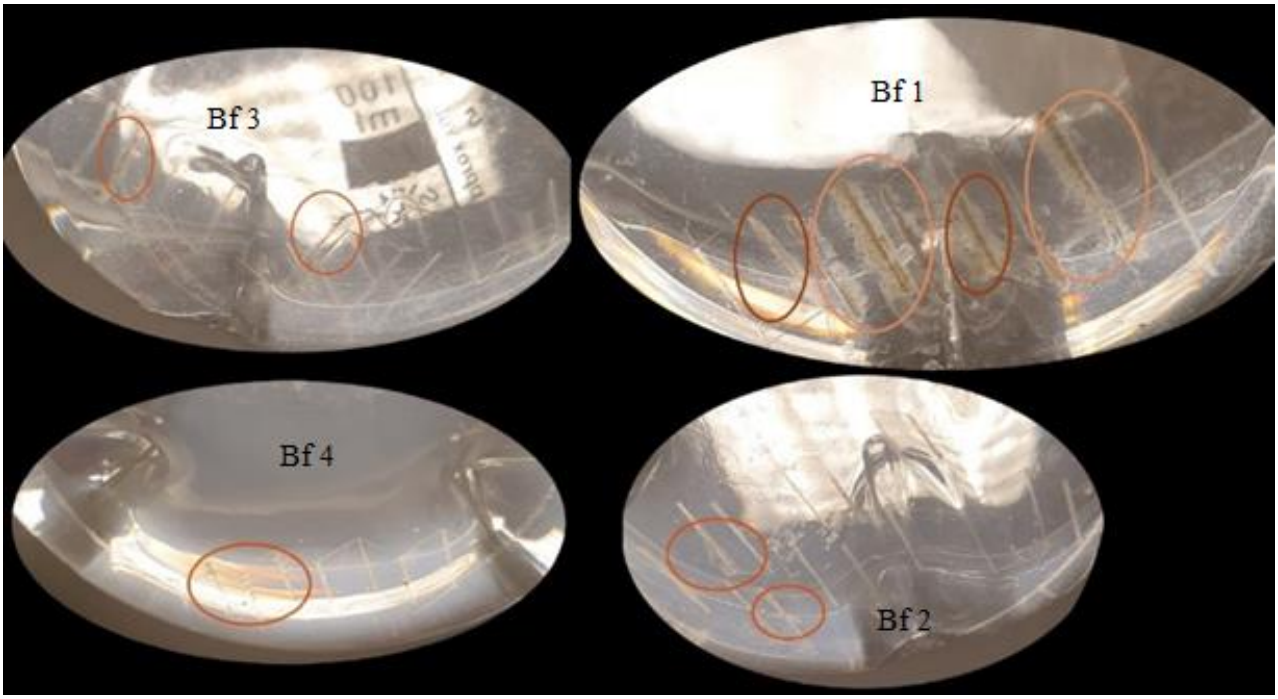


Figure 13. Biofilm formation on PET films

This process was repeated for 8 weeks. Every 2 weeks streaks of the biofilm were tested for sequencing, however the only results obtained were that it was a mixed consortium of *Pseudomonas sp* and *Bacillus* sp.

MC agar plates of the biofilms were produced in order to try and sequence the individual *Pseudomonas* species; however, all of the results came back as overlapping. The reason for this was to identify the individual components of the consortia to determine if the biofilm was valuable for further investigating or if it is just a collection of cells surviving on the minimal amount of yeast extract available in the YSV media.

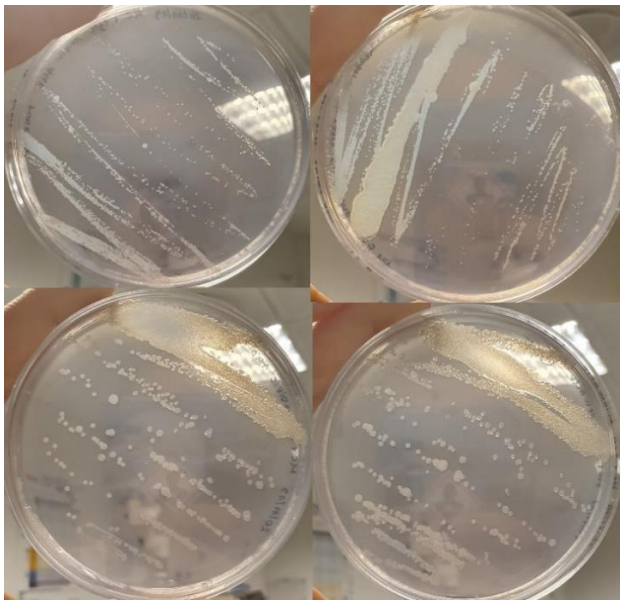


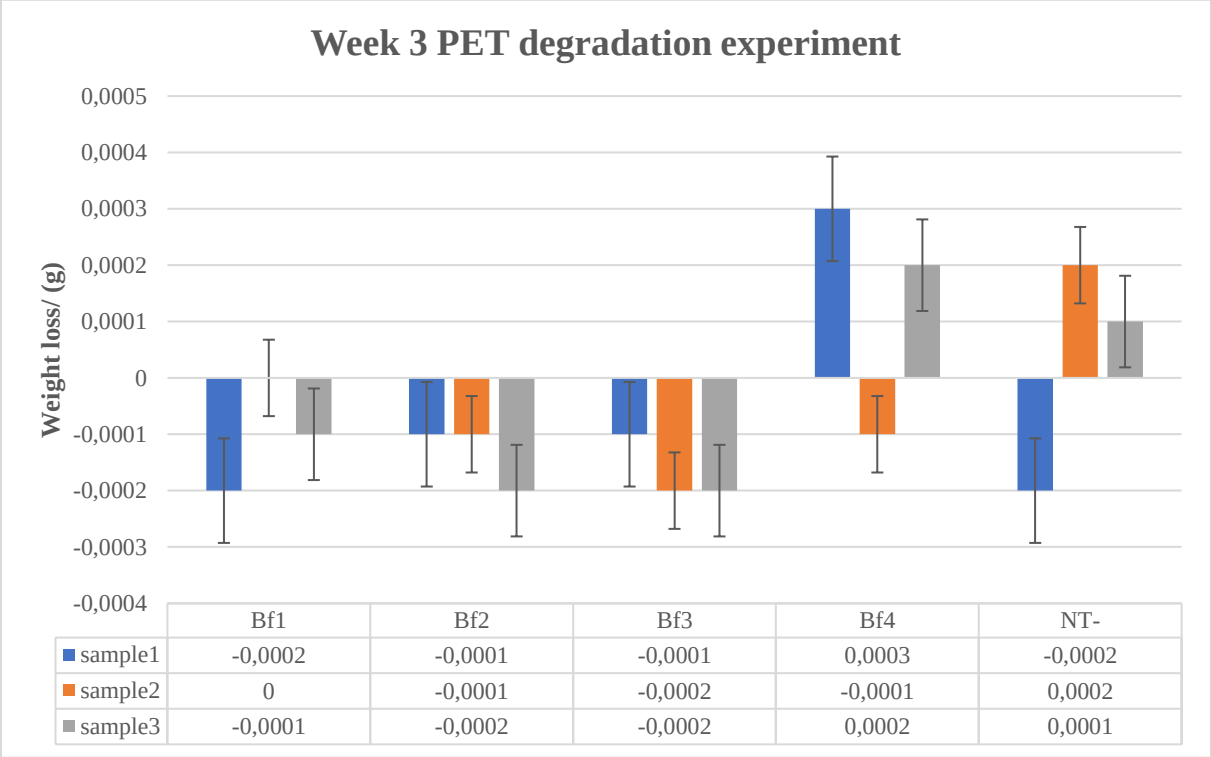
Figure 14. MC agar plates of biofilms

Some *Pseudomonas* species, as mentioned before, are known plastic degraders, and therefore this consortium could prove to be valuable for assessment. As of March 2020, the biofilms were supposed to be left alone for the duration of the project to be tested at the end, as a part of a long-running enrichment process. It was constructed as a long-term enrichment process that may lead to strains adapted for plastic degradation. Specifically, to enrich this biofilm to degrade PET. However due to the COVID-19 crisis the biofilms were left unsupervised and are now unusable.

3.2.1 PET bottle degradation experiments

As mentioned above, 2 degradation experiments were conducted, at various ages of the biofilm to test if they had any plastic degradation properties; at 3 and 8 weeks. These were set-up as according to (Yoshida et al., 2016b), however, strips of PET bottles of unknown crystallinity were used instead of low crystallinity (1.9%) PET as per the original. These were of a lower crystallinity than the PET film used for the biofilm formation and therefore more easily accessible for degradation. These tests were running for a duration of 3 weeks, and the plastic was measured gravimetrically. The degradation that was recorded all fell into the detection limits of the analytic scale and was considered insignificant as it equalled to the control strips as well. Therefore, it was prescribed to any mechanical degradation over time. The biofilms remained stable and after the decision to leave them for the maximum amount of

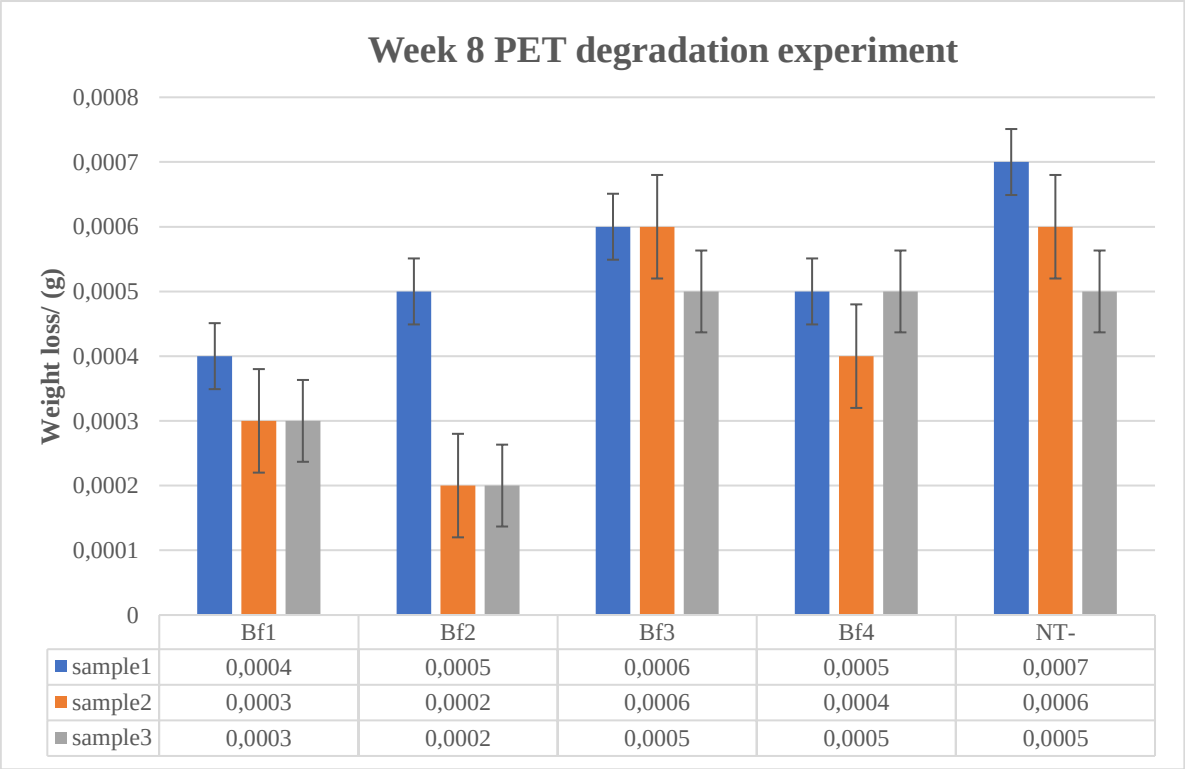
time available, a final degradation experiment would be run, however, the time for this ran out.



	Bf1	Bf2	Bf3	Bf4	NT-
sample1	-0,0002	-0,0001	-0,0001	0,0003	-0,0002
sample2	0	-0,0001	-0,0002	-0,0001	0,0002
sample3	-0,0001	-0,0002	-0,0002	0,0002	0,0001

Figure 15. First PET degradation experiment results

As visible in the figure above, the sampled biofilms (Bf) 1,2, 3 and 4 all displayed negative weight loss, meaning that the samples gained mass. This error can be attributed to 2 distinct reasons: observational error by incorrectly weighing due to human error, and greatest possible error of the analytic scale which has a limit of ±0,0005g. All of the measurements above fall below this measurement and therefore cannot be taken into account as valid results. This was the first experiment where high precision of washing and weighing the PET strips was required, and therefore human error must be taken into account. As the results are too low it is unable to compare to any published data. The conclusion from the numbers available is that there is no plastic degradation activity present in the samples.



	Bf1	Bf2	Bf3	Bf4	NT-
sample1	0,0004	0,0005	0,0006	0,0005	0,0007
sample2	0,0003	0,0002	0,0006	0,0004	0,0006
sample3	0,0003	0,0002	0,0005	0,0005	0,0005

Figure 16. Second PET degradation experiment results

The results displayed in the figure above are from the 8-week-old biofilms in another PET degradation experiment set-up the same as the previous one. While it is apparent that the results have increased in terms of weight-loss, the negative control sample (NT-), has the highest weight-loss comparatively. The human error was less present at this time due to development of skills while handling the PET strips. Even with this increase, these results are not comparable to literature, and this would be considered as no PET degradation activity present in samples.

The plan was to do another test with the 4 biofilms at the end of the project when they will be 6 months old, and observe if any PET degrading strains have been selected for. However, due to lockdown, this was not done.

3.3 Optimisation of the PET powder production

Creating PET powders proved to be an instrumental part of the project, as buying a variety of different crystallinity PET is not possible. Therefore, a method for easy production in-house was high on the list of requirements. As particle size has shown to be important to hydrolysis rates, and the smaller the powder, the higher the degradation rate, it was paramount to produce a method that could be easily reproducible with good fidelity(Gamerith et al., 2017). This method had to cover a variety of stipulations: any shape of PET could be converted, any

crystallinity as well. The powder can be collected and separated into several particle sizes. It is reliable and highly reproducible. While different machines were used to test the production, a central preparation technique for the plastic was cooling it in liquid nitrogen for 10 minutes, insulated in a freezer bag to prevent condensation on the plastic itself, as the introduction of moisture interfered in the milling process. Preliminary tests were done with the FRITSCH® Pulverisette 6 planetary ball mill. The feed material were PET films and PET bottles cut into 1 cm^2 squares. The top speed of this machine was 600 rpm, and even with modifying the cycle lengths, cooling down the plastic as well as the milling chambers, this style of milling proved unyielding and ineffective. The plastic would heat up very readily, turning into liquid and adhering to the milling balls as well as the milling chamber. The produced powders also had a grey colouring which suggested the continued friction inside the milling chamber was burning it. It was possible to separate the obtained powder into different size categories, and it was used to produce the original Plate X, however it would be unusable for the degradation experiments. It was unsure what kind of chemical changes happened to the plastic due to this aggressive milling.

Figure 17. PET milled in a planetary ball mill

When PET pellets were obtained, it was very quickly apparent these turned into powder a lot more readily. A high-powered blender was tested to produce a powder and it was successful however the powder clung to the sides of the blender once it collided with the blades, and it no longer fell down to be further broken down. This method also produced discoloured powder as it had to be re-blended to obtain finer grades. After the FRITSCH® Pulverisette 14 was obtained, the powder production was more fine-tuned. This proved to be the optimal type of mill for a small-scale production. The mill has an open construction which creates air flow into the rotor area. The plastic falls into the centre of the rotor, is rapidly sent toward the rotor ribs where it collides and breaks down, then it is sent into the sieve behind the ribs and with the collision force further broken down, before being flung into the collection chamber. This high-speed mill ensures that the milling process is very short and the plastic is not overly heated up. The powders produced were white or very slightly discoloured, and in a variety of

sizes. However, the drawback was a very low loading amount. Only a few squares of plastic at a time could be fed into the mill, and the milling had to be stopped after approximately 1 minute as the whole system would heat up and cause the plastic to start melting onto the rotor and forcibly jam the entire system. The smallest grade of powder produced was below 250 μm and therefore, the collection of the powder was very tedious and thorough as any leftover would interfere with the next batch of milling in terms of clogging the rotor. PET powders are highly electricized and stick readily to any surface, as well as there being a high risk of inhaling the powder therefore a face mask is required while producing it. After each batch of milling, a stiff bristled brush was used to sweep as much powder as possible onto a prepared collection surface, and a vacuum cleaner was used to completely clear the machine. This way it was possible to produce powder from PET films, bottles and pellets. The PET film presented the largest amount of work as it was extremely thin and more readily melted onto the rotor ribs. It also produced the more discoloured powder. The powders were checked under a light microscope to see if there was any difference in their shape, and the powders created from flat shapes were more jagged and torn, while the powder produced from the pellets was more rounded and uniform. This coincides with the fact that a flatter shape will less readily create a spherical particle. It was also discussed the possibility that the milling process itself interfered with the crystallinity of the powders, and that it would make the plastic more amorphous and serve as a mechanical pre-treatment. There were plans to examine the crystallinity and size distribution later in the project however there was no more time for it.

However, in literature there is evidence of the amorphous aspect of PET increasing by using cryomilling by a significant amount. As time of milling, type of input, the starting crystallinity and temperature of the PET all affect the amorphous proportions, it would be necessary to test all of them with a method such as differential scanning calorimetry (DSC)(Bach et al., 2009). This would definitively show the crystallinity of the powders.

3.4 Determining comprehensive quantification of degradation of PET powder

Gravimetric quantification is the most straightforward way of determining how much plastic was consumed. However, if the amount of plastic degraded is very low, the gravimetric change may not be measured accurately. The use of powder with a full cell system also

presented an additional step as the biomass forms a biofilm on the powder and needs to be separated. HPLC detection of the PET monomers in the media is a more accurate way of quantifying degradation. However, in a system with a cell which is possibly consuming the monomers, it is less reliable, and therefore needs to be paired with multiple quantification methods. For this project, it was decided a well-rounded list of methods would be used to quantify the degradation and this consisted of: total protein quantification, enzymatic activity assay, and a biomass and powder gravimetric quantification. Samples were also kept for HPLC; however, those would be analysed if the other tests showed degradation and therefore warranted the more sophisticated quantification method.

3.4.1 BCA and enzyme assay experiments

These experiments were conducted on the biofilms that had formed on the PET strips and were being refed approximately every week. They were chosen as a general cost-effective way to test activity of the biofilms instead of HPLC testing, as it was unclear as to if they were productive, and therefore no resources would be wasted on HPLC testing. This way, a BCA protein assay, with an enzyme assay would give insight into how the biomass growing was behaving towards the inert carbon source. A Pierce™ BCA Protein Assay Kit was used to determine the total protein present in the sample. It uses bovine serum albumin (BSA) for calibration, and works on the biuret reaction principle with a reduction of copper from Cu^{2+} to Cu^{+}. It is a colorimetric method where a colour change occurs which is then read by the microplate reader. This was initially tested on the biofilm samples.

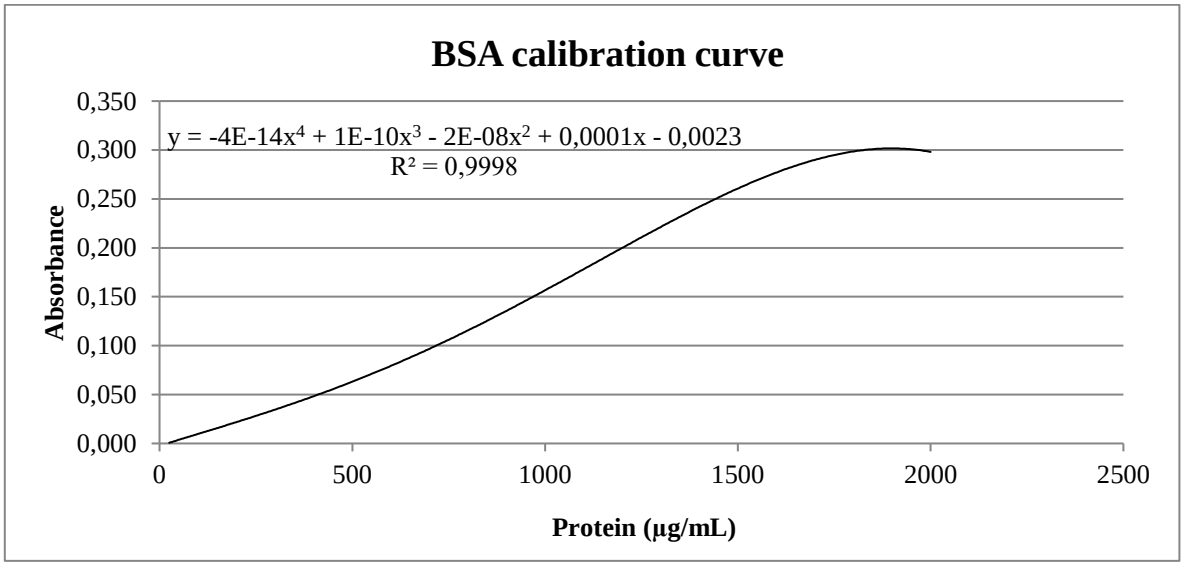


Figure 18. BSA calibration curve for the BCA assay

The calibration curve puts the limits of the kit between 20 and 2000 µg/mL of protein in a sample. It is calculated using a 4th order polynomial as per instruction of the manufacturer.

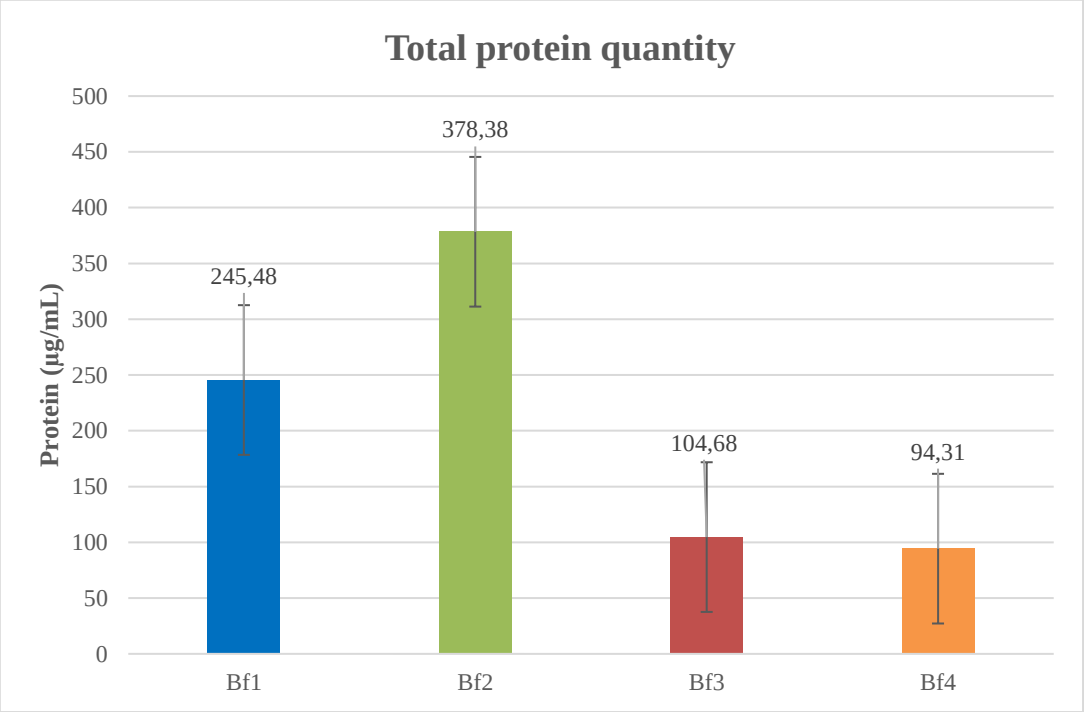


Figure 19. Total protein quantity as calculated from the calibration curve

There is no research to compare these results in terms of what this means for the amount of enzyme available. As these results are based on biofilms that formed from an unknown source, these experiments were used as a learning opportunity for using later with a pure *Ideonella* sample. What is apparent from the results is that biofilm 1 and 2 are for some reason more productive than 3 and 4. It is visible in the pictures of the biofilms that this seems to be in alignment with biomass visible growing on the PET film. The samples for the test were taken from the surrounding media, and not directly from the biofilm attached to the PET strip, therefore the low results could be attributed to the fact that very few cells were sampled. In order to make a proper comparison, there needed to be another set of experiments to be able to describe these results correctly. As this was the first time this test was personally done in a non-classroom setting, it served as a learning experience into the technique and data collection. Firstly, the homogeneity of samples is of upmost importance so as to keep variation low across samples. This is done by sampling everything in a consistent way. The test should be conducted at minimum in triplicates as to give a more statistically relevant set of results. These results were obtained from doubles. As the method for the kit used is standardised, these are the areas where improvement is needed when repeating this experiment. There was no time to retest this on the new *Ideonella* samples.

The enzyme assay used was from (Salgado et al., 2019), and its purpose was to determine the unites of enzyme per mL of solution. This, coupled with the BCA protein assay would give a clearer picture into the activity of the biofilm growing on the PET strip. This enzyme assay used was suggested as it had been used to determine esterase activity which would be present if the biofilm was degrading the PET. The assay was colorimetric and functions on a principle of cleaving the ester bond in the p-nitrophenyl butyrate(p-NPB) which was the substrate. This would produce p-nitrophenol which colours yellow and can be detected with a microplate reader.

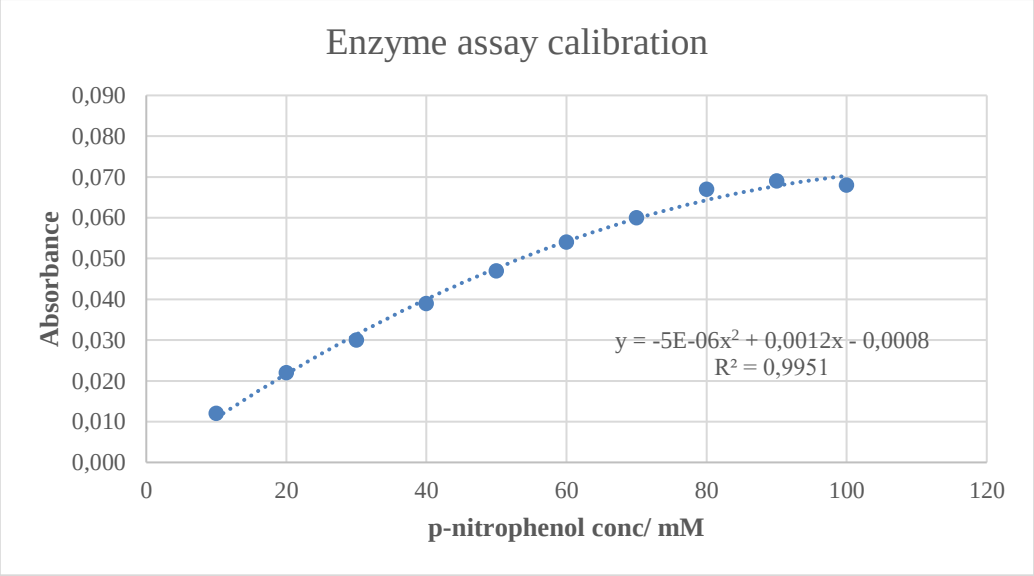


Figure 20. Enzyme assay calibration curve

The calibration curve was created to measure between 10-100mM concentration of p-nitrophenol. This was decided on after a discussion, both to test how the biofilms would react to it, and to learn how to perform the method.

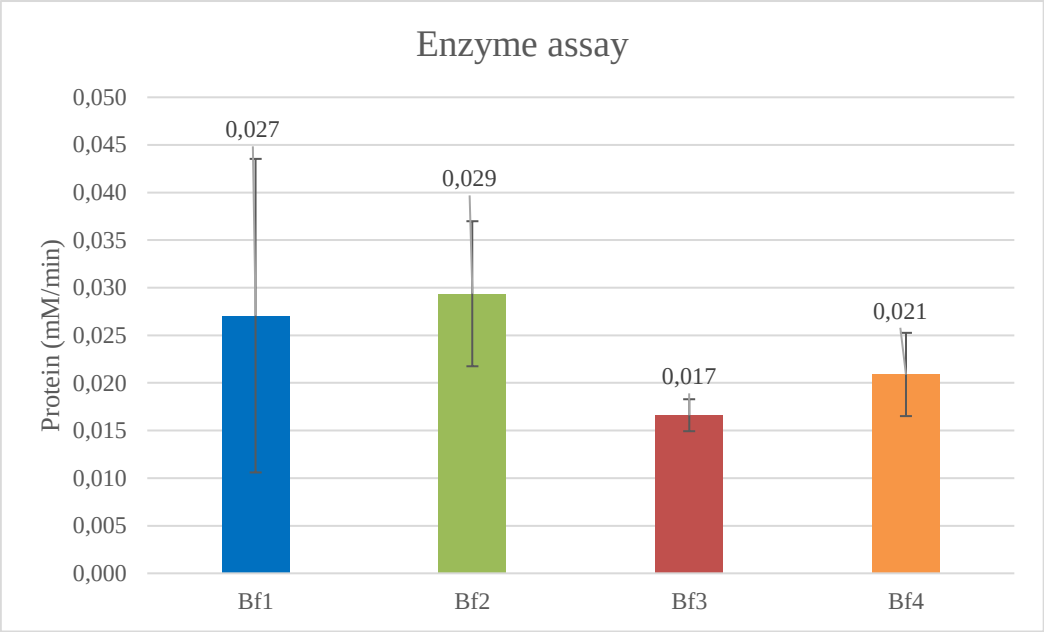


Figure 21. Results of the enzyme assay

This again was tested on the formed biofilms. The samples were once again taken from the surrounding media and not the biofilm itself meaning that the amount could have been too small to start with. There is also a possibility that there were no esterases present in the sample, and the small colour change that did occur was due to the degradation of p-NPB at room temperature. The results are below the 1,5 mM/min reported in (Liu et al., 2019), and lead to the conclusion that there was probably no esterases present in the sample. Later on, several modifications to the assay were suggested. Firstly, the buffer solution needed to be modified to better fit the PETase profile. This test initially was done on the biofilm, to try and confirm if there was any degradation happening, or if the biofilm was inert and feeding solely on the very small amount of yeast extract present in the YSV media. For the test that were supposed to be done on *Ideonella*, the pH would be moved to more alkali at 8, and the incubation temperature would be 30ºC. The method would be conducted by placing the microplate on ice while all the components were added. This would prevent any degradation of the substrate at room temperature, and ensure the incubation period was accurate. This assay was designed to confirm the enzyme activity, which would be then coupled with HPLC results which would directly quantify degradation products. This would then give a complete picture from biomass formation to how much PET can be degraded in a specific time frame.

3.4.2 Solvent experiment

The purpose of the solvent experiment was to gravimetrically determine the degradation of PET powder which would be used in future experiments. As well as quantifying the biomass that would form around it. As other research has shown, the particle size of the PET is, next to crystallinity, one of the most important factors in speeding up degradation. The smaller the particle size, the higher the surface area for degradation, and also the higher the chance that more of the crystalline structure was affected and become more amorphous, and therefore more available. Due to the plan being to use cells in degradation experiments, and the fact that *I. sakaiensis* forms a biofilm, there was an additional step needed to separate the biomass from the PET powder. As shown in the powder production, the powder produced was as fine as down to 50µm, and would not be visually distinguishable from the biomass surrounding it. (Farzi et al., 2019) suggested using an organic solvent to separate PET powder from the biomass. The organic solvent would disrupt the cell walls and leave the powder which would be retained on the filter paper. There were several considerations to be had in this situation. Firstly, organic solvents can dissolve PET. In this case, chloroform was used as it is a widely available organic solvent in the laboratory, as opposed to the toluene suggested in the original paper, and would give a starting point in testing other organic solvents. A simple test of just the PET powder and chloroform was done as described in the methods, to test the recovery of the powder and how it reacts with the powder in general. During the experiment, the PET powder did not show signs of dissolving. A Teflon filter was used in conjunction with a vacuum pump to filter the chloroform from the powder. The PET powder proved very cumbersome to work with as it distributed along any surface it touches. It was difficult to flush out from the glass container it was in with chloroform, and it was difficult to flush down the sides of the filtering funnel. The test was conducted in triplicates and all of them had issues in powder recovery. The necessity of working in a fume cupboard due to the toxicity of chloroform, proved as unwieldly and restrictive. The powder was not recovered in a capacity that can be quantified. And after the experiment it was decided that using an organic solvent does not align with the sustainable goals of this project. As the waste produced would require special management and would waste many resources. It was suggested that for further experiments a different approach to separating the biomass from the PET powder could be done with a simple lysing buffer that would not be in danger of reacting with the PET.

3.5 *I. sakaiensis* experiments

The fresh cryo stock of *I. sakaiensis* arrived at the end of February and it was extensively checked and activated to ensure multiple sources would be available in case any additional contamination happened in the future. The activation media suggested by the supplier was NBRC 982 in both liquid and solid form. For both the solid and liquid media, the incubation temperature was 30ºC, and for the liquid media it was shaken at 150 rpm. The methodology for the experiments involving *I. sakaiensis* was the culmination of knowledge acquired while testing the biofilms. The activated culture would be monitored every 24 hours by microscopy and re-plated. This way, it could be monitored how it behaves on a solid and liquid media.

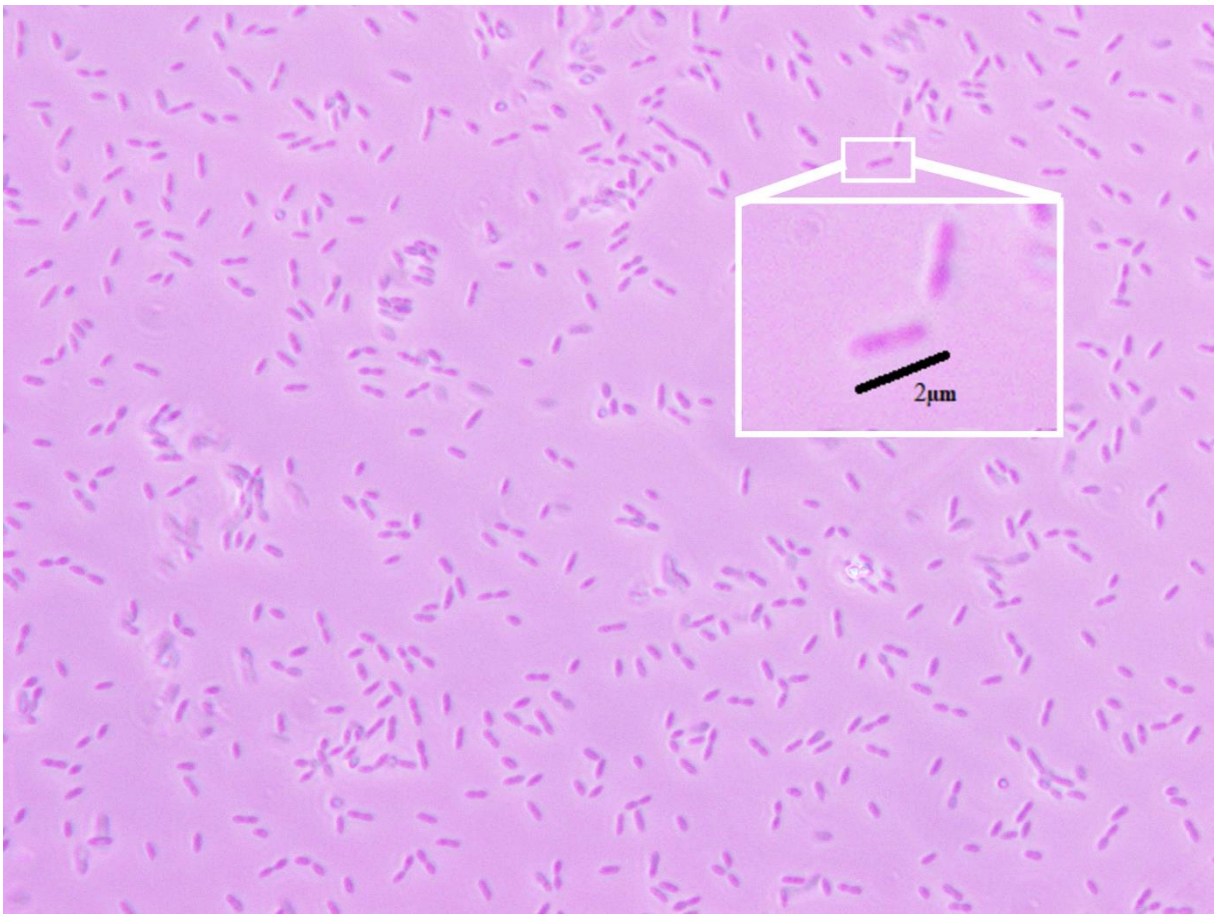


Figure 22. Light microscopy picture of an *I. sakaiensis* colony 100x magnification

After 2 weeks of reviving the culture and testing out how it behaves in terms of OD_{600} after 24 hours, it was agreed to start the experiments as originally planned. Several things were agreed upon. Any OD experiments would be conducted with a fresh culture that was activated the day before. The OD_{600} measurements after 24 hours was 4, more prolific than reported in literature however, it is unreported for this particular media. This remained consistent with 4 separate tests. Due to it reaching this high reading after 24 hours, the inoculum for further

testing would be diluted to a 0.1 reading as a starting point. And with different liquid media tested, the solid equivalent would be used as well. This way it could be monitored if the morphology of cells changed depending on the availability of carbon sources in the media.

To be able to test multiple media over a short span of time, microplates were used. It was possible to conduct 2 separate experiments. The first one ran for 24 hours, and the second for 16 hours as it was started the day before the lockdown officially started in Denmark. These first experiments were done to determine which media *I. sakaiensis* could be activated in, in a large enough number, and would be the activation media of choice moving forward. Additional tests using PET powder in the liquid media were scheduled to see how that affected the growth rate, but were unable to be conducted. All of the samples were tested in triplicates, including every blank for each media type. These values were then subtracted from the values given by samples in order to have a true reading of the samples.

In the first microplate experiment, 12 variations on media were tested. As addition to the minimal medias, an addition of 1g/L of adipic acid, or maltose were added as that was stated as 2 of the simple carbon sources *I. sakaiensis* can assimilate. The media were the following: LB, NBRC 982, YSV, M9+, M9+ and adipic acid, M9+ and maltose, M9, M9 and adipic acid, M9 and maltose, H_2O, H_2O and adipic acid and finally H_2O and maltose. The microplate was spun at 180 cpm, 30ºC, read at 600 nm every 15 minutes for 24 hours. The organism was activated from a swab on a plate in liquid NBRC 982 24 hours before the start of the experiment, when checked it had a reading of OD_{600} 4. The inoculum was the calculated to dilute down to 0.1 when added to the microplate wells. Inoculum size was 10% in a 200µL working volume. This left 60% of headspace in each well. The microplate was covered in an optically compatible anaerobic film. An aerobic film was tested with just water in the wells and it obstructed the readings as it was not clear. Therefore, it was decided the headspace available would be sufficient for the duration of the experiment. A sample of the activation

media was taken and centrifuged and washed for each subsequent media. This was done 3 times to insure no highly nutritive media would interfere with the readings for minimal media.

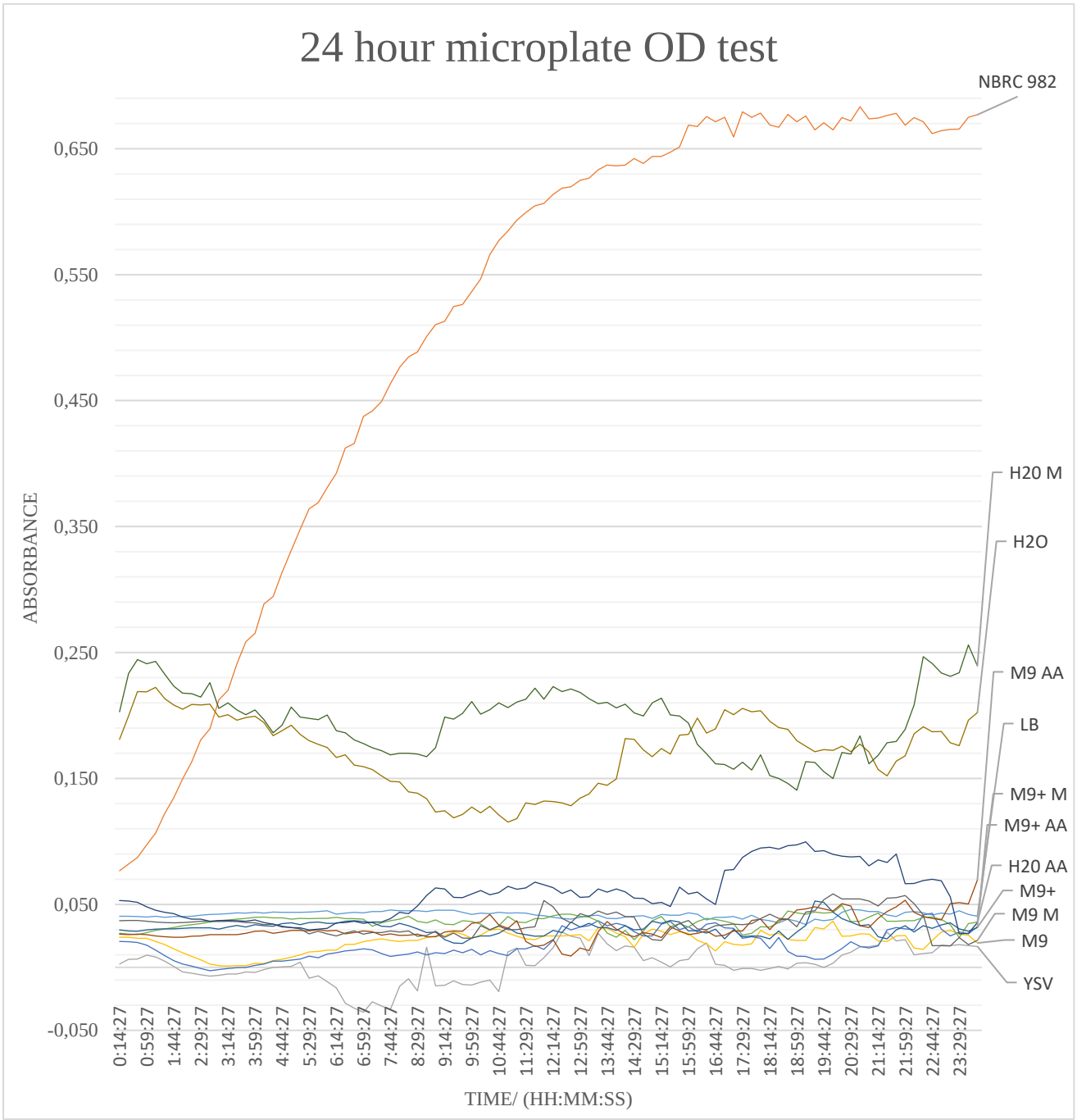


Figure 23. Results for the 24-hour microplate OD test

Firstly, the starting measurement is not the calculated 0.1 and that is a human error that was mitigated in the following experiment. Just at first glance it is very apparent that *I. sakaiensis* favours the NBRC 982 above all other media, finishing at a reading of 0.7. Water and water with 1g/L of maltose are the second-best performers. However instead of a growth curve, the growth is almost stagnant for the entirety of the experiment. This can be attributed that the

starting value for them was close to 0.2, while for the rest of media was at an average of 0.03. This low of a number could not have been enough to establish a proper growth curve.

The second experiment lasted only 16 hours as it had to be stopped due to the start of the lockdown in Denmark.

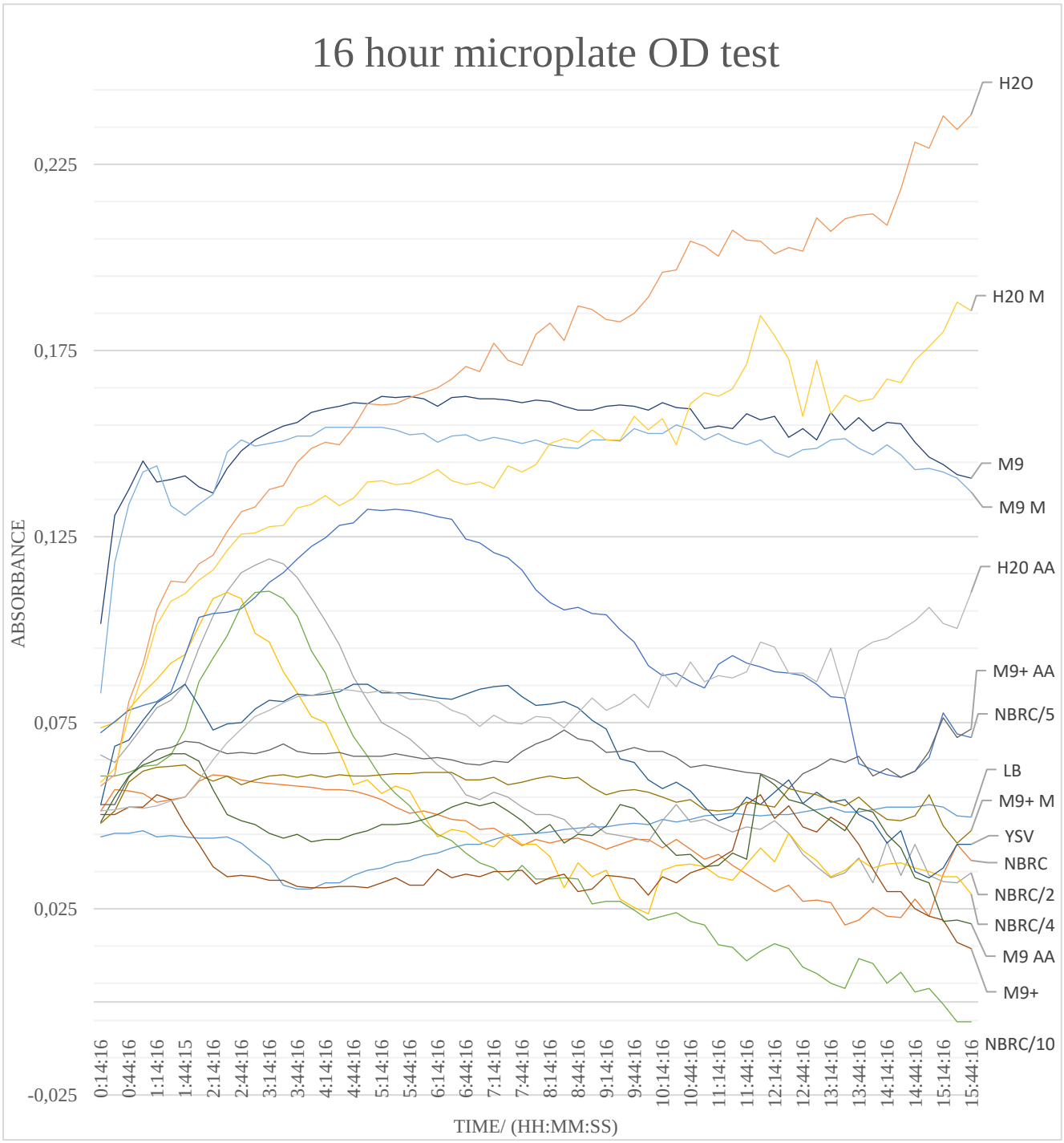


Figure 24. Results for 16-hour OD experiment for *I. sakaiensis*

The starting reading for this experiment was an average of 0.06 and much more uniform for all samples. In this case the inoculum was activated by transferring a 48-hour culture into

fresh NBRC 982 media and left for 24 hours. It had a reading of OD 4 the day of the experiment. However, it seems to not have been as active. If this experiment was repeated the activation would be done as it was done for the 24-hour experiment. As NBRC 982 was the preferred media in the first experiment, this time it was diluted 2, 4, 5 and 10 times. This was done to determine at which point *Ideonella* would maintain the growth but the media could be diluted. It seems to increase in biomass for the first 3 hours when it peaks and starts a steep decline. However, for the original NBRC it maintains the starting reading and starts to decline after 2 hours. The inoculum for NBRC samples was not centrifuged and washed like the others but taken directly from the activation vessel. This could have affected the reading as some cell debris and inhibiting factors produced by the original media would not be washed out. It seems to respond to the 2 times diluted media well however the 10 times diluted is not significantly enough low to be discounted. These experiments would have to be repeated with these corrections. The highest results this time come from the water and water with 1g/L maltose samples. It seems to be able to sustain itself in just water very well, however, this can be due to it being activated in a highly nutritive media for 24 hours previously. In this setup it does not seem to respond positively to the YSV and minimum medias constructed. *I. sakaiensis* was originally isolated from a soil sample on a plastic waste landfill. This would imply a lack of nutrients available in its surroundings. An improvement to this experiment would be the inclusion of CFU and dry cell weight to correctly identify the number of cells present in a sample. OD in this case was used as a quick approximation from which other experiments could be established.

4 Future perspectives

Due to the untimely end of the project, the future perspectives do not only reflect the developments in the *I. sakaiensis* research, but what this project in particular was supposed to develop into as well.

4.1 Project plans

This project was supposed to continue onto testing how particle size affects the degradation rate. Previous research has shown that the smaller the size of the PET powder, the higher the degradation rate, however, these studies were done using the purified PETase enzyme. This project in particular would test how the rate would change when the whole organism was

utilised instead. The crystallinity of PET powder would also change based on what the powder was made from. This would also give insight into how the degradation rate changed based on crystallinity. This was again tested on the purified PETase, and it showed the lower the crystallinity, the higher the degradation rate. Additionally, it was planned to test how changing the pH and salinity of the media would affect the degradation rate. It was generally acknowledged that increasing the biomass of *I. sakaiensis* would lead to a higher degradation rate due to a higher number of biofilm constituents. Therefore, the research would focus on optimising the growth conditions for the organism. As *Ideonella* forms biofilms, there were plans on exploring how quorum sensing (QS) functions as a mechanism of social interaction between bacteria, especially in biofilms(Y. H. Li & Tian, 2012). Research on QS would provide insight into if the activity of the biofilm may be influenced to increase biodegradation of PET. Finally, there were plans on looking into the exact mechanism with which *I. sakaiensis* uses its special appendages to attach itself to the PET. In terms of how do they form, and how the protein composition changes in the early and late stages of the biofilm. This project would aim to make a more complete picture of how *I. sakaiensis* grows and can be influenced to better degrade PET, without using molecular methods to enhance it.

4.2 PEF as a substitute for PET

PEF is a polyester equivalent to PET. It is constructed with 2,5-furandicarboxylic acid and can be produced from polysaccharides and sugars. It may present a 100% bio-based polyester as opposed to petrochemically produced PET. It has also proven to be both food safe, and more appropriate for storing both carbonated and noncarbonated drinks(Hoppe et al., 2018).

PEF is also reported to be hydrolysed 1.7 times faster than PET. Alongside being more readily hydrolysed, it seems to have a more gradual decrease in degradation and crystallinity rises as opposed to PET which has a drastic fall in degradation as the crystallinity rises(S. Weinberger et al., 2017).

Due to the structural similarities between PEF and PET it was hypothesised that IsPETase may be able to hydrolyse it. And as expected, during experiments, it was not only able to hydrolyse the amorphous regions of PET, it seemed to have an even greater effect on the PEF samples(Austin et al., 2018).

Future perspectives of PEF would involve both optimising its production, while also focusing on developing a polymer that would at the end of its life be more susceptible to enzymatic degradation. And simultaneously, optimising enzymes that would more readily affect the

crystalline structure of the backbone. These lines of research are imperative in order to close the circular economy loop.

As stated before, the future of research as it pertains to *I. sakaiensis* and PETase and MHETase is leading towards using molecular biology tools in order to optimise degradation ability. This includes improving thermal stability and affinity for substrate. As well as testing which other polymers would also be affected by the enzymes. An important aspect of future research is developing an enzyme with a new substrate in mind, a parallel research line of creating new material which will then be processed by it. This way solving a problem before it is created.

5 Conclusion

The goals this project set out to meet were not accomplished. Starting from the contamination and trying to separate the *I. sakaiensis* from the numerous contaminants which did not work. The biofilms that did form on the plastic were not confirmed to be plastic degraders, and by the point the lockdown started, the decision was to leave them to enrich till the end of the project, in hopes that they will produce an consortia of interest that would be enriched for PET degradation. The true value of the experiments done in that time frame was the personal development in terms of planning experiments, gaining practical understanding of making media, plating technique, and efficiency of time. As well as updating the understanding of classical techniques and how they can be used to compliment molecular techniques.

During this time, gaining better understanding into methods such as protein and enzyme assays, and how the method changes based on the need of the project, and what parameters of the method must change to fit the scope of the project. Some kits were standardised, such as the BCA total protein assay, but personally conducting the tests was valuable experience. The enzyme assay first suggested was due to it being widely used in the laboratory, but upon experimenting with it, it was apparent it would have to be adapted to fit this project. However, some parts were transferable in terms of handling, order of operations, and the importance of precision and consistency were highlighted. The use of the microplate in these tests gave raise to the idea of using the microplate reader to conduct a large series of OD tests using it as well. As it would be possible to test many variables in a shorter amount of time. This means a development of knowledge of the machines used in the laboratory as well.

Creating PET powder from an array of sources also proved a long process. This particular aspect of the project involved research into specific chemistry and physics of polymers, how they were affected by abiotic conditions and different mechanical forces. However, this also involved research into the mechanics of different types of mills and the forces that they exerted on specific material. This led to several conclusions. PET must be cooled to its brittle point with liquid nitrogen in order to properly shatter when milled. This cooling process must also be done in a way where the PET is not exposed to moisture as it will start absorbing it, making it harder to mill. The mechanical forces produced by small scale mills are enough to generate thermal friction that brought PET to its glass transition point and melted it onto the sides of the milling components. A small-scale ball mill is an inappropriate type of mill to use as it does not have the correct shearing forces necessary to mill plastic. PET powder sticks to surfaces after it is milled, be it the surface of the mill itself, or any container it is deposited in, making it extremely difficult to contain properly. The shape of the powder is determined by the shape of the feedstock. If powder is produced from a film, it will come out more jagged, but if it produced from pellets it will be more rounded. If the powder is improperly milled, it will burn and turn a grey colour. And finally, the crystallinity of PET is affected by the milling process. It needs to be retested after the powder is produced to ensure which crystallinity was produced.

When the fresh batch of *I. sakaiensis* was acquired, it was very necessary to keep a number of cryo stocks to insure there was always a pure culture available. As well as, each step of the way, the culture would be streaked onto the plate, and it would be monitored for any morphological changes. According to the OD test that were conducted on it, *I. sakaiensis* very clearly prefers NBRC 982, which is the suggested activation media. However, it seems to positively respond to water and water with added maltose, but these experiments must be repeated to validate completely. Due to a small amount of data available, it is unclear if it prefers this because it is an oligotroph that can survive in very low nutritive media, or simply because the components of the minimal media used were actively impeding on its growth and in water it was just surviving on leftover energy from the activation media. One would suggest that water and additional carbon source with PET would be good enough for growths, while the other suggests that the minimal media was impeding on growth and therefore artificially enhancing the perception of water as a suitable media.

In conclusion, even as the goals of the project were not made, in the larger overview of the topic of plastic waste management, a system using *I. sakaiensis* as a set-up for plastic

degradation, without any molecular biology tools used to enhance it in some way, is not a sustainable system. The bacteria degrade the plastic by breaking it down into monomers and then utilising the monomers for its own metabolism, and producing CO_2 as an end product. This does not align with a circular economy plan, as the bacteria does not produce monomers that are released into the media and can be collected. The cell system should be explored fully to uncover all the details on how it operates, and give insight into how it can be enhanced for the purpose of obtaining the monomers. As plastic production shows no signs of stopping, providing the market with monomers extracted from plastic that was already created, is an example of a circular economy system that will ultimately present a more sustainable approach to an industry that is so fully integrated into and integral for the functioning of modern society.

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