Towards the biological degradation of plastics: Genetic engineering of *Saccharomyces cerevisiae* to secrete *Ideonella sakaiensis* derived PETase.

A thesis submitted to the University of Kent for the degree of

M. Sc. Microbiology

2019/2020

Noor Issa

School of Biosciences

Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or institute of learning.

Noor Issa

December 2020

Acknowledgements

First and foremost, I would like to thank my supervisor Dr Tobias von der Haar for all the great and ongoing support he has given me in this project. Without his teaching and incredible knowledge, I would not have been able to get this far in my studies. I would also like to thank my colleague Charlotte for her support in the lab, as well as to all of the Kent Fungal Group and the School of Biosciences for continuously providing a wonderful and supporting environment. Last but not least, I would like to thank my mother Mbarka Elabaya for all of her support which kept me going throughout my entire academic pathway.

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1. Abstract

In this project we demonstrated that two strains of *Saccharomyces cerevisiae,* were able to produce the heterologous protein Polyethylene Terephthalate (PET) hydrolase from the novel bacterium *Ideonella sakaiensis*. This novel enzyme has been shown to have the capability of degrading PET into its subsequent monomers, Ethylene Glycol and Terephthalic acid. The original native form of PET hydrolase was mutated by the Beckham lab (Skaf et al. 2018) to increase efficiency of degradation, the two residues changed were at the active site which resulted in the narrowing of the cleft. The residues changed were Tryptophan at position 159 to a Histidine residue; and Serine at position 238 to a Phenylalanine. In this study the double mutant form of the enzyme was used. In this project we also demonstrated the capability of PETase expressed by *Saccharomyces cerevisiae* to cause surface changes on waste PET samples. Lastly, we have also shown that *Saccharomyces cerevisiae* has the ability to form cell clusters similar to biofilms on a PET surface. All of these findings point to the viability of using *Saccharomyces cerevisiae* as a novel system for plastic waste removal in an industrial setting.

2. Introduction:

2.1 Plastic as a pollutant

As of 2018 annual global production of plastics has reached 359 million tonnes, and despite global campaigning the demand for plastic continues to increase by approximately 3.5% annually. Since the patenting of the first synthetic plastic, Bakelite™, in the 1940's the accumulative production of plastics surpassed 7.8 billion tonnes in 2015 (Geyer, Jambeck, and Law 2017). The associated increase in plastic pollution has resulted in the entry of plastic into several ecosystems, most notably marine environments, as well as entering the human food chain.(Campanale et al. 2020) Over 8 million tonnes of plastic were recorded entering the oceans in the year 2019 alone. Although it seems that the solution to remove plastic pollution is to recycle, only 10% of all plastics produced have been recycled, hence leaving a large proportion of plastics produced in several environments and ecosystems. With the increased awareness for plastic pollution several small businesses and individuals have tried to remove plastic, especially single use plastic from their daily lives. Currently, 20% of plastic is produced by large corporations, which is why their efforts are also required to reduce plastic pollution and production, contrary to popular belief that consumers are mostly responsible for plastic waste accumulation. (Jambeck et al. 2015)

Plastic is not only an environmental polluter in its solid form, but also a large contributor to greenhouse gas emission. This is a result of its derivation from the petrochemical industry, hence crude oil is required as a starting product. These factors further drive environmental damage, and plastic production is one of the key drivers for the demand of crude oil. Of all the oil and gas reserves, 14% is used to

produce plastics. And due to the increased demand for plastics this is expected to increase by 20% in 30 years' time. This production rate would create 2.75 billion tonnes of greenhouse gas emissions, further exacerbating the pollution and climate change issues we currently face. (Pang et al. 2016)

In addition to visible pollution that plastic creates, increased lifetime of plastics in the environment results in the formation of microplastics and nanoplastics. Microplastics are classed as fragments of any plastic type equal to or smaller than 5mm in length. Nanoplastics form part of this subgroup of plastic fragments and the term is used to characterise fragments which are less than 1µm in length.(Gigault et al. 2018) These fragments can cause bioaccumulation in the food chain and ultimately consumption by humans due to their small size. This bioaccumulation is the result of plastic fragments being consumed by marine and terrestrial wildlife, which is then consumed by humans, as well as unfiltered fragments introduced into water mains which accumulates in several food chains and food webs. (Cole et al. 2011) Further studies have also shown that a large and neglected effect of microplastics is also prevalent in terrestrial environments, most notably those in agricultural and farmland soils, which increases likelihood of contamination of plants and domestic farm animals by these pollutants. They are often leached from wastewater plants and can pose a risk of pathogenic microorganisms and harmful chemicals to be introduced into the food chain. This has been shown through studies of gut contents of several organisms, such as birds and earthworms. In the case of birds which display foraging behaviour, 94% of those analysed had ingested microplastics. (Zhao, Zhu, & Li, **2016**).

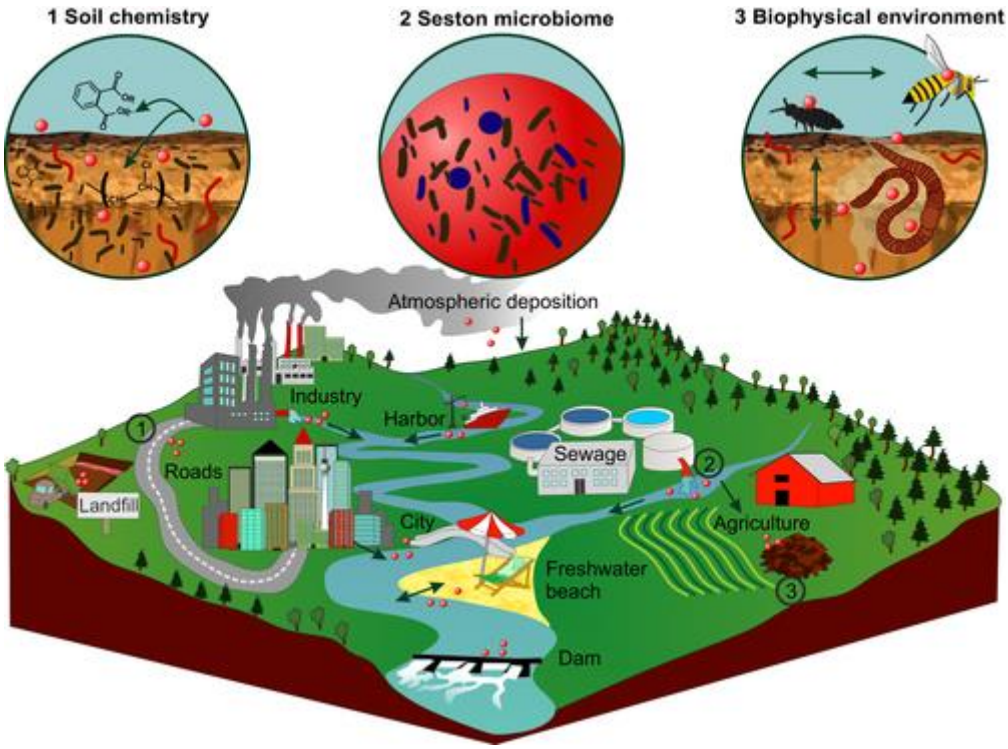


Figure 1: A diagram showing different ways in which microplastics can leach and enter different ecosystems. The red dots in the diagram symbolise microplastic concentration points. The three circles show the different factors which can be affected by microplastic presence. (de Souza Machado et al. 2018) Seston microbiomes refers to the micromaterials in a body of water, these micromaterials can be classified as both organic and inorganic.

Several chemicals involved in the synthesis of plastics has been known to be leached following long periods of sedentation. One of these chemicals are classified as endocrine disrupting chemicals (EDCs), these are involved in the production of plastics as antioxidants and packaging stabilisers. Examples, include octylphenol, nonylphenol, and bisphenol A. (Gao et al. 2015) EDCs are a concerning group of chemicals, as they are known to disrupt endocrine pathways, by mimicking oestrogen and binding to oestrogen-specific receptors, resulting in abnormalities of cell growth, apoptosis, and cell migration, which has been linked to the development

of cancers, among other pathologies.(Wright and Kelly 2017) Other leached chemicals which have a cause for concern is antimony, this chemical has been shown to be released by plastic, as it is often used as a catalyst during the production of plastic in the form of antimony trioxide (Sb_2O_3) alongside antimony acetate and antimony glycolate. Antimony has been shown to be toxic following long exposure to this chemical, although the exact long-term effects are still being researched this compound has been linked to disrupt several organ functions and is also thought to be carcinogenic.(Filella 2020; Sundar and Chakravarty 2010)

Separation of microplastic fragments from cells and tissues to gain a deeper understanding of their effects has been difficult to achieve, hence novel methods to do this are still being researched. It has currently been shown that there is a potential of nanoplastics entering cells through various mechanisms. (Gopinath et al. 2019) This can lead to intracellular damage and conformational changes in proteins. In addition to this, plastic fragments can become reservoirs of pathogens due to their hydrophobic nature, hence increasing susceptibility of disease transference. (Campanale et al. 2020)

Overall, the impacts of plastic pollution span from general ecological disruption to being a factor in severe diseases (such as cancer), which is why the need to find a solution to this problem has become a worldwide cause for concern.

The largest industry use of plastic is packaging, and accounts for approximately 40% of annual production. This can be seen from the graph in figure 1 (Geyer, Jambeck, and Law 2017)

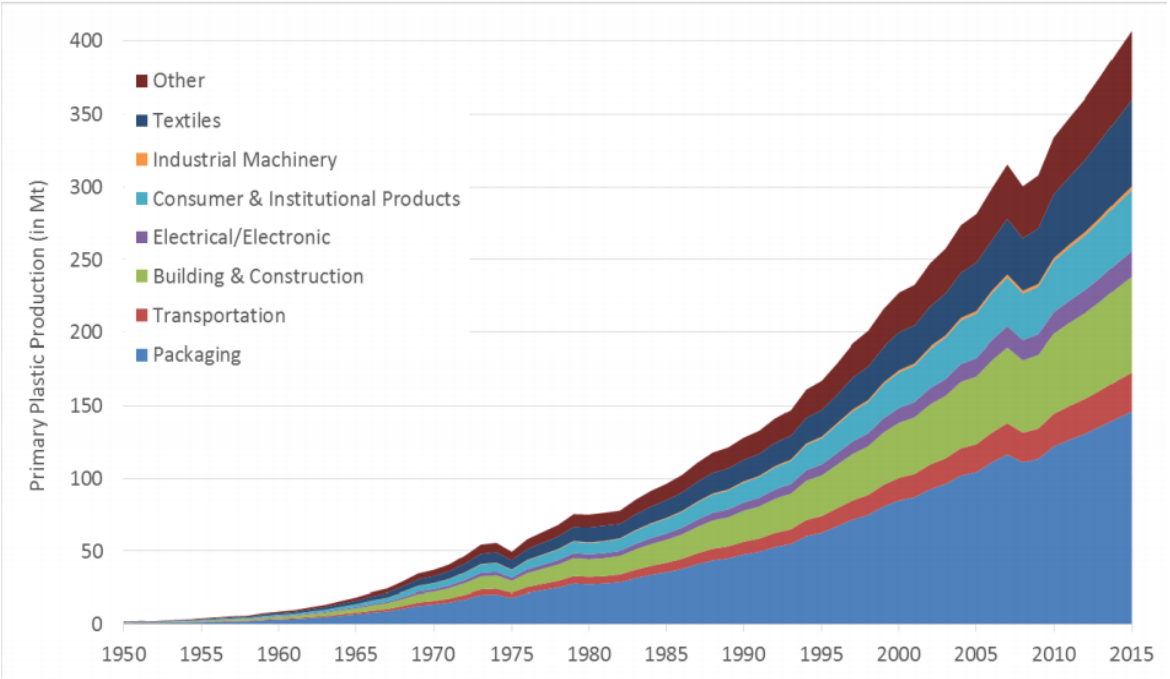


Figure 2: A representation of plastic production over a 65 year period, showing the proportions of each industry use. Each industry will have a different type of plastic which is most commonly used to suit the needs of that industry.

Polyethylene Terephthalate (PET) is the most commonly used plastic in the packaging industry. PET is classed as an aromatic polyester, due to its ring structure as well as ester linkages in between monomers. It is produced from two monomers: Ethylene Glycol (EG) and Terephthalic acid (TPA). These two monomers are extracted from crude oil production. PET is also used in the textile industry for the formation of fibres, subsequently called polyester, and in other manufacturing processes. There are two ways in which PET can be synthesised. The first is through the addition of dimethyl terephthalate to excess ethylene glycol in a transesterification process, shown in figure 2. (Köpnick et al. 2000)

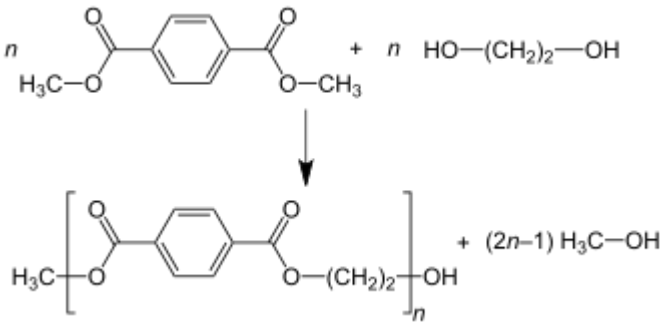


Figure 3: Transesterification of PET

The second method of synthesis is through polycondensation shown in figure 3, which involves the addition of terephthalic acid to excess ethylene glycol.

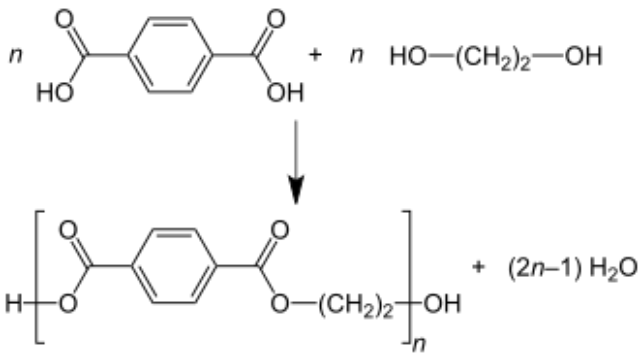


Figure 4: Polycondensation reaction of PET

After its synthesis PET can remain intact for several hundred years. UV rays and heat (above 300°C) can result in PET degradation; however, these are usually only surface-level changes. These changes were observed in accelerated weathering studies, and analysis of plastic collected from the environment, these showed that reduced crystallinity and increased surface hydrophilicity occurred following environmental exposure as the functional groups on the PET strands changed. (Ioakeimidis et al. 2016)

2.2 Biological degradation of plastics

Following the global concern of increasing plastic pollution, other novel methods for removing plastic from the environment have been investigated. Such as the Ocean Cleanup project set up by Boyan Slat, which uses engineering and technology to filter plastic from oceans and other large bodies of water. Recycling is usually the first solution that springs to mind, however these efforts seem to be insufficient, as only 12% of plastics are recycled annually. Hence biological degradation is being discussed and researched as a potential solution.

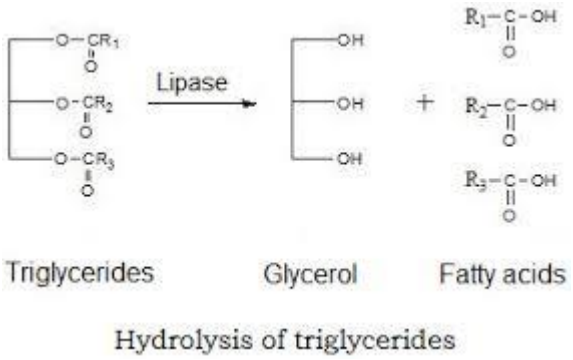
Natural polymers already exist in nature, this can be in the form of starch, wax, cellulose, cutin, and chitin, to name a few. Although these naturally occurring polymers are generally also resistant to enzymatic attack, some natural enzymes have evolved that can degrade them efficiently. This includes for example cutinases, which are secreted by plant pathogens in order to penetrate the waxy leaf covering (the cuticle) during the infection process. Plant waxes and plastics share common chemical features, such as being long hydrophobic polymer chains with varying levels of crystallinity, these often have ester bond linkages within the polymer chain as well as additional hydroxyl and aromatic groups. Due to these similarities, enzymes that can degrade natural polymers have been researched for applications into synthetic plastic degradation, specifically PET. (Joo et al. 2018)

Several factors should be considered before discussing the viability of enzyme degradation of synthetic plastics these are: crystallinity, molecular size, surface topography, and hydrophobicity. One of the mostly focused on factors is crystallinity, which is defined as the degree of long-range order within a material, hence the more ordered the polymer chains, the higher the crystallinity. PET crystallinity can vary between 8% to 40% depending on its industrial use. Fibres synthesised from PET

tend to have higher levels of crystallinity compared to packaging products. This factor is important to consider as a higher crystallinity would reduce the efficacy of enzymatic degradation as there would be fewer chains for the enzyme to access. PET is also highly hydrophobic and does not contain many branches, which further adds to the difficulty of enzymatic degradation as non-polar enzymes would face difficulties binding to the surface of PET. These are all factors which would have to be tackled when finding enzymes viable for the degradation of PET.

Currently there have been several applications of bacterial and fungal enzymes classified as polyesterases which have shown the ability to degrade PET. This ability is due to the presence of ester bonds within the polymer chain of PET which links the monomers in the chain. The hydrolytic enzymes which have been researched are classed as either cutinases or lipases. (Joo et al. 2018)

Lipases are found in all living organisms they are responsible for the breakdown of triglycerides into fatty acids and glycerol. These enzymes are necessary for the digestion and delivery of dietary lipids and are a sub-class of esterase enzymes. They are able to carry out this function through the cleavage of ester bonds found between the three fatty acids and the glycerol molecule, as shown in the figure below.



Hydrolysis of triglycerides

Figure 5: shows the enzymatic action of lipases.

Lipases are used commercially for several uses, including the application for biological detergents to remove fatty acid stains, oleochemical industry, food industry, biosensors, and cosmetics. Previously, lipases were extracted from animals, mostly through pancreatic excretions, however in recent years microbial lipases obtained from yeast and bacterial cells have been favoured. Microbial lipases have been shown to have a higher catalytic efficiency, easier to produce, and are more thermostable, these are useful qualities for industrial applications. (Patel et al. 2019)

Lipases have an alpha and beta hydrolase fold structure, they function through the use of aspartic acid, a histidine base, and a serine nucleophile at the active site to hydrolyse the ester bond in a similar fashion to chymotrypsin. One example of a lipase which was found to have the ability to cause surface modification of PET is from *Thermomyces lanuginosus.* (Khan et al. 2017)

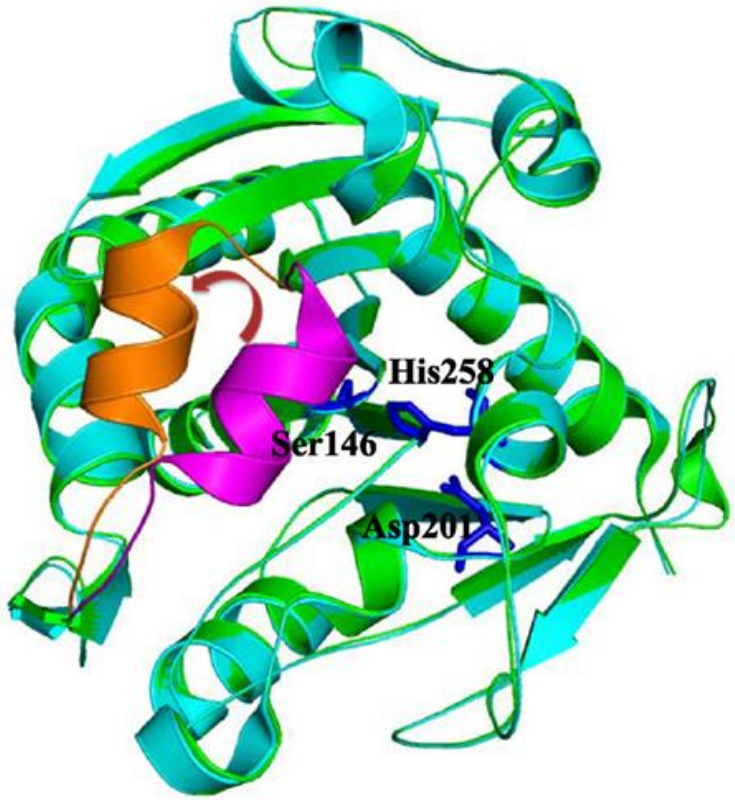


Figure 6: This ribbon structure diagram represents a lipase from *Thermomyces lanuginosus.* This schematic diagram shows the closed lid formation with the orange ribbon obstructing the catalytic triad of Ser-Asp-His.

The lid structure of lipases is the controlling factor of this enzyme group's catalytic activity. The activity is influenced by the environment in which the enzyme is in. Due to its lipid-water association, the lid remains closed when in highly aqueous environments and opens in areas with higher lipid concentrations. Hence, in the correct environment the lid opens and allows the substrate to bind to the active site of the lipase. Lid structures will vary in size depending on the function of the lipase, thermostable lipases have been shown to have larger lid structures, whereas mesophilic lipases display loops or helices rather than a lid. (Khan et al. 2017)

The second group of enzymes to have been researched is cutinases, these are usually synthesised by fungi and secreted out of the cell to aid in cutin degradation, found in plant cell walls. Although some bacterial species have also been found to

secrete this enzyme. Similar to the lipases, these also employ alpha-beta folds and have a conserved Ser-His-Asp triad at the catalytic site. (Skaf et al. 2018)

The most popular cutinase to have been researched into for the degradation of PET is derived from *Thermobifida fusca.* This enzyme is synthesised by the thermophilic soil dwelling bacterium. Several other bacterial and fungal cutinases as well as lipases have shown sequence homology with this specific enzyme. Apart from the characteristic arrangement of alpha helices and beta folds, this superfamily of enzymes possess a conserved catalytic triad which is assumed to aid with binding to a hydrophobic chain of polymers, the Ser^{170}-His^{248}-Asp^{216} triad which is thought to perform a serine hydrolase mechanism which involves a number of intermediate states. The structure of this enzyme is shown below in Figure 7. (Skaf et al. 2018) (Nikolaivits et al. 2018)

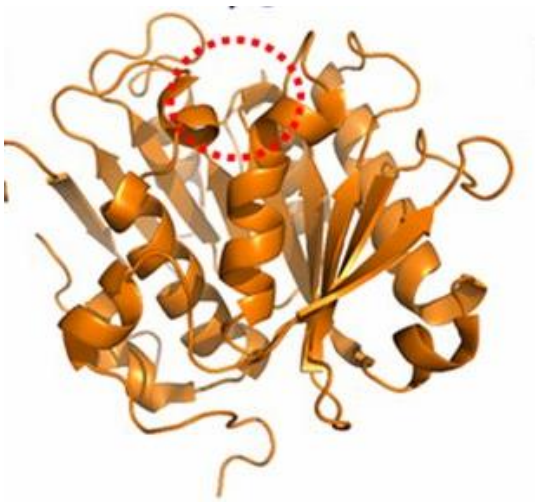


Figure 7 : *T. fusca* cutinase ribbon structure.

Cutinase enzymes are thought to overlap with lipase enzymes, which hydrolyse ester bonds in triglycerides to release fatty acids, however in comparison to cutinase enzymes these have a characteristic “lid” structure, a lack of this structure is what allows the serine residue to be exposed, which allows binding to the hydrophobic cutin. In lipases a conformational change is required within a partially aqueous environment in order to access polymer chains, known as “interfacial activation”. This leads to the hydrolysis of long chain, hydrophobic triglycerides. (Grochulski et al., 1993, Pleiss et al., 1998, Fojan et al., 2000).

In 2016 Oda’s lab in Japan isolated a PET hydrolase enzyme, dubbed PETase, from the novel bacterium *Ideonella sakaiensis.* The bacterium was isolated from sludge samples taken from the nearby recycling plant. This bacterium was found to assimilate PET which had leached from the plant nearby. It is thought that the high levels of PET within the environment of *Ideonella sakaiensis* resulted in the enrichment and evolutionary drive to assimilate and utilise PET as its main energy source. The bacterium is able to do so via a complex biosynthetic pathway. Firstly, both PETase and MHETase are secreted out of the cell and the PET is degraded by the joint action of these two enzymes into Terephthalic acid (TPA) and Ethylene Glycol (EG). The TPA produced is then transported into the cell via a novel tripartite aromatic acid transporter (TPATP). Once in the cell TPA is further catabolised into an open ring structure after oxidation by PCA dioxygenase. This pathway results in the formation of the co-enzyme NADPH, which is assumed to then be used in other metabolic processes within the cell. (Yoshida et al. 2016)

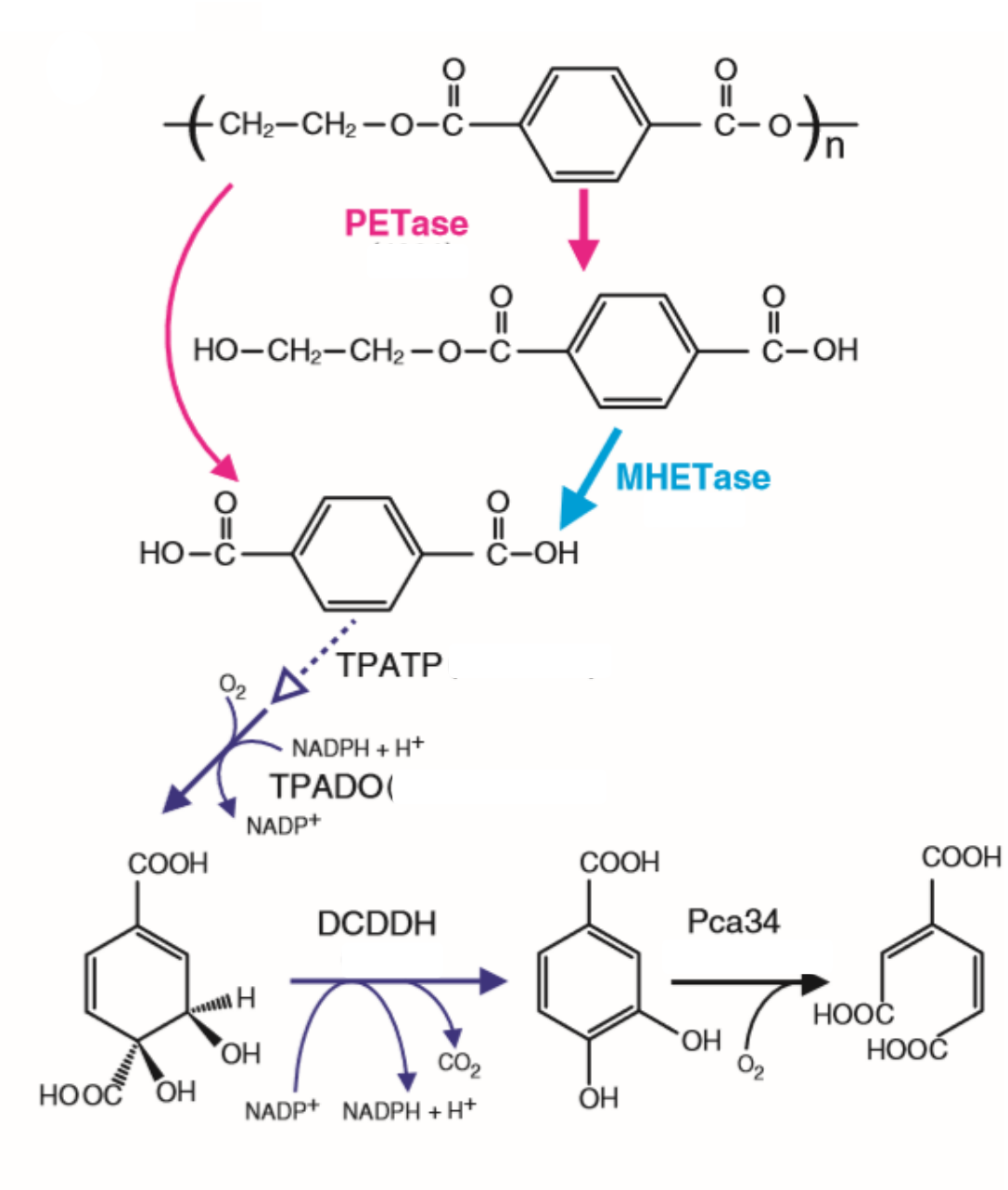


Figure 8: This diagram shows the metabolic pathway of PET following degradation by *Ideonella sakaiensis.* As shown in the diagram this pathway also includes the involvement of a second enzyme MHETase. The only by-product of the degradation which is then used by the bacterial cell is TPA. TPA enters the cells through TPATP where it is then converted to a tri-carboxylic acid. The pink arrows show the by-products of PETase degradation. The blue arrows show the by-products of

MHETase degradation. The dotted arrow represents the entry of TPA into the cell via TPATP.

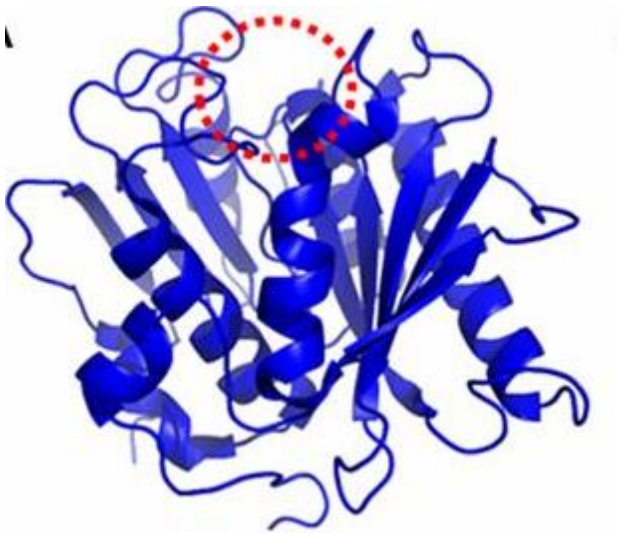


Figure 9: *Ideonella sakaiensis* PETase ribbon structure with a ring showing the catalytic triad. (Skaf et al. 2018)

Upon further investigation it was found that the structure of PETase was similar to that of the α/β hydrolase family. PETase possessed the same number of a/B helices and folds as well as an additional disulphide bridge, by the active site and at the C terminus of the protein, which is thought to increase thermostability and attachment to the PET chains. PETase holds a 52% structural homology with *T. fusca* cutinase, as well as sharing a conserved catalytic triad.

Although there are conserved similarities, other differences in the PETase structure allows increased PET degradation. One of them being the dipole which is spread over the entire enzyme surface, compared to the distribution of acidic and basic residue hotspots within the cutinase. This leads to a change in the isoelectric point, with PETase having a higher PI. This aids with binding to the hydrophobic surface of polyester chains of PET. This is shown in figure 10 below. (Kawai, Kawabata, and Oda 2019)

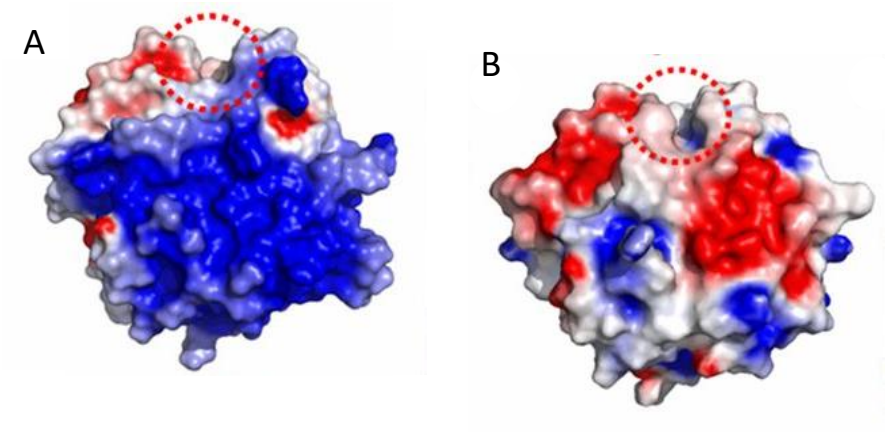


Figure 10: This shows the differences in charges between PETase (A) and *T. fusca* cutinase (B). The electrostatic potential distribution mapped to the solvent-accessible surface of PETase compared with the *T. fusca* cutinase as a colored gradient from red (acidic) at −7 kT/e to blue (basic) at 7 kT/e (where k is Boltzmann's constant, T is temperature and e is the charge on an electron). The dotted circle shows the active site cleft. (Austin et al. 2018)

When observing the ability of PETase to degrade PET films, reduced crystallinity was shown to have a better degradation rate. This is due to the chains being less stacked and allows increased interaction between ester bonds and the enzyme's catalytic site. These factors also resembled the action of other polyester degrading enzymes.

Since the discovery of PETase there have been several applications of enzyme engineering to enhance the activity. One of them being from the Beckham lab, which engineered a double mutant which showed increased degradation of PET films. This was through the substitution of two residues at the active site, where Tryptophan at position 159 was substituted for a Histidine residue and Serine at position 238 was substituted for a Phenylalanine. These substitutions changed the electrostatic interactions between other residues within the active site, which resulted in the

narrowing of the active site cleft and improved the degradation of PET. (Skaf et al. 2018)

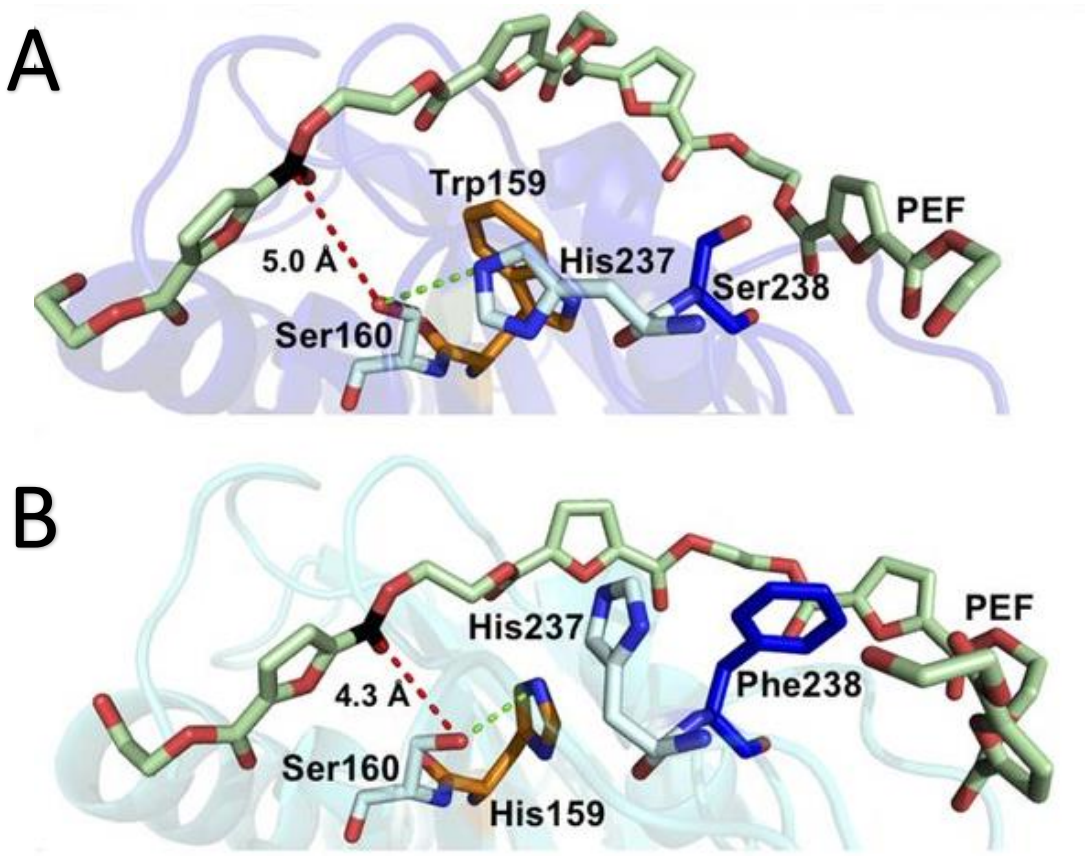


Figure 11: This image shows a schematic representation of the differences in the residues in the active site between the wild type PETase (A) and the double mutant (B). As well as the change in the size of the opening. (Skaf et al. 2018)

In addition to engineering the enzyme, other labs have been researching the application of using better understood host organisms which would be able to synthesise the enzyme to suit different conditions. The first example is the production of PETase in *E. coli* using sec-dependent signal peptides carried out by *Seo et al*. In this study, they were able to transform the bacterial cells by using an expression vector with the PETase gene in addition to signal peptides to promote the translocation of the protein using of maltose/maltodextrin binding periplasmic

protein, which is used in the export of maltose in bacterial cells. Using this secretory pathway, the group were able to extract and purify between 3.0mg to 6.2mg of PETase per 1L of culture media. They were also able to show that the secreted form of the protein retained activity when compared to the cytosolic protein without the added secretory peptides. Using HPLC the production of MHET and terephthalate, the by-products of PET degradation, were measured as an indicator of enzyme activity. (Seo et al. 2019)

A second group who used bacteria as a host for recombinant protein secretion of PETase was *Huang et al.* In this group *Bacillus subtilis* was used as a result of being previously used to produce large quantities of heterologous proteins which are used to degrade pollutants such as organophosphates and arsenic, another reason for choosing this organism is its lack of outer membrane, which in theory would increase the chances of secretion as a result of fewer barriers to leave the cell. One difference in this study compared to *Seo et al* was the secretory pathway used and they observed that the by removing TAT-dependent signal peptides in addition to the Sec-signalling peptides increased secretion almost 4-fold. This was speculated to be the result of PETase being secreted independently of the TAT pathway, as well as the preprotein being negatively impacted by TAT chaperone proteins. One negative aspect which was highlighted by this study was the effects of culture temperature on the activity of PETase, as higher temperatures were thought to have a negative impact on the folding of PETase. Bacteria are usually cultured at 37 degrees which may have a negative impact on folding and decreasing this temperature would result in lower growth rates and reduced protein secretion.(Huang et al. 2018)

One group also looked at transforming an organism to produce the enzyme recombinantly, however this time microalgae was used instead of bacteria, to find an ecological use of the enzyme. In this study the photosynthetic microalga *Phaeodactylum tricornutum* was used as a chassis for PETase production and grown in a saltwater environment to observe the breakdown of PET within this growth media. They observed that lower densities and smaller sized fragments of PET were degraded more efficiently. SEM imagery and concentrations of the by-products MHET and TPA were used to analyse the efficiency of degradation by the enzyme. (Moog et al. 2019)

Overall, these studies show that a wide range of organisms can be transformed to secrete working forms of PETase and that the treatment of plastics with the enzyme is viable, however all the studies noted that the efficiency of the enzyme could be further improved, and several factors should be considered when growing the organisms, such as temperature and media. This leads to further work required to be done to improve efficiency and activity of PETase secretion by different host organisms.

2.3 Applications of *Saccharomyces cerevisiae* in Biotechnology

Yeast is a group of organisms which fall under the Fungus kingdom of which there are at least 150,000 species. They are eukaryotic unicellular organisms which are able to reproduce asexually via a budding process. They can sometimes form multicellular structures by producing hyphae, although this does not account for all species. When yeast is mentioned this usually refers to *Saccharomyces cerevisiae,* also known as bakers' yeast. However, other commonly used yeast species include *Candida albicans* and *Komagatella phaffi* (previously known as *Pichia pastoris*), *Candida* species are typically used in research on anti-fungals, biofilms in

pathogenic strains, and metabolic processes, whereas *Komagatella* species have been used in research for the production of foreign heterologous protein production, similar to *S. cerevisiae.* (Tran et al. 2017) (Żymańczyk-Duda et al. 2017)

Following their full genome analysis, yeast have been used in several different aspects of biotechnology, mostly in the realm of synthetic biology, and the production of pharmaceutical and heterologous proteins in several industries. They have also been utilised to research cellular processes in human cells, to understand metabolic pathways involved in the cause of human pathologies. They have also been used in remediation of water waste. Yeast have been vastly utilised for a host of reasons. Mostly due to the fact that several techniques have been developed to easily genetically engineer yeast, as well as being easy and cheap to grow. Additionally, due to being eukaryotic cells they can form post-translational modifications and easily export proteins, which is something that bacterial cells are unable to do and hence need an in-vitro process following protein expression to add these in, especially when the protein in question is from a eukaryote. Furthermore, they can produce a high yield of highly productive proteins, whilst remaining easy to grow and store. (Mattanovich, Sauer, and Gasser 2014)

2.4 Recombinant protein expression

Yeast have been utilised for the production of heterologous proteins for medical and scientific research for several years due to the several advantages they have as host organisms. Recombinant protein expression is achieved through genetically modifying standard strains of yeast using vectors containing the gene of interest, CRISPR/cas9, or using drug resistant markers to tag or delete a gene. Firstly, there are three main types of vector plasmids which can be used: centromeric; episomal; integrative. Centromeric and episomal plasmids do not result in the gene of interest

being incorporated in the genome of the yeast, whereas integrative plasmids do. Hence, when transforming yeast with non-integrating plasmids selection markers are necessary. Usually cloning work is undertaken in *E. coli;* thus all of the plasmids contain an *ori (*origin of replication*)* site for maintenance in the bacterial cells. *Ori* sequences also determine the number of copies made for the gene, the most commonly used one is *ColE1* which allows for a high copy of genes. (Panchal 1987)(Taxis and Knop 2006)

Yeast have been used in the synthesis of several heterologous protein secretion as extensive research has been carried out to understand their secretory pathway. Yeast employ two main methods of translation and translocation when synthesising proteins. One is post-translational translocation, whereby the newly formed peptide is translated by the ribosome and then transported into the ER. The second is co-translational translocation, in this case the peptide chain is translated whilst in contact with the ER translocon pore. (Delic et al. 2013)

Leader peptide sequences are vital in the recognition and translocation of newly synthesised peptide chains. The most common being MAT-α (alpha mating factor). This sequence is formed of a 19 amino acid chain called the pre-signal, and a 67 amino acid chain called a pro-signal. Following transcription, the pre-signal is vital for its interaction with the signal recognition particle, which results in the subsequent translocation of the nascent peptide to the endoplasmic reticulum (ER). This part of the leader sequence is recognised as a molecular chaperonin which aids in the nascent peptide to be transported to the correct part of the cell during synthesis. Following translocation to the ER the pre-peptide is cleaved by signal peptidases within the ER. And is then packaged into vesicles by COPII. The MAT-α leader sequence results in a post-translational translocation. This has certain limitations as

nascent proteins which require folding within the cytosol can be poorly secreted as a result not being efficiently translocated through the ER. Aggregation can also occur if the protein self-associates during translocation. (Aggarwal and Mishra 2020)

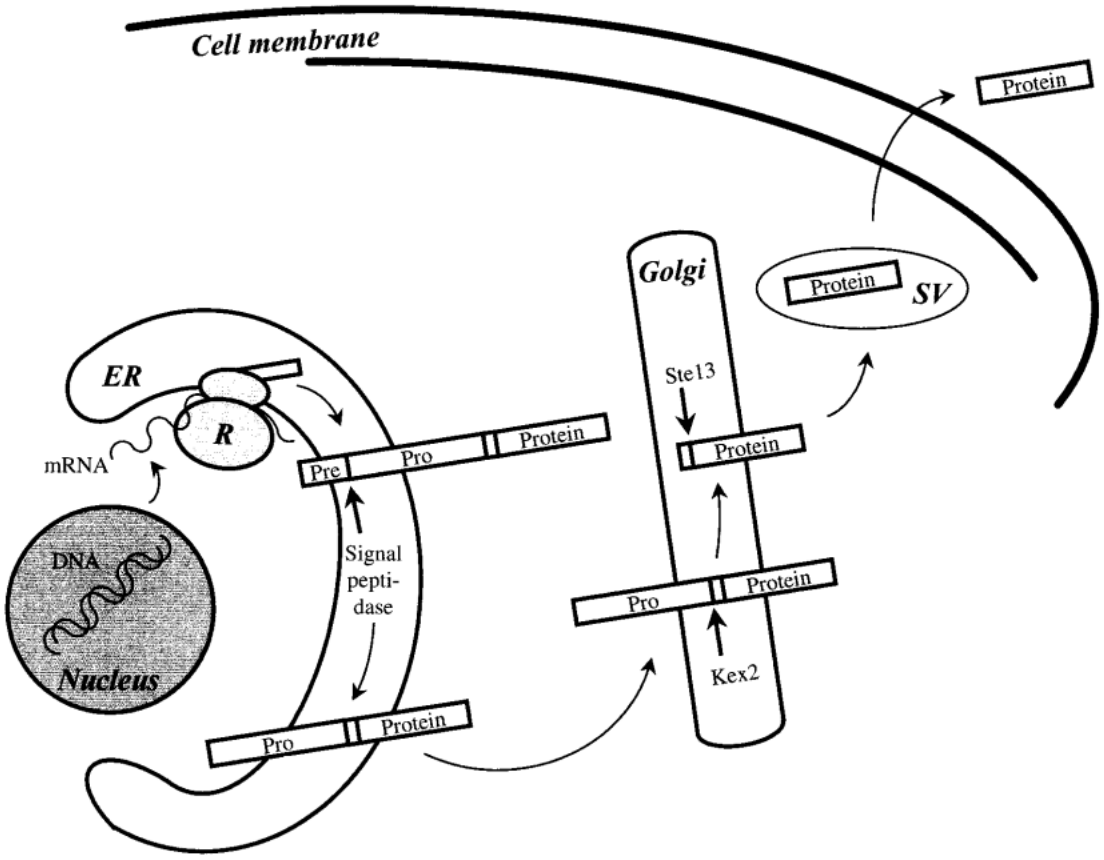


Fig 12: A schematic diagram representing the secretion pathway of a nascent protein with the alpha-mating leader peptide. R- Ribosome. ER- Endoplasmic Reticulum. SV-Secretory Vesicle (Ostergaard, Olsson, and Nielsen 2000)

To combat the issues with post-translational translocation another leader peptide sequence OST1 has been employed. This leader peptide sequence is often used to by-pass issues with nascent peptide processing seen in post-translational translocation. This leader sequence does this by utilising a co-translational translocation mechanism whereby the nascent peptide chain is synthesised as the protein moves through the ER, which then allows folding of the protein after being

transported through the ER. This reduces any misfolding and aggregation. The OST1 sequence is able to direct this due to being hydrophobic enough. (Barrero et al. 2018)

In several studies both OST1 and the Mat-α leader sequences were used to increase heterologous protein production in *Saccharomyces cerevisiae* and *Pichia pastoris.* (Fitzgerald and Glick 2014)

Although the two different leader sequences can be used to improve secretion of recombinant protein production in yeast, several other factors within the protein expression machinery can have an impact on secretion, which still requires some more research. In addition to the different secretory pathways used, other factors can impact protein secretion by yeast, including growth conditions, nutrient supply, and aeration, to name a few. (Delic et al. 2013)

2.5 Adhesion and formation of Biofilms by *Saccharomyces cerevisiae*

Yeast cells have the ability to attach to surfaces and other cells through the use of adhesins found on their cell walls. This allows cells to remain adhered to an area with high levels of nutrition, or to shield and protect the cells from a hazardous condition. We thought biofilm formation on plastics might be a useful mechanism for targeting PETase-secreting yeast to the plastic – hence our interest in this topic. These adhesins vary between species of yeast and can also depend on the pathogenicity of the yeast type. Factors affecting adhesion in yeast has been widely studied due to their presence in various industries, from food production, to fermentation, medical prostheses. Biofilm formation can either be a limiting factor or can be advantageous, depending on the industry.(Zara et al. 2020) In the case of medical prostheses, the formation of biofilms can lead to the growth of opportunistic

pathogens, such as *Candida albicans,* which can become drug-resistant, leading to persistent fungal infections in already immune-compromised patients. In the case of the fermentation industry, following growth of yeast, biofilm formation allows an easier method of separating the product of interest from the cell biomass which accumulates at the end. (Reynolds 2001)

Adhesion to surfaces by fungi is mediated by specialised surface proteins on the cell. These are able to adhere to other cells, as well as abiotic surfaces such as plastics, and silicone. Adhesins mediate extracellular binding of yeast cells, these are split between sugar binding adhesins which have a lectin domain able to bind to carbohydrate groups on other cell surfaces. The second group of adhesins are sugar-independent adhesins, these bind to peptides or they increase the cell-surface hydrophobicity, thereby promoting hydrophobic interactions between the cells and certain abiotic surfaces. (Verstrepen and Klis 2006)

The adhesion of yeast to a solid surface, as well as to each other provides a great advantage. Once they grow as hyphae and attach to one another they form an extracellular matrix, giving them increased cellular motility, nutritional sources, and protection. (Flemming and Wingender 2010)

Cell adhesion is mediated by several environmental factors, usually stress inducing. These include depletion of glucose/nitrogen sources; changes in ethanol concentration; changes in pH; activation of pathogenesis. These factors are then sensed by the cells and adhesion specific genes are activated. In *S. cerevisiae* adhesion (or flocculation) is mediated by the FLO genes, whereby FLO11 is responsible for adhesion to substrates and abiotic surfaces, and FLO1-10 are

responsible for cell-to-cell adhesion. In the case of the more pathogenic *C. albicans* cells adhesion is mediated by ALS and EPA genes. (Purevdorj-Gage et al. 2007)

The FLO11 gene has been shown to be activated by several signalling pathways. These include the TOR pathway, cAMP/MAPK pathways, and the Cyc8p/Tup1p complex. (Van Nguyen et al. 2020)

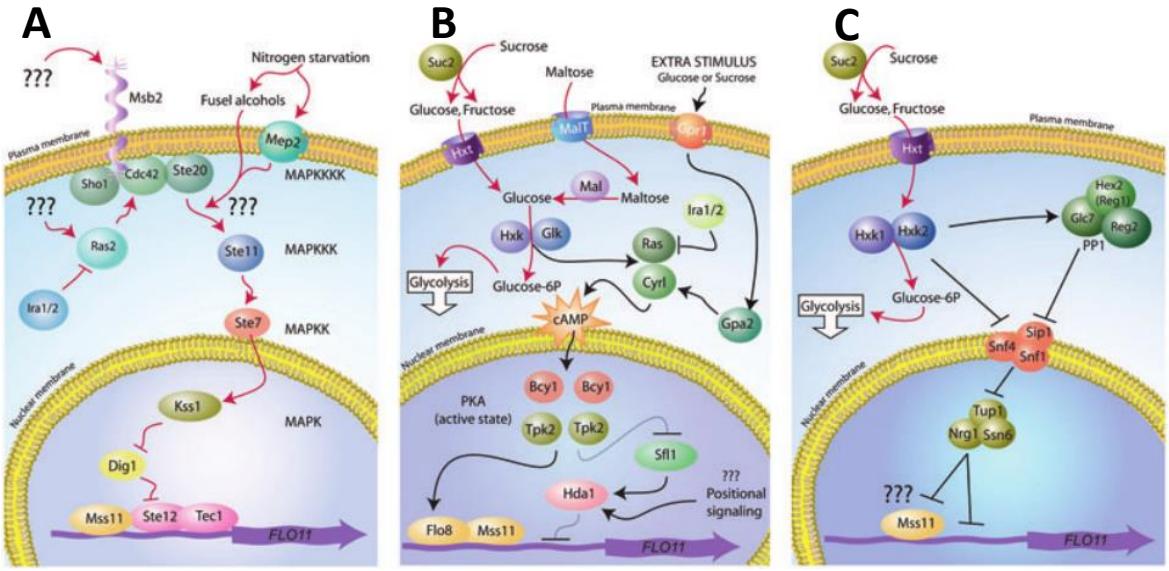


Figure 13: A diagram showing three studied signalling pathways which mediate FLO11 activation. (Left to right) (Verstrepen and Klis 2006) A- MAPK-dependent filamentous growth pathway. B- Ras/cAMP/MAPK pathway. C- Glucose repression pathway.

The first pathway shown is the MAPK-dependent filamentous growth pathway. This pathway is influenced by the depletion of nitrogen, which has a secondary effect of increasing fusel alcohols in the cell, and results in filamentous and invasive growth of cells.

The second pathway is the Ras/cAMP/MAPK pathway mediated by Glucose and sucrose levels in media available. This pathway is linked to stress, cell growth and glucose metabolism.

The third pathway is the glucose repression pathway, the reduction in glucose results in the activation of PP1 which stops the suppression of FLO11. It is thought that other yeast cells, such as *C. albicans* also use similar pathways to mediate adhesion.

These factors are important to consider when developing an industrially viable system of biologically degrading plastic, whereby the host organism (yeast) is able to secrete PETase directly onto the surface of the plastic. In order to have directed secretion by yeast, biofilms have been shown to be a stable and solid structure in which the yeast cells are able to proliferate in a stable manner whilst achieving the goal of degrading the plastic, which is the aim of this study. Hence it was necessary to explore all the components of achieving this system.

3. Materials and Methods

N.B All materials used are sourced from Sigma-Aldrich unless stated otherwise.

This section outlines the procedures and materials used for this project.

NAME OF CHEMICAL	ABBREVIATION	STOCK CONCENTRATION
2-MERCAPTOETHANOL	2-ME	2% (w/v)
ACETIC ACID	-	4M
AMMONIUM PERSULFATE	APS	40% (w/v)
BROMOPHENOL BLUE	-	Powder
ETHANOL	EtOH	>99% (v/v)
ETHIDIUM BROMIDE	EtBr	1mg/ml
ETHYLENEDIAMINETETRA ACETIC ACID	EDTA	0.5M EDTA, pH 8
METHANOL	MetOH	>99% (v/v)
SODIUM HYDROXIDE	NaOH	Powder
***TRIS*(HYDROXYMETHYL)AMINOMETHANE**	Tris-Base	Powder
***TRIS*(HYDROXYMETHYL)AMINOMETHANE**	Tris- HCl	Powder

Table 1: Lists the chemicals used in this project.

NAME OF BUFFER	COMPONENTS
4X LOWER TRIS	1.5M Tris-Base, 0.4% SDS, pH8.8
4X UPPER TRIS	1.0M Tris-HCl, 0.4% SDS, pH6.8
4X SAMPLE BUFFER	80mM Tris-HCl, 20% GlyOH, 2% SDS, 0.05% Bromophenol Blue.
ACRYLAMIDE SOLUTION (30% W/V)	29 Acrylamide:1 Bisacrylamide
ECL SOLUTION I	25mM Luminol, 0.4mM Coumaric Acid, 100mM Tris, pH8.5
ECL SOLUTION II	6mM H_2O_2, 100mM Tris, pH8.5
5X ISOTHERMAL BUFFER	5% PEG-8000, 500mM Tris-HCl pH 7.5, 50mM $MgCl_2$, 50mM DTT, 1mM each of the 4 dNTPs, and 5mM NAD
10X PBS	100mM PO_4^3, 1.37M NaCl, 27mM KCl, pH7.4
1X PBS-TWEEN	50mM Tris, 150mM NaCl, pH7.8, 0.05% Tween-20
SDS RUNNING BUFFER	25mM Tris-HCl, 180mM Glycine, 6.5mM SDS
1X TAE	40mM Tris, 20mM Acetic Acid, 1mM EDTA, pH7.5
1X TRANSFER BUFFER	180mM Glycine, 20mM Tris-Base, 4mM SDS

Table 2: Lists the buffers, and their components, used in this project

NAME OF MEDIUM	COMPONENTS
LYSOGENY BROTH (LB)	Difco Bactotryptone (1% w/v), Difco Yeast Extract (0.5% w/v), Sodium Chloride (1% w/v).
YEAST EXTRACT PEPTONE DEXTROSE (YPD)	Glucose (2% w/v), Difco Yeast Extract (1% w/v), Difco Bactopeptone (2% w/v).
SYNTHETIC COMPLETE (SC) URA DROPOUT	Glucose (2% w/v), Difco Yeast Nitrogen Base without Amino Acids (0.67% w/v), Yeast Synthetic Complete Drop-out Media Supplement -URA (0.2% w/v)

Table 3: List of media used to culture cells in this project.

3.1 Molecular Biology protocols

3.1.1 Oligonucleotide design

The following oligonucleotides were used to amplify the PETase gene in cloning procedures. The primers used contained sequences complimentary to the template sequence, as well as additional sequences that added restriction enzyme digest sites and complementary sequences to the cloning plasmid for Gibson assembly.

OLIGONUCLEOTIDE NAME	SEQUENCE (5'-3')
PTH798 FORWARD	GCGGCGAAAATTTCCGCGGCGGGTCGTCCGGTC***GACAACTTTCCGAGAGCGTCCAGATTGATGC***
PTH644 REVERSE	TCGAGGTCGACGGTATCGATAAGCTT***GCTTTTCATAGGGTAGGGGAATTTC***
PRS306 FORWARD	GGGCTGCAGGAATTCGATATCAAGCT***TCATTATCAATACTGCCATTTCAAAGAA***
PRS306 REVERSE	TCGAGGTCGACGGTATCGATAAGCTT***GCTTTTCATAGGGTAGGGGAATTTC***

Table 4: Lists the oligonucleotide name and sequence, nucleotides in bold italics represent the sequence complimentary to that of the target plasmid to be used in cloning.

3.1.2 Polymerase chain reaction (PCR) for DNA amplification

The PETase gene was amplified by PCR alongside the appropriate forward and reverse primers. These were pipetted into Eppendorf tubes on ice alongside other reagents before being processed by thermocycling.

Reagent	Volume from stock (µL)	Final concentration
GoTaq Green MasterMix	12.5	1X
Oligonucleotides	2	1.0 µM
Template DNA	1	<250ng
ddH_2O	9.5	-

Table 5: Lists components used in a PCR reaction to a total volume of 25µL.

Stage	Temperature (°C)	Duration (minutes:seconds)
Preheated Lid	105	5:00
Denaturing	95	2:00
Annealing	55	1:00
Extension	72	5:00
Hold	12	Forever

Table 6: Describes the standard settings used in PCR on a thermocycler.

3.1.3 TAE (Tris Acetic acid EDTA) DNA gel electrophoresis

Analysis of DNA was conducted by running a 1%(w/v) TAE Agarose gels at 80V and 200mA for 45-60mins. Ethidium Bromide (EtBr) was added at a 1% (w/v) concentration as an intercalating agent to later visualise and document banding patterns using a GeneSys Gel doc (Syngene).

3.1.4 DNA extraction from Agarose Gels

Following DNA analysis on an agarose gel, DNA was extracted and purified for future use. This method was used to remove any excess salts, oligonucleotides, and other components prior to homologous recombination. This was achieved using the QIAquick Gel Extraction Kit, QIAGEN following the manufacturer's protocol.

3.1.5 Restriction enzyme digest

Restriction enzyme digests of DNA were used to linearize plasmids in preparation for Gibson assembly, and for analysing products of cloning procedures for correct integration. All enzymes and buffers were provided by NEB. 50 µl reactions

consisting of 5 µl 10x buffer, 1 µl of each enzyme and 1-2 µg of DNA were used in for preparative digests, whereas 20 µl reactions consisting of 2 µl 1x buffer, 1 µl of each enzyme and 0.2-0.5 µg DNA were used for analytical digests. This was carried out using enzymes provided by NEB according to manufacturer's protocol.

3.1.6 Gibson Assembly

Gibson assembly reactions were a modification of those described by Gibson et al ((Gibson et al. 2009)**)**. The amplified gene of interest (PETase) was inserted into a linearized vector plasmid following this protocol. A Mastermix is made prior to addition of other components. The mixture was pipetted into an Eppendorf tube on ice, to prevent any denaturing, then incubated at 50°C for 1 hour. The mixture can then be stored in a freezer at -20°C for any future use.

Reagents	Volume (µL)	Stock Concentration
5x Isothermal Buffer	160	5X
T5 Exonuclease	3.2	1U/µL
Phusion Polymerase	10	2U/µL
Taq Ligase	80	40U/µL
ddH_2O	346.8	-

Table 7: Outlines the reagents used to prepare the Master mix used in the Gibson Assembly reaction.

Reagents	Volume (µL)
Master Mix	15
Linearized vector plasmid	2
Gene of interest	1
ddH_2O	2

Table 8: Lists the reagents and the quantities used in a single Gibson Assembly reaction with a total volume of 20µL.

3.2 Cell Culturing

3.2.1 Liquid and Agar media preparation

The different liquid media outlined in table 3 were prepared under sterile conditions, these were then autoclaved in a Prestige Medical Bench-top autoclave at 121^0C, 15lb/sq. Inch for 11 minutes. For solid agar plate preparation 2% granulated agar was added to the original liquid components prior to autoclaving.

3.2.2 Escherichia coli (E.coli) uses

The strain of *E. coli* used as a chemically competent cell in this project is outlined in the table below. These cells were grown either in liquid LB media or LB agar plates at 37°C either shaking or statically, respectively.

Strain	Genotype
***E. coli* Top10**	mcrA (mrr-hsdRMS-mcrBC) 80lacZ M15 lacX74 recA1 ara 139 (ara-leu)7697 galU galK rpsL (StrR) endA1 nupG

Table 9: Describes the strain name and genotype of the competent cells used in this project.

3.2.3 Transformation of chemically competent E. coli

Chemically competent *E.coli* cells were prepared using the CCMB80 method as outlined in the protocol by Hanahan et al(Hanahan, Jessee, and Bloom 1991). Following this, an aliquot was taken to thaw on ice for 20-30minutes, in the meantime a water-bath was prewarmed to 42°C. During the thawing process 1µL of mini-prep grade DNA or 8 µL of ligation reaction was pipetted into an Eppendorf tube and placed on ice. Once the competent cells had thawed 100µL of was added to the DNA on ice and left to incubate for 20-30 minutes. The mixture was then heat-shocked at 42°C in the pre-warmed water-bath for precisely 60 seconds, and then immediately placed on ice. Sterile LB media, 1-2ml, was added to the mixture and put to shake at 1800 RPM at 37°C for 30-60 minutes. The incubated mixture was then placed in a microcentrifuge for 5 minutes at 3000RPM, following this 100-200µL of the mixture was plated onto a selective plate and left to grow in a static incubator overnight at 37°C.

Successful transformants were inoculated into LB media containing Ampicillin at a concentration of 100mg/L and grown overnight at 37°C in a shaking incubator for subsequent DNA extraction.

3.2.4 Mini-Prep following competent cell transformation

Following transformation, individual colonies were picked into 2ml of LB medium and grown in a shaker at 37°C overnight. DNA was then extracted using the QIAprep Spin Miniprep Kit, QIAGEN, according to manufacturer's protocol.

3.3 Yeast growth

3.3.1 Strains used

This project was conducted using two strains of *S. cerevisiae* outlined in the table below. These strains were used as they are commonly used for recombinant production and both BY4741 and SK1 are strains known for high levels of secretion.

Strain	Genotype
BY4741	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*
SK1	MATa *ura3Δ0*

Table 10: Outlines the strains used in this project.

3.3.2 Growth conditions

Working cultures of yeast strains were taken from stock plates and grown in liquid media prior to use in experiments. Standard strains were grown overnight in YPD at 30°C in a shaking incubator, and then diluted and regrown to a logarithmic phase measured at an OD_{600} of 0.7-1.0.

Transformed yeast were plated onto -URA agar plates and incubated statically at 30°C overnight. Following this they were inoculated and regrown in the same manner as standard strains in liquid -URA media.

3.3.3 LiAc (Lithium Acetate) yeast transformations

The plasmid of interest was transformed into BY4741 and SK1 strains following the LiAc/PEG4000 method. All reagents were pipetted into a 1.5ml Eppendorf tube, containing a yeast cell pellet harvested from an overnight culture, in the quantities and order outlined in table 11.

Order	Reagent	Volume (µL)
1	50% PEG 4000	240
2	1M Lithium Acetate	36
3	1mg/ml Single stranded DNA	10
4	14.3M 2-ME	2.5
5	Plasmid	2
6	ddH_2O	69.5

Table 11: Outlines reagents used for the LiAc transformation for a total volume of 360µL.

Following this the mixture was vortexed and left to incubate for 20 minutes at room temperature. Meanwhile a water-bath was pre-warmed to 42°C, which the mixture was then placed in for a further 20 minutes. After incubation, the mixture was placed in a microcentrifuge for 2 minutes at 2000RPM and the pellet was resuspended in 200 µL of sterile water using a blue pipette tip to ensure that the fragile cells were not shorn. The resuspended cells were then spread onto a selective -URA agar plate and incubated statically at 30°C for 2-4 days.

3.4 Protein work

3.4.1 Yeast complete protein extraction

For analysis on SDS-PAGE gels, proteins were prepared as described in von der Haar, 2007 (von der Haar 2007) a heating block was pre-warmed to 96°C prior to starting. Logarithmically growing cells were pelleted in a microcentrifuge for 1 minute and maximum speed and the supernatant discarded. Extraction buffer was supplemented with 2% 2-ME. The pellet of yeast was resuspended in 200 µL of supplemented 2-ME and incubated at 96°C for 10 minutes. Following this 5 µL of neutralisation buffer was added to the mixture and vortexed for 30-60 seconds. Lastly 50 µL of loading buffer was added to the mixture.

3.4.2 Native protein extraction using Yeast Protein Extraction Reagent (Y-PER)

When extracting the recombinant protein without denaturing Y-PER, ThermoFisher, reagent was used with logarithmically growing yeast cells following manufacturer's protocol.

3.4.3 Preparation of secreted proteins from growth supernatants

Supernatant samples were taken from the transformants overnight media after centrifugation at 13000RPM for 60 seconds. From the supernatant 15µL was concentrated using a VIVA spin with a cut-off of 10kDa. Sample buffer was then added to the concentrate and analysed with SDS_PAGE gel.

3.4.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS_PAGE)

Proteins of interest were separated using a 12% SDS-PAGE which enabled high resolution for products with a molecular weight ranging from 10-70kDa. Two components were prepared to cast the gels: Resolving and Stacking. These were prepared using the reagents outlined below:

Reagent	**Volume**
Acrylamide:Bisacrylamide (29:1)	4.5 ml
Lower Tris buffer	2.7 ml
APS	40 (µl)
TEMED	5(µl)
ddH_2O	3.6 ml

Table 12a: Lists the reagents and volumes used to cast the Resolving gel

Reagent	**Volume**
Acrylamide:Bisacrylamide (29:1)	1 ml
Upper Tris buffer	1.75 ml
APS	40 (ul)
TEMED	5(ul)
ddH_2O	4.2 ml

Table 12b: Lists the reagents and volumes used to cast the Stacking gel

The resolving gel was first cast in an empty cassette, where it was filled to ¾ of the height and left to polymerise. Following this, the stacking gel was added to the cassette with a divider inserted and left to polymerise.

Samples were then added to the wells, alongside a protein plus ladder and run with TGE buffer for approximately 1-1.5 hours at 100V, 2.0mA.

3.4.5 Western Blot

The SDS-PAGE gel was then transferred by electrophoresis onto a nitrocellulose membrane cut to 9cm by 9cm, this was then assembled in a sandwich with components pre-soaked in Transfer Buffer in the following order: Paper, Nitrocellulose Membrane, Gel, Paper onto the Trans-Blot Turbo Transfer System, Bio-Rad at 2.5A, 25V, for 30 minutes. Following this the membrane was blocked for 1 hour in 5% milk/PBS-T. The primary anti-HA rabbit antibody was then added at a concentration of 1µL/ml and left shaking overnight.

After the overnight the membrane was washed with 1x PBS-Tween (PBS-T), and the secondary HA antibody was added at a concentration of 0.1µL/ml in 5% milk/PBS-T. This was left for 1-2 hours. Finally, the membrane was washed again with PBS-T.

3.4.6 Enhanced Chemiluminescence Detection of Horseradish Peroxidase (ECL)

The nitrocellulose membrane containing the protein was developed with equal parts of ECL solution 1 and solution 2. This was used to detect Horseradish Peroxidase (HRP) conjugated antibodies. The signal was then visualised using the GeneSys Gel Doc software.

3.5 Plastic Work

3.5.1 Biofilm formation of Saccharomyces cerevisiae

Both strains of *S. cerevisiae:* BY4741 and SK1, were diluted to an OD of 0.1, measured at 600nm (OD_{600}) following overnight growth in YPD at 30°C. The cells were then grown to a static phase after 4 hours of incubation. Prior to this PET fragments, obtained from Innocent Smoothie™ bottles, were cut into 1cm^2 pieces and incubated in 70% ethanol overnight for sterility. A used smoothie bottle was used to simulate plastic waste in the environment and the impacts of PET degradation on non-standardised PET. Ethanol was used to sterilise the plastic as PET is resistant to degradation by ethanol if stored for a short period of time. The PET fragments were then washed in sterile ddH_2O to remove any residual ethanol prior to incubation with *S. cerevisiae.*

After incubation yeast cells were grown to static growth, 1ml of cell cultures were added to fully immerse each PET fragment in a 24 well plate. This was done for both BY4741 and SK1 strains in separate plates. The individual plates were then left to incubate at 30°C overnight.

Following overnight incubation, the PET fragments were removed from the wells and washed in 1x PBS solution prior to microscopy analysis.

To observe cells, an Olympus IX81 inverted microscope was used. The light source was provided by a CooLED pE4000 illumination system, and all images were captured with an Andors Zyla 4.2 PLUS sCMOS camera. Cells were viewed using an olympus 100x objective lens. A small droplet of Olympus Immoil- F30CC immersion oil was applied to the lens, and the sample slide inverted and placed upon the stage, and microscopy was carried out

3.5.2 PET hydrolase activity analysis

In order to assess the activity of recombinant production of *Saccharomyces cerevisiae* derived PETase production a series of PET samples were treated with the enzyme.

Intracellular PETase was extracted from previously transformed BY4741 strains using the extrachromosomal plasmid pTH644_mPETase. This was achieved by

using Y-PER reagent from Thermo-Fisher according to manufacturer's protocol. A total volume of 500µL of extract was added to PET fragments for activity analysis.

PET samples were cut to 1cm^2 size obtained from an Innocent Smoothie bottle and sterilised in 70% ethanol and left to dry overnight. Following the paper published by Furukawa et.al a group of the PET fragments were treated with 0.025% SDS and left to dry overnight, this application of SDS increases PETase adhesion by acting as a surfactant.

Following incubation with the enzyme the fragments were analysed using a scanning electron microscope. The SEM used was a Hitachi S3400-N scanning electron microscope. We utilised a variable pressure mode (with a gas pressure of approximately 50 Pascals) in order to image the non-conductive samples without needing to coat the samples. Images were taken in backscattered electron mode.

This Hitachi SEM also had the capability of detecting elements by using Energy Dispersive X-ray spectroscopy (EDX, EDS, XEDS). This method detects elements based on each element's specific excitation X-ray spectrum when exposed to a high energy beam of X-ray or electrons. For the samples used the integrated EDX function, and the spectra was analysed using the user interface created by Horiba, Ltd., Hitachi, Ltd., and Oxford Instruments, plc, as described in (YURUGI et al. 2001). The EDX was used to analyse the different elements within and outside of the pits observed on the surface of the PET being treated with the PETase.

The following table is a schematic representation of the 24 well plate used to incubate the 1cm^2 fragments of PET. Each fragment of PET was treated with either water (control), PETase, or a combination of PETase and SDS.

H_2O	**H_2O**	**H_2O**	**H_2O**	**H_2O**	**H_2O**
PETase + SDS	**PETase + SDS**	**PETase + SDS**	**PETase + SDS**	**PETase + SDS**	**PETase + SDS**
PETase	**PETase**	**PETase**	**PETase**	**PETase**	**PETase**
Blank	**Blank**	**Blank**	**Blank**	**Blank**	**Blank**

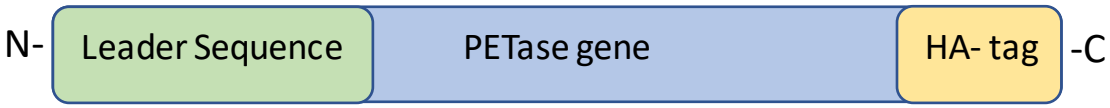
Table 13: A schematic representation of the 24 well plate and each component added to the PET fragment within the well.

4. Results:

4.1 Codon optimisation

An initial aim of my work was to express recombinant PETase in *S. cerevisiae* in a way that would result in the protein being secreted from the yeast cells. The first step to achieve this was optimising the original *I. sakaiensis* PETase sequence to fit with *S. cerevisiae* codon usage, this allows increased efficiency of protein transcription and translation. Codon optimisation was performed by replacing each codon with the one decoded most efficiently in yeast, using decoding time data published in Chu et al. (2014). This sequence was then inserted in a suitable vector to allow optimal secretion. The open reading frame was preceded by an optimised leader sequence consisting of elements from the yeast *OST1* and MAT genes, as described by Fitzgerald et. al(Fitzgerald and Glick 2014)

In addition to optimisation of codons the *S. cerevisiae* specific PETase gene was added an additional HA (haemagglutinin) tag at the C-terminus of the sequence to allow identification of the protein using an anti-HA antibody in future western blots.



4.2 Extrachromosomal plasmid transformations

4.2.1 pTH644_mPETase

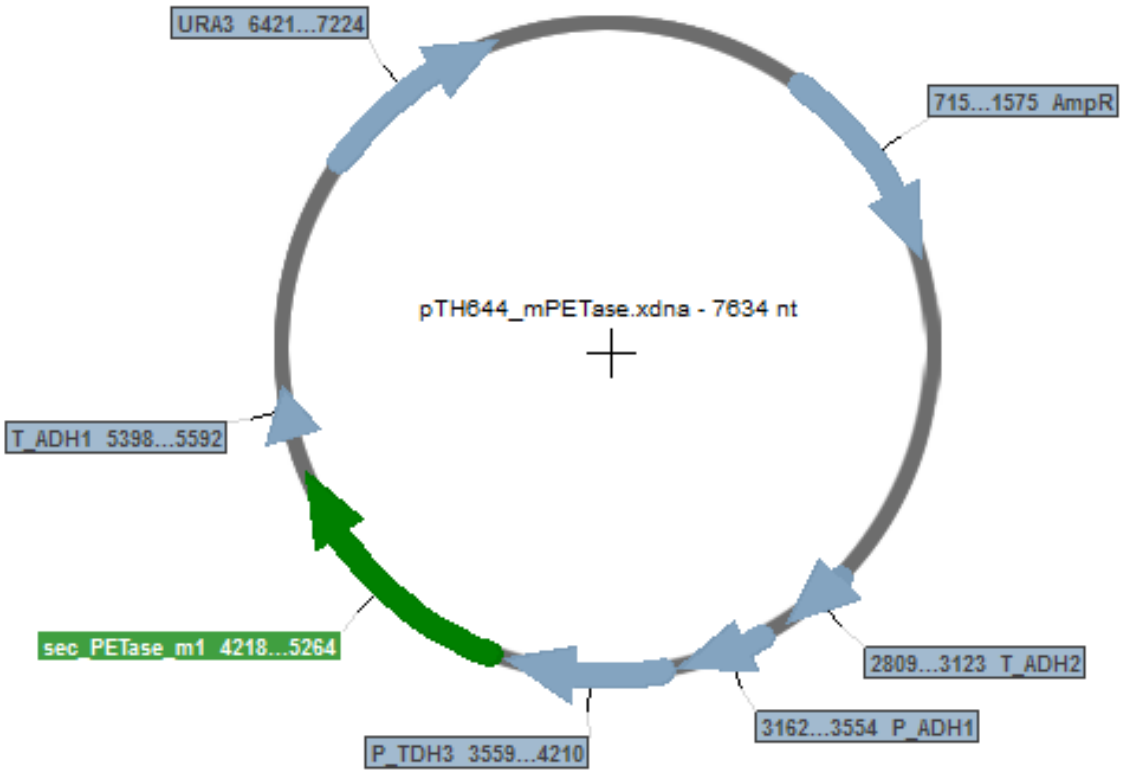
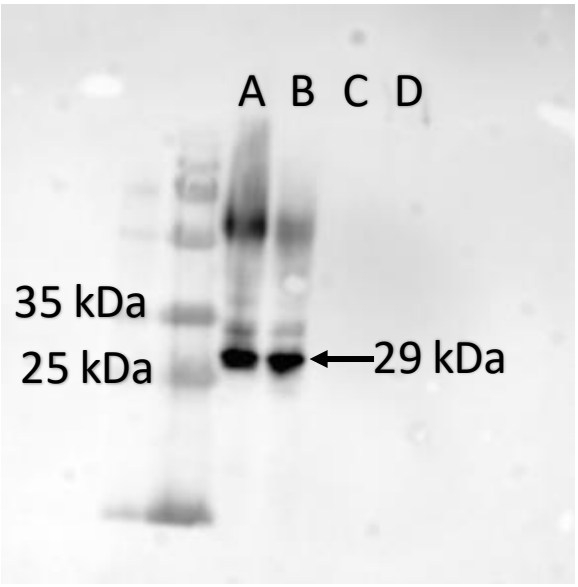


Figure 14: Display of the yeast centromeric plasmid vector pTH644_mPETase used to transform BY4741 and SK1 *S. cerevisiae* strains.

Figure 14 shows the first vector plasmid containing the optimised PETase sequence used to transform *S. cerevisiae* strains. This vector plasmid contains a leader sequence to allow secretion as well as other features to aid in the induction of the PETase gene and the selection of successfully transformed cells. This plasmid contains the constitutive promoter TDH3 of the highly expressed gene encoding glycerol-3-phosphate dehydrogenase (GPD), a significant enzyme involved in the Krebs cycles, lipid metabolism, and other respiratory functions of yeast cells. This makes GPD a strong promoter to utilise and increases efficiency of expression as this gene requires no external induction. This vector also includes the addition of the URA3 gene, which aids selection of transformed yeast cells in minimal media. Lastly the ampicillin resistance gene is also present for the selection of transformed bacterial cells during cloning steps.



A- BY4741 cell lysis products

B- SK1 cell lysis products

C- BY4741 supernatant

D- SK1 supernatant

Figure 15: Shows a western blot for the detection of PETase in two transformed strains BY4741 and SK1.

BY4741, a commonly used lab strain, and SK1, a strain that showed higher expression of secreted protein than BY4741 in previous work, were transformed using pTH644_mPETase, shown in figure 14, following the protocol outlined in (insert section of materials and methods). The two transformed strains were then grown in -URA media overnight. Supernatant samples were obtained from the overnight cultures. Intracellular protein samples were obtained from logarithmically growing cells following overnight culturing.

The samples were then tested using SDS-page gel separation and probed using an anti-HA antibody on a western blot. The western blot shows two bands between 25 and 35kDa in lanes A and C. This corresponds to the known molecular weight of PETase of 29kDa, confirming intracellular production of PETase in both the BY4741 and SK1 strains. However, no presence of PETase was observed for secreted protein in the supernatant for either strain.

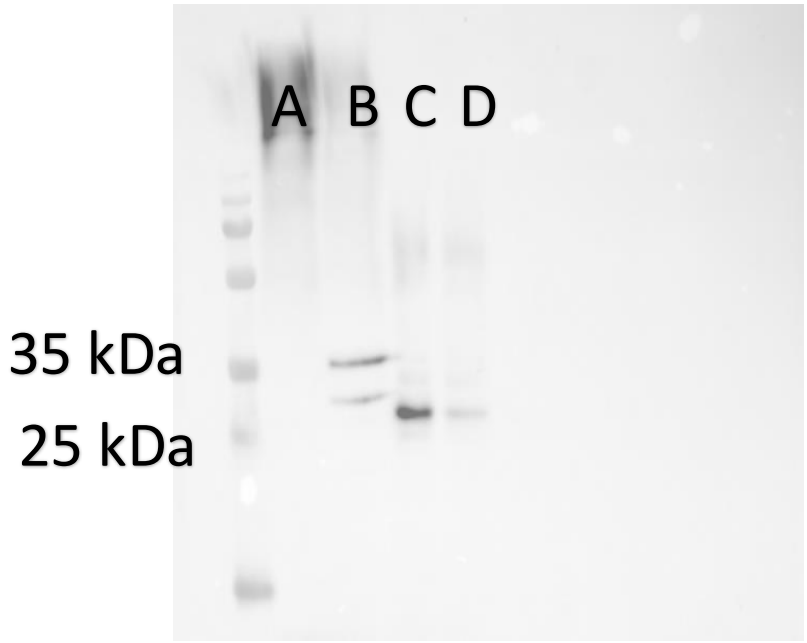


Figure 16: Shows a repeated western blot for the detection of PETase using pTH644_mPETase in BY4741 and SK1 strains.

Transformation of the two strains was repeated, in this case growth conditions were changed. Previous work in the lab had shown that secretion of some proteins is improved when grown in YPD rather than minimal media. However, using YPD with our vector system would be problematic as extrachromosomal plasmids are lost at rates of up to 5% per generation in the absence of selection. In order to minimise plasmid loss, we inoculated transformants from selective plates into YPD medium to a starting OD of ~0.1 and grew them to a stationary phase in a single round of growth overnight.

Following this supernatant samples from both overnight YPD cultures were taken and concentrated using a centrifugal concentrator with a molecular weight cut-off of 10kDa. This was done to increase the likelihood of protein presence in the samples. The protein samples were normalised by including a control sample which did not have the gene for PETase inserted.

Intracellular protein samples were obtained from logarithmically growing cells following overnight growth in YPD. A lower volume of cell lysis product was used in

the western blot analysis shown in Figure 16, compared to provide a clearer result by the reduction of lipid interference which can be seen in Figure 15.

At the top of band A, it is suspected that the secreted protein has aggregated, and hence resulted in a 'smudged' appearance. A higher band for PETase can be seen in lane B, corresponding to the supernatant of the SK1 strain, the apparent higher molecular weight is suspected to be the result of post-translational modifications to the PETase protein, such as glycosylation, previously observed in other studies. (Vieira Gomes et al. 2018) Or this could also be secreted protein with the leader peptide not having been cleaved prior to secretion. No secreted PETase is observed for the BY4741 strain. Similar to Figure 15, intracellular PETase is observed in both BY4741 and SK1 strains. This is shown in lanes C and D with a band shown between 25kDa and 35kDa.

4.2.2 pTH798_secPETase

Due to pTH644_mPETase not showing sufficient secretion, a second episomal plasmid was used and the optimised PETase gene was inserted into an empty pTH798 plasmid. This new plasmid had a different leader peptide sequence to pTH644_mPETase and we hypothesised that this change may result in improved secretion.

The PETase gene was amplified using PCR using pTH644_mPETase as template DNA, prior to inserting the gene into pTH798 using homologous recombination. A 1% agarose gel was then run to confirm the amplification of the PETase gene.

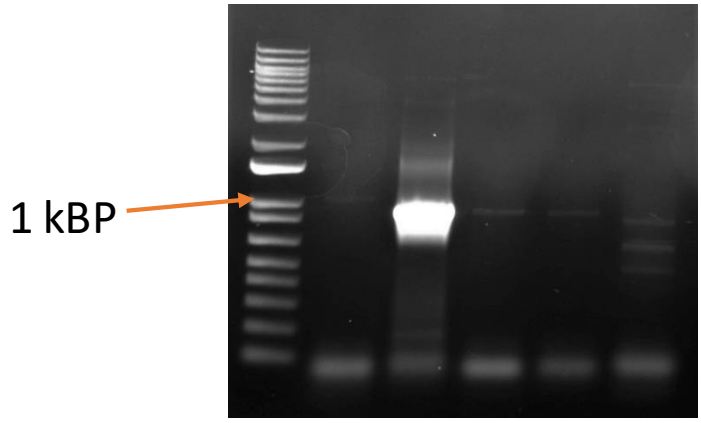


Figure 17: A 1% agarose gel image of bands seen at 1kBP, which was approximate the expected size of the PCR product.

Following this the two strains (BY4741 and SK1) were transformed using this new vector plasmid and a western blot was carried out to analyse PETase production both in the cell and as an extracellularly secreted product in the supernatant.

A- BY741 cell lysis products
B- BY4741 concentrated supernatant sample
C- SK1 cell lysis products
D- SK1 concentrated supernatant sample

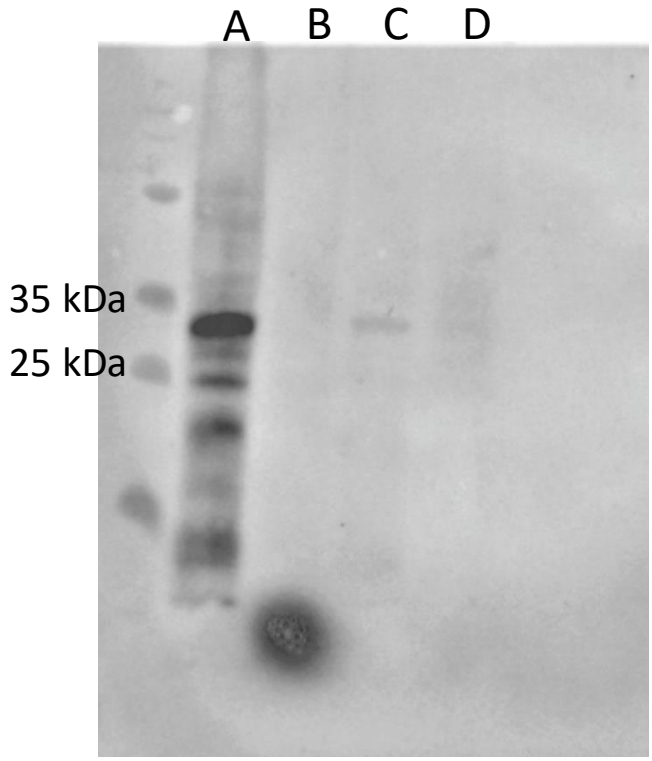


Figure 18: Shows a western blot for the detection of PETase in BY4741 and SK1 strains transformed with the pTH798_secPETase plasmid.

From this western blot we observed that there was a strong band between 25kda and 35Kda, which would be expected for the PETase gene in lane A, and a faint band at the location for lane C. This is the expected band size to be seen for the PETase gene.

No bands were seen for the supernatant samples, which suggests that the enzyme was not secreted out of the cell.

4.3 Integrated plasmid transformation

Following difficulties faced with the retention of extrachromosomal plasmids in rich media (YPD), alongside the necessity for optimal conditions to ensure the secretion of PETase, an alternative method of transformation was used. In this section we looked at creating a yeast integrative plasmid in order to overcome these issues and allow transformants to grow in YPD without losing the PETase gene.

To begin this a suitable plasmid was chosen, which did not contain an origin of replication (ORI), as well as important features to allow selection of transformants. The pRS306 plasmid is from the commonly used and well characterised pRS series of vectors from Sikorski and Hieter (Sikorski and Hieter 1989). The plasmid used is shown below.

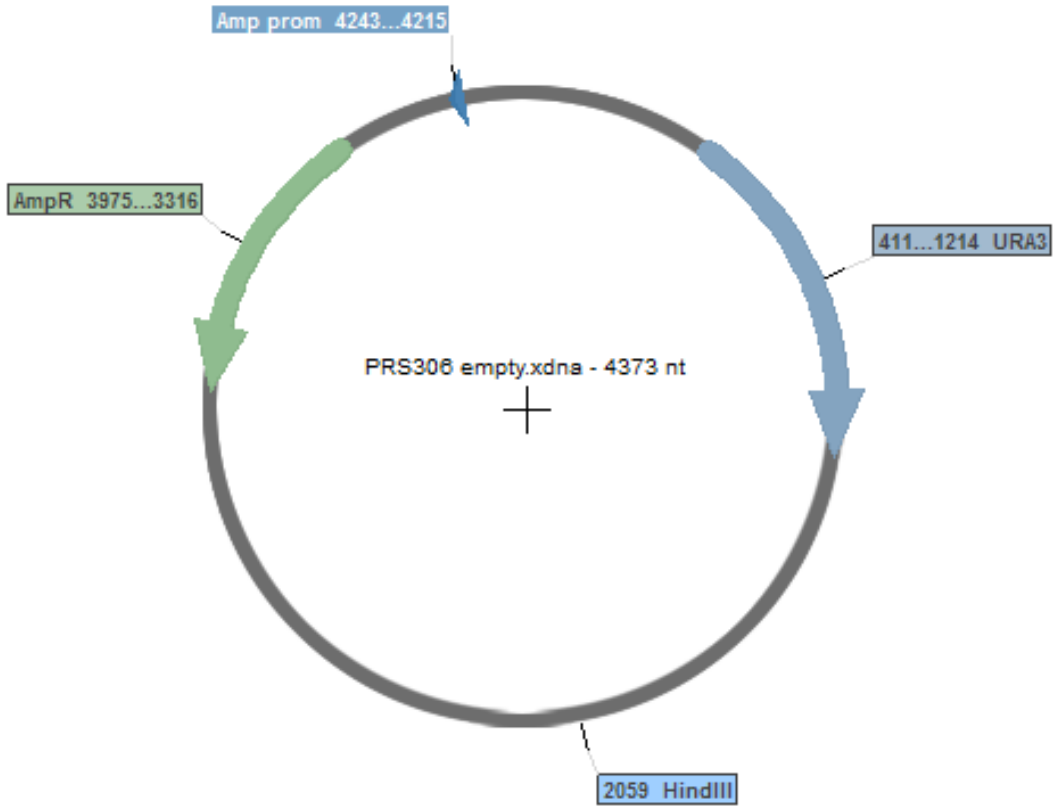


Figure 19: Display of the empty yeast integrative plasmid used to start a construct containing the PETase gene.

Similar to the extrachromosomal plasmids used, PR6306 contains an Ampicillin resistance gene to allow selection of competent cell transformants during cloning. The *URA3* gene is also present to allow initial selection of successfully transformed *S. cerevisiae* cells and allows integration into the locus of this gene. The (double

check) restriction site is used to linearize the plasmid prior to Gibson assembly and is used to confirm the correctly formed plasmid containing the PETase gene.

The optimised PETase gene, including the promoter and terminator, from pTH644_mPETase was inserted into PRS306. The promoter-gene-terminator cassette was amplified by PCR and additional ends were added to allow homologous recombination during a Gibson assembly reaction.

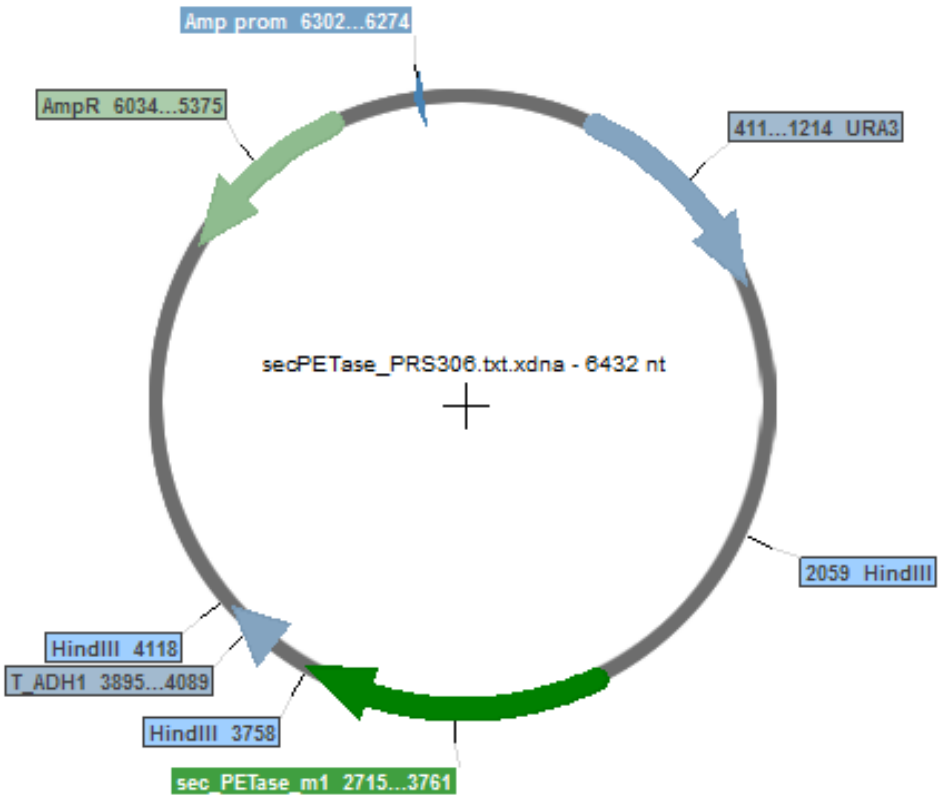


Figure 20: Display of the yeast integrative plasmid PRS306 now containing the secretory PETase gene, as well as additional HINDIII sites.

Following Gibson assembly, the newly formed plasmid was transformed into competent *E. coli* cells and the DNA extracted from successful transformants. To identify correctly cloned plasmids a sample was sent for sequencing as well as a restriction enzyme digest using HINDIII was also carried out to allow analysis of the plasmid on a 1% agarose gel.

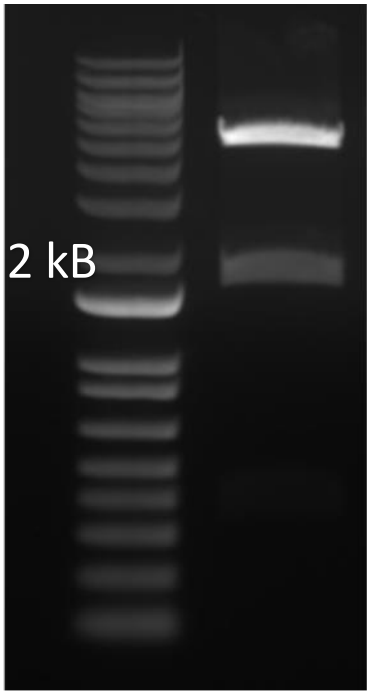
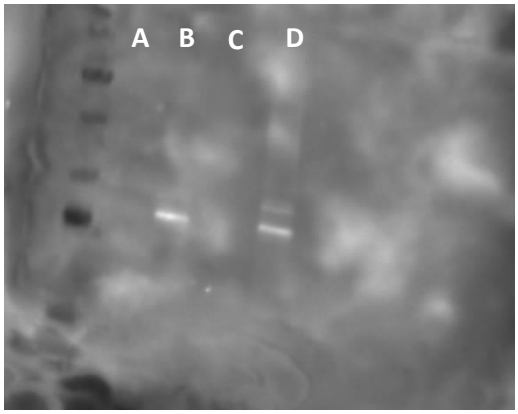


Figure 21: A DNA agarose (1%) gel showing two bands one at approximately 2kb and one between 5kb and 8kb.

The bands show a successfully cloned secPETase_PRS306 linearized integrative plasmid for the secretion of PETase after a restriction enzyme digest with HINDIII. The band at 2kb represents the secretory PETase gene. Although there were 3 restriction enzyme sites only 2 bands are shown as the third fragment which would have been generated is too small (360bp) to be seen on the gel.



A- BY4741 concentrated supernatant sample
B- BY741 cell lysis products
C- SK1 concentrated supernatant sample
D- SK1 cell lysis products

Figure 22: Shows a western blot for the detection of PETase in BY4741 and SK1 strains transformed with the secPETase_PRS306 integrative plasmid.

The successfully cloned integrative plasmid was then used to transform BY4741 and SK1 strains in the same manner as the extrachromosomal plasmids. In this case upon linearization the gene integrates into the yeast *URA3* locus.

Following transformation, the two strains were grown in YPD overnight before being tested for the protein. Supernatant samples in lanes A and C were concentrated using a centrifugal concentrator at a molecular weight cut off of 10kDa. Intracellular protein samples were obtained from logarithmically growing transformants.

In this western blot two bands were seen between 25 and 35kDa in lanes B and D. This confirms intracellular production of PETase in both the BY4741 and SK1 strains. However, no presence of PETase was observed for secreted protein in the supernatant for either strain.

4.4 Biofilm formation of *S. cerevisiae* on PET

With the goal of engineering *S. cerevisiae* to secrete PET hydrolase onto PET it was important to explore the adhesion of yeast onto PET to assess the viability of this being applied as a solution to plastic waste in the future. Hence a series of PET fragments were prepared, and yeast cells were inoculated into 24 well plates containing YPD media and a submerged fragment of plastic to test this. In this experiment both BY4741 and SK1 strains were tested to identify whether there would be a strain which showed an ability to adhere better. The following images represent a 48mm^2 area of the 1cm^2 PET fragment being analysed.

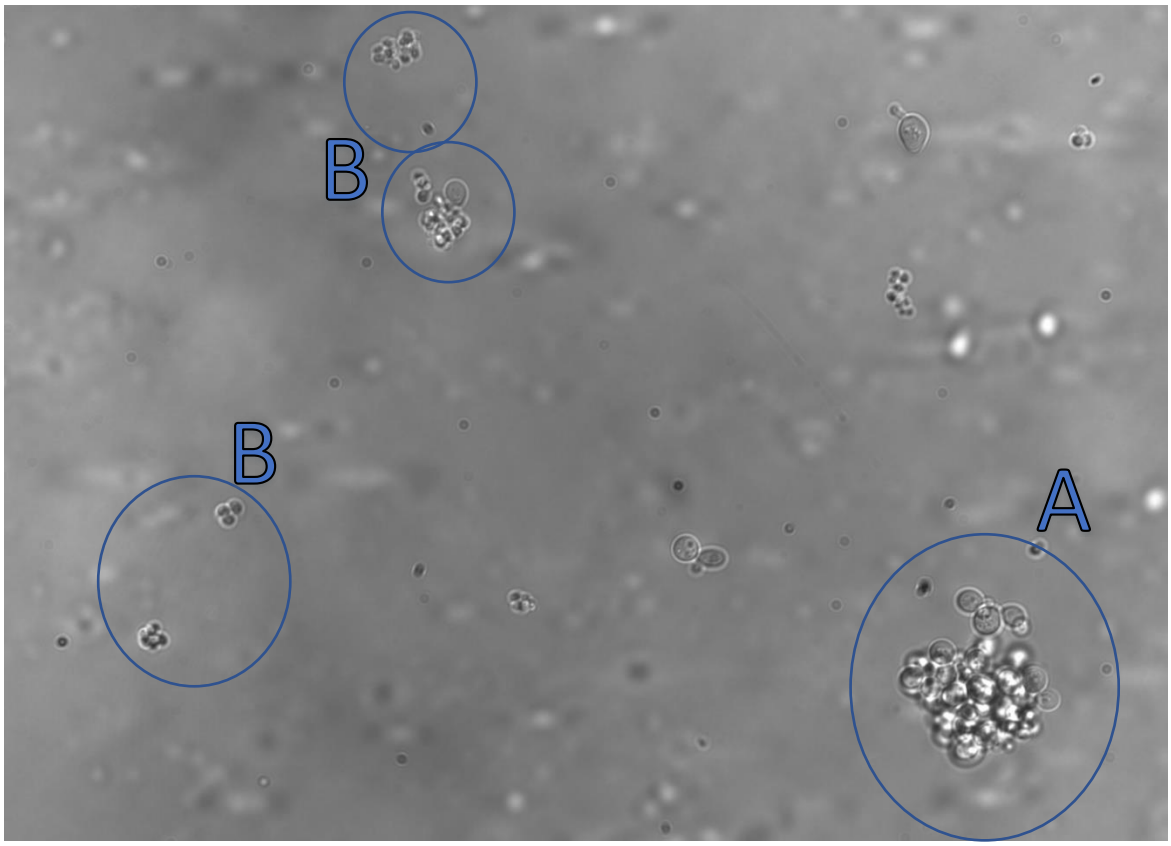


Figure 23: A microscope image showing clusters of BY4741 cells growing on a PET fragment.

Clusters of yeast cells were observed growing on this section of the PET fragment in the area circled in blue labelled A. Other smaller clusters are seen starting to form on the other parts of this fragment labelled B.

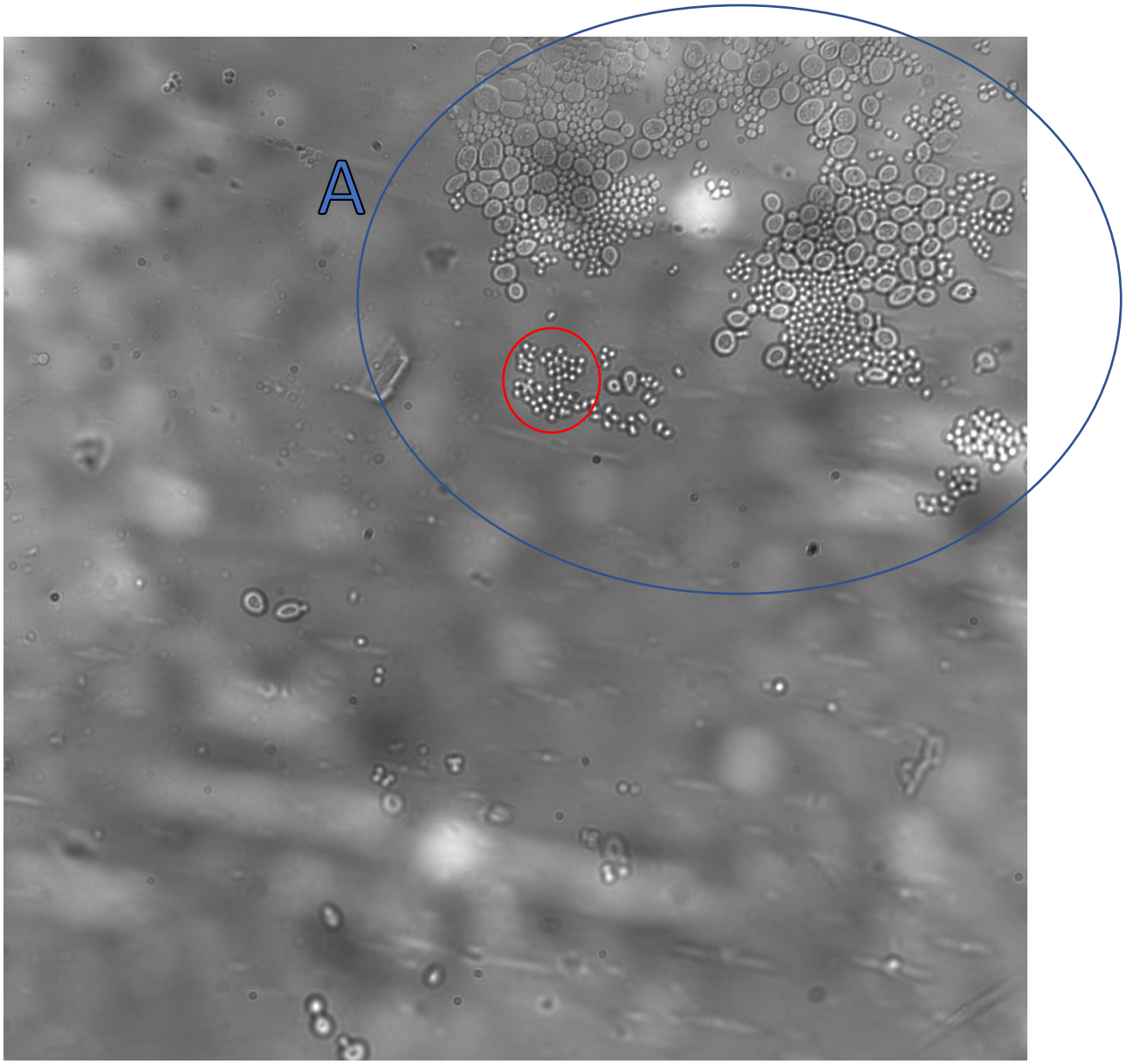


Figure 24: A microscope image of a second PET fragment with clusters of growing BY4741 cells.

In this figure larger clusters of cells can be observed in comparison to the previous figure. These clusters can be seen in the region circled and labelled A. Unlike the previous image there are not as many smaller clusters growing nearby. It is not known what the smaller structures circled are, it is likely that they are bacterial cells as the result of a contamination.

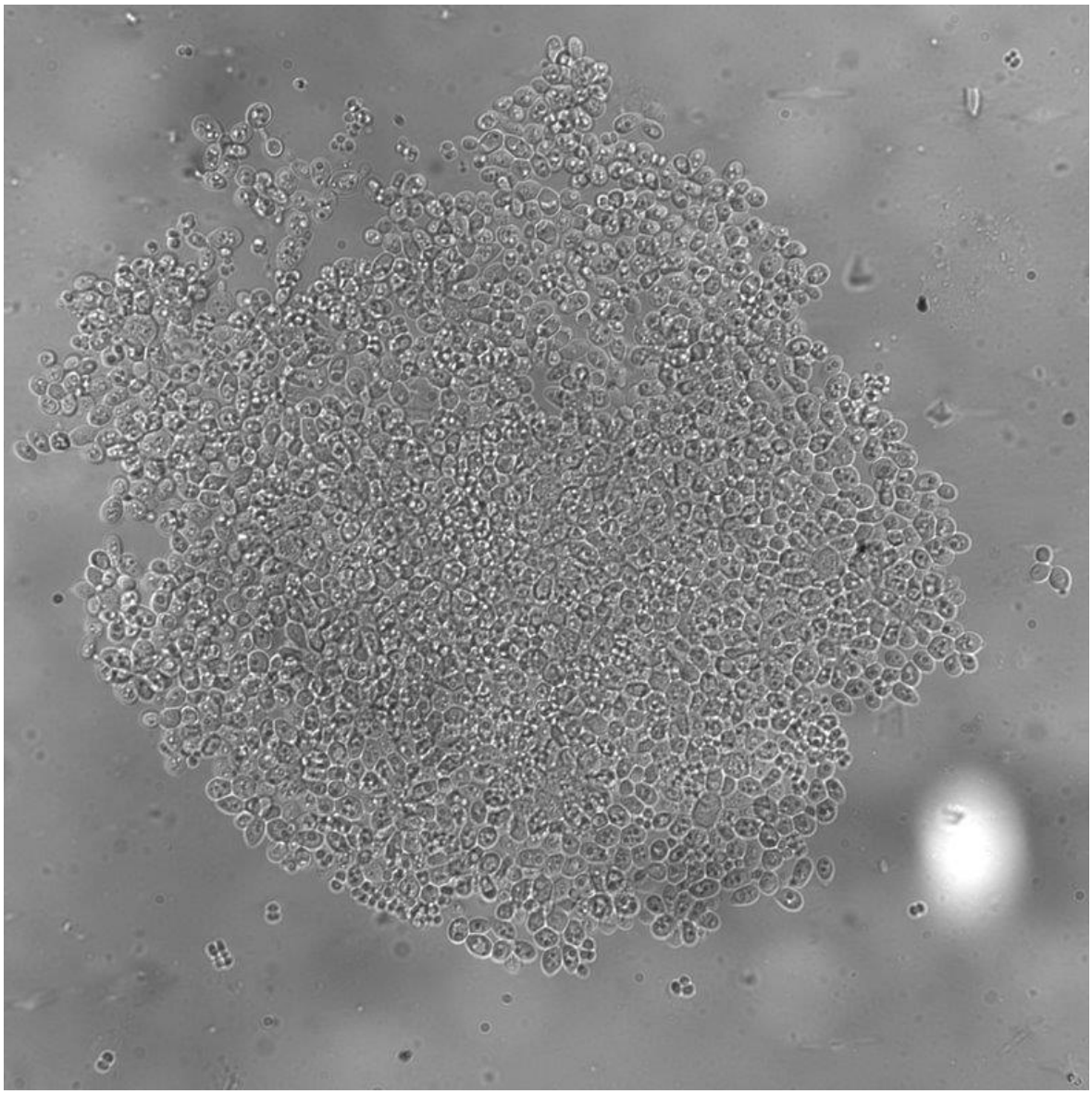


Figure 25: A microscope image of a PET fragment on which a large cluster of SK1 cells are shown to be growing in a biofilm like formation.

In this figure a large proportion of the 48mm^2 area of the 1cm^2 square is occupied by a large cluster of *S. cerevisiae* cells.

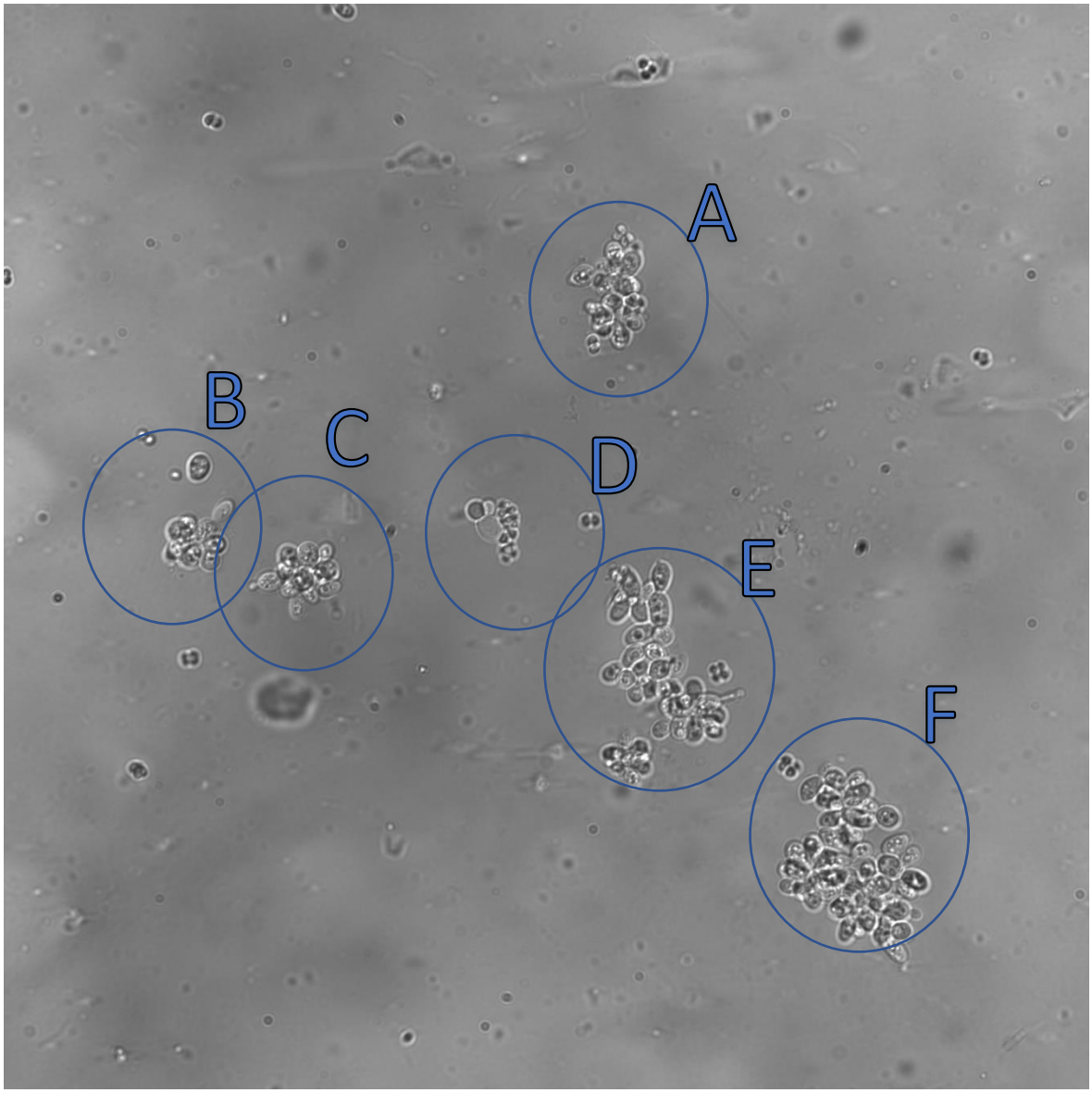


Figure 26: A second microscope image of a PET fragment with SK1 cells growing on the surface.

Unlike figure 25 in this image the fragment shows numerous smaller clusters of cells growing on the PET. These are circled in blue and labelled A-F.

All the above images were taken following static growth of yeast cells incubated with the PET fragments as described in section 2.3. These were viewed using MetaMorph® after gentle washing of the PET fragments with 1X PBS. These images suggest that *S. cerevisiae* cells are capable of growing on PET and are able to form biofilms under the right conditions. Furthermore, from these images we can see that there is an improved rate of biofilm formation in the SK1 strain, which may suggest that this would be a more viable strain to use in the future.

4.5 PETase activity analysis

In order to assess the viability of using *S. cerevisiae* as a host organism to produce recombinant PETase, the intracellular recombinant PETase was extracted and applied to PET to test for its activity. PET fragments obtained from Innocent™ Smoothie bottles were treated with the PETase extract and left to incubate for 72 hours at 30°C, as described in section 3.5. Following this the fragments were analysed using Scanning Electron Microscopy (SEM) and the following images were produced. Control PET fragments were incubated with water. Some fragments were treated with 0.025% SDS, which was shown in previous studies to act as a surfactant (Furukawa et al. 2016), prior to incubation with PETase.

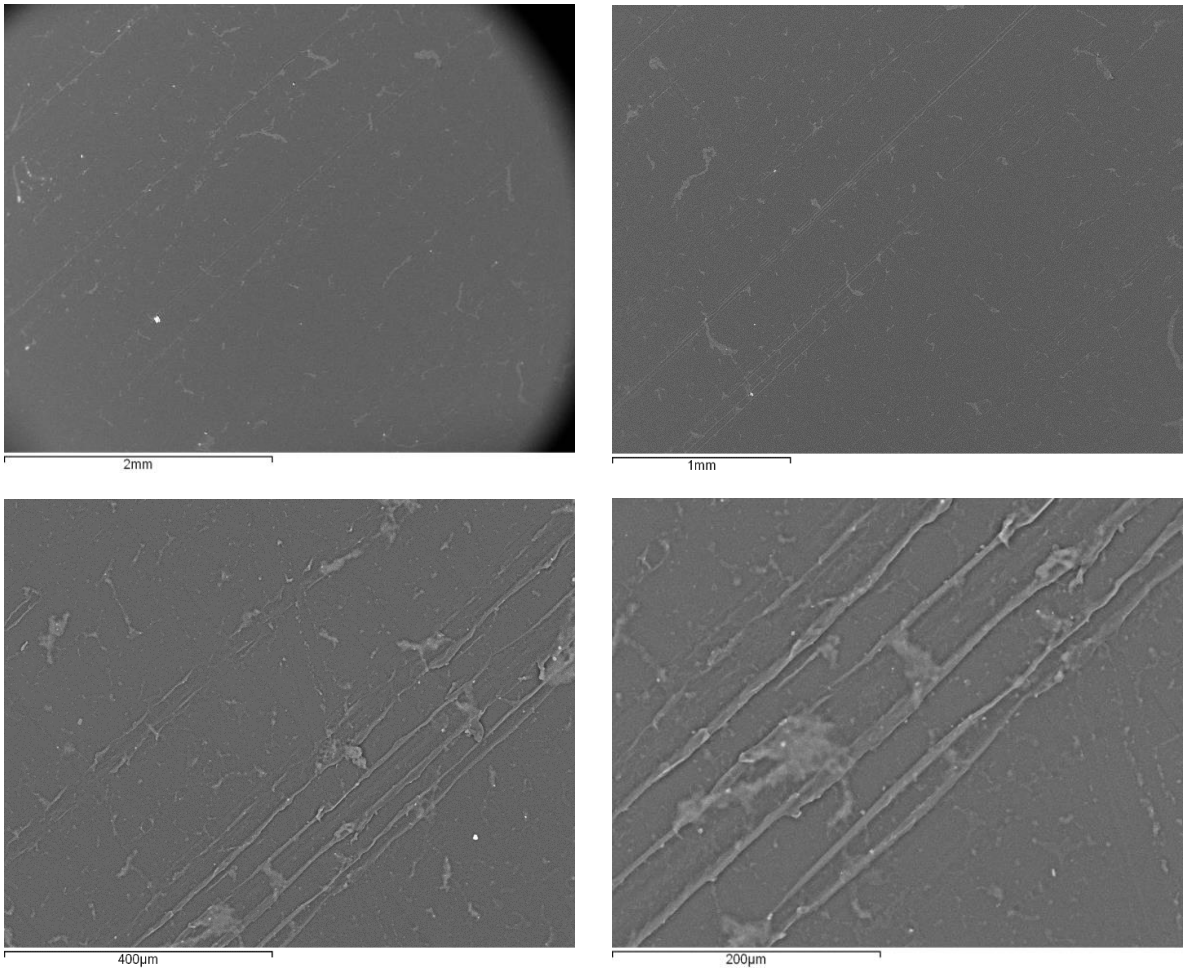


Figure 27: The figure above shows the control fragments in PET which were incubated with water. This is one image shown at different magnifications with increasing magnifications in a clockwise manner.

Controls with the PET fragments were made by the incubation of PET in water within the 24 well plates. As observed in figure 25 at different magnifications long striations are expected to be seen in the PET fragments as PET from bottles are not microscopically smooth and are subjected to scratches and microtears during production and use. Another observation is that, apart from the striations, most of the scratches seem irregular in nature.

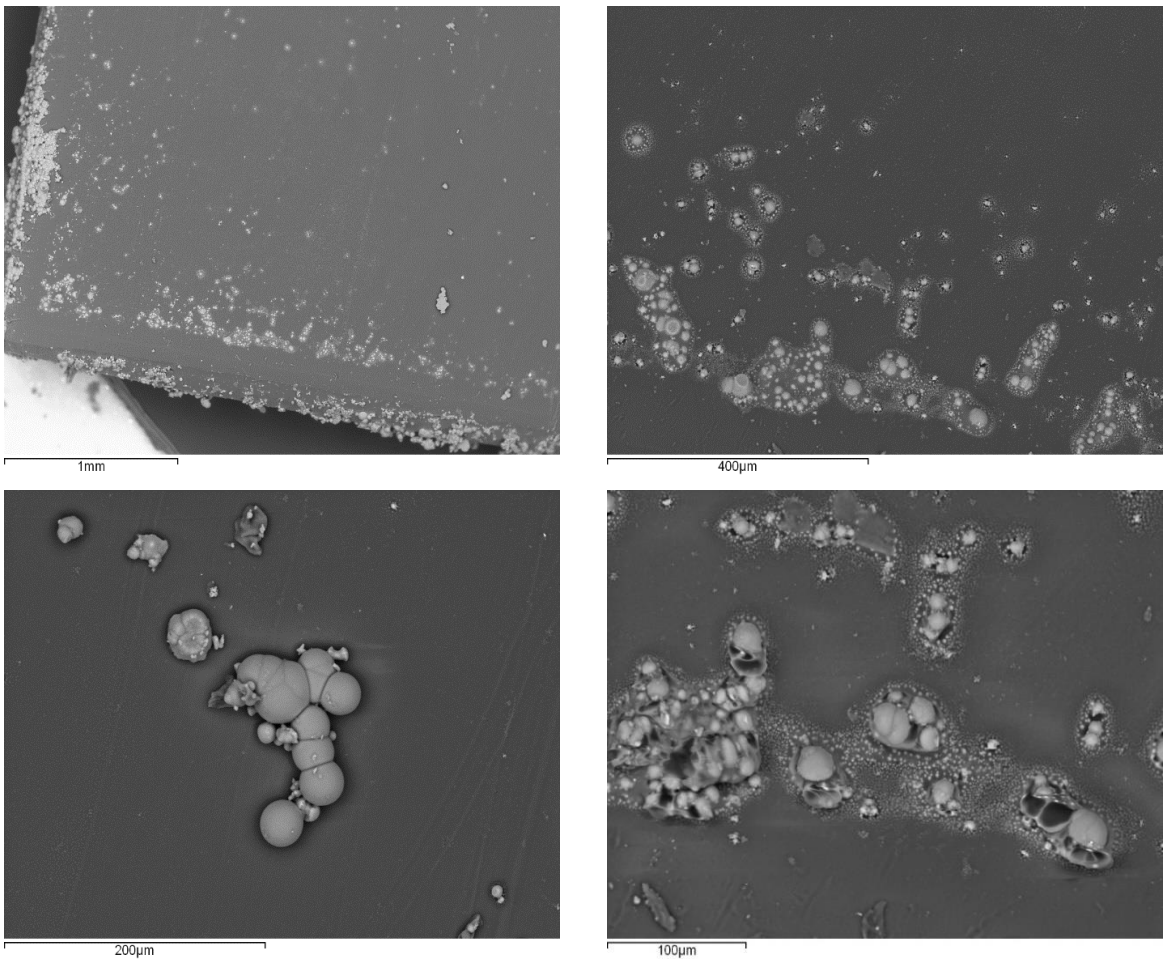


Figure 28: The images above show PET fragments analysed by the SEM following incubation with PETase for 72 hours.

When waste PET fragments were incubated with the enzyme extract after 72 hours pitting of the surface was observed on the surface at different magnifications. Unlike the scratches seen in the control as well as those seen in figure 27, the pitting is consistently circular in nature.

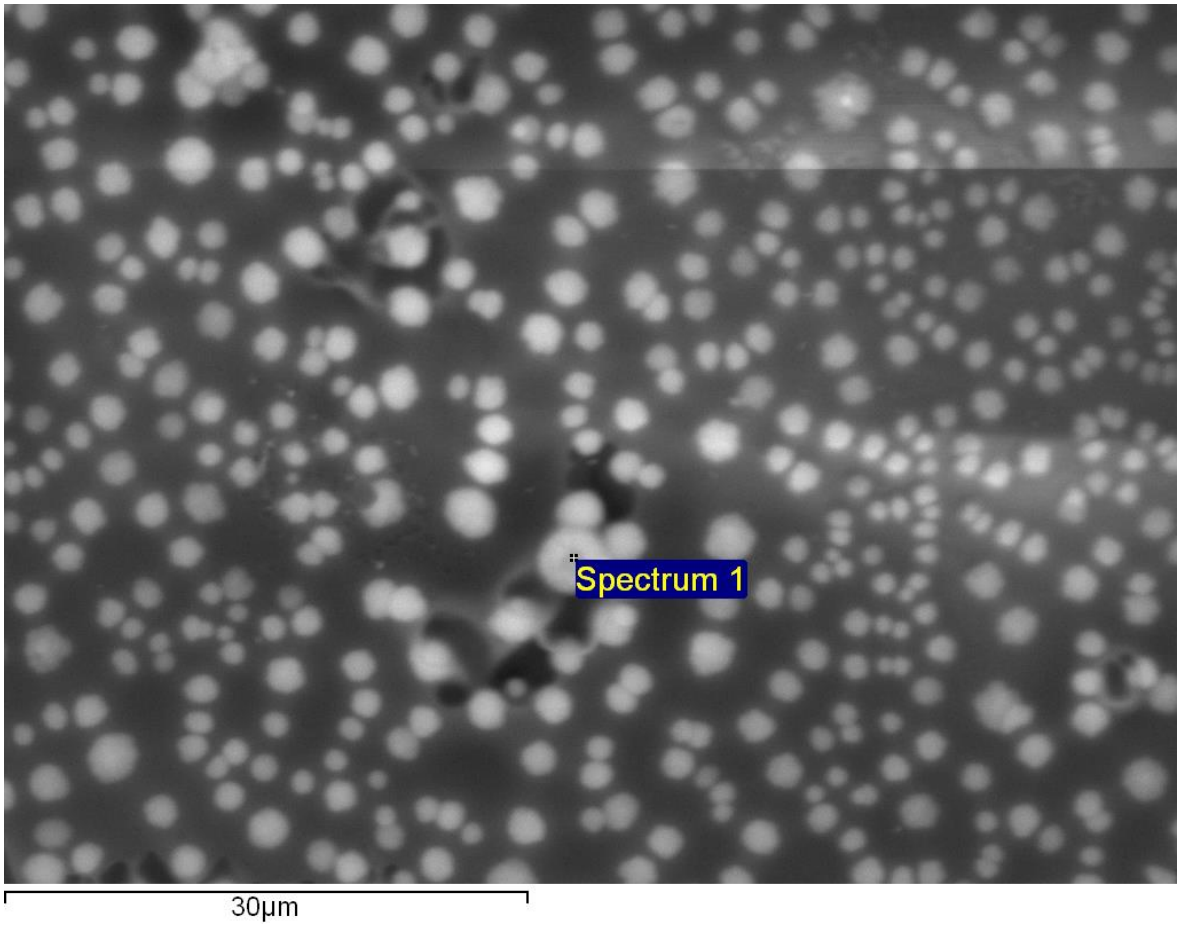


Figure 29: Shows a PET fragment incubated with PETase but no surfactant.

When viewed at a higher magnification the circular nature of the pitting becomes more apparent.

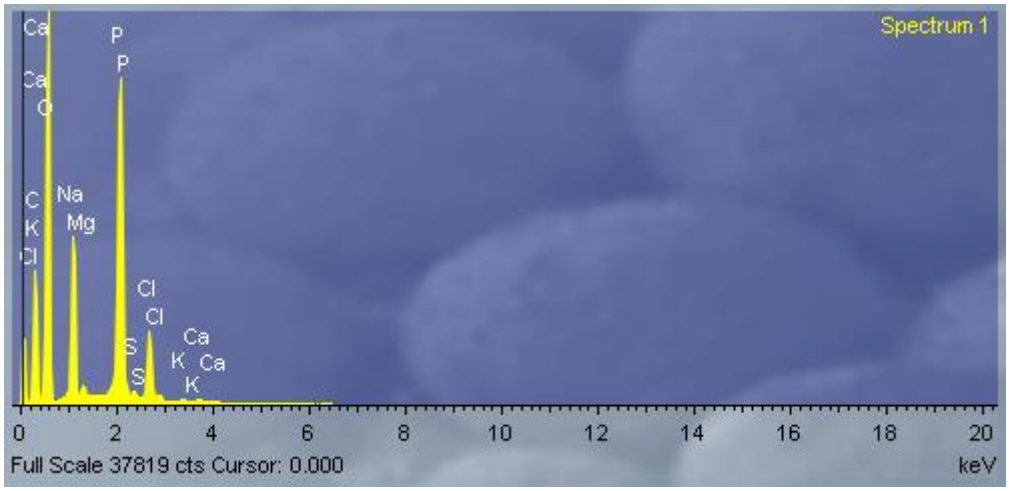


Figure 30: An EDX spectrum of the observed elements found within the pitting from the figure above.

The spectrum shown in Figure 30 shows that the only elements present within the pit correspond to the enzyme as well as the elements which would be found in the buffer used to extract the PETase protein, this further reinforces the likelihood that these pits would have been a result of the enzyme activity. The elemental

Overall, all SEM images with PET treated with recombinant *S. cerevisiae* PETase showed pitting, this is similar to those observed in (Yoshida et al. 2016).

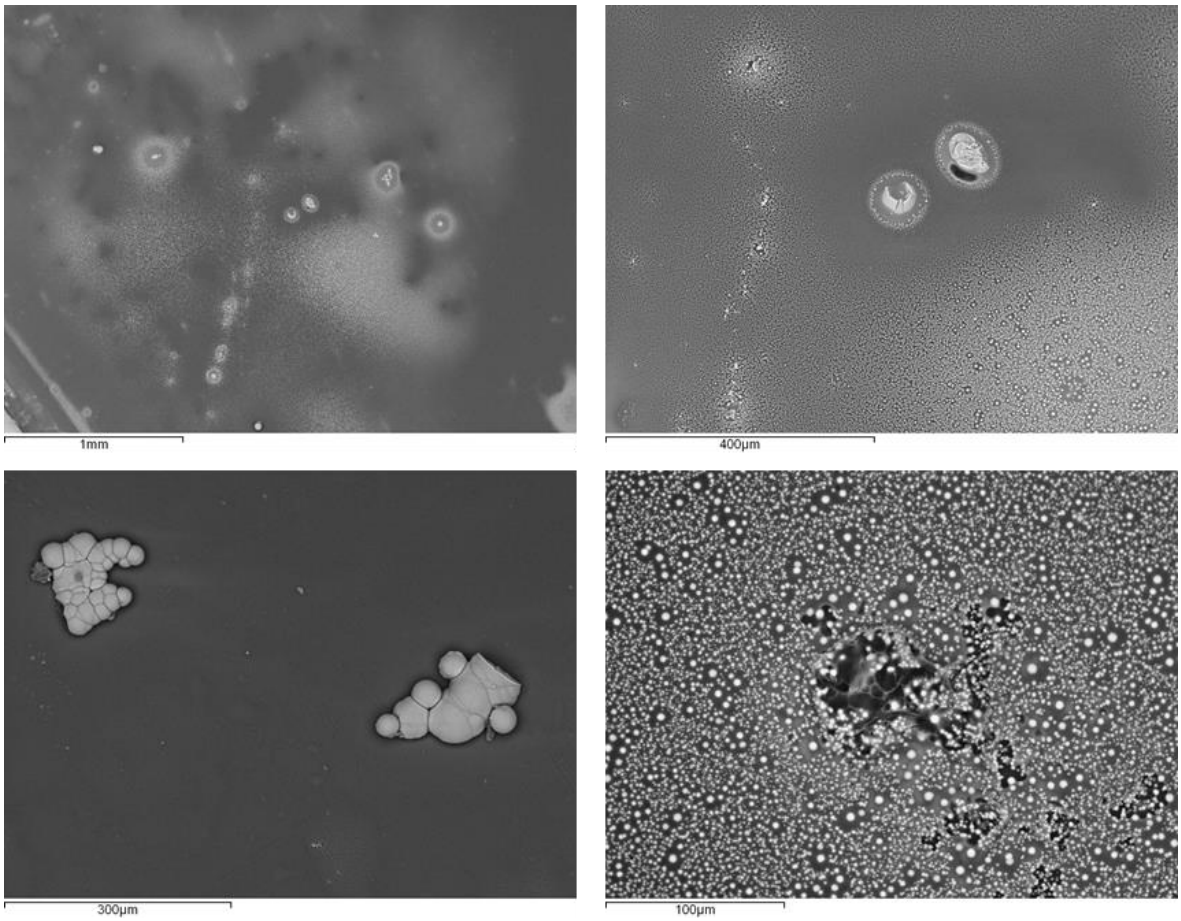


Figure 31: Shows a PET fragment treated with surfactant and incubated with PETase shown at different magnifications.

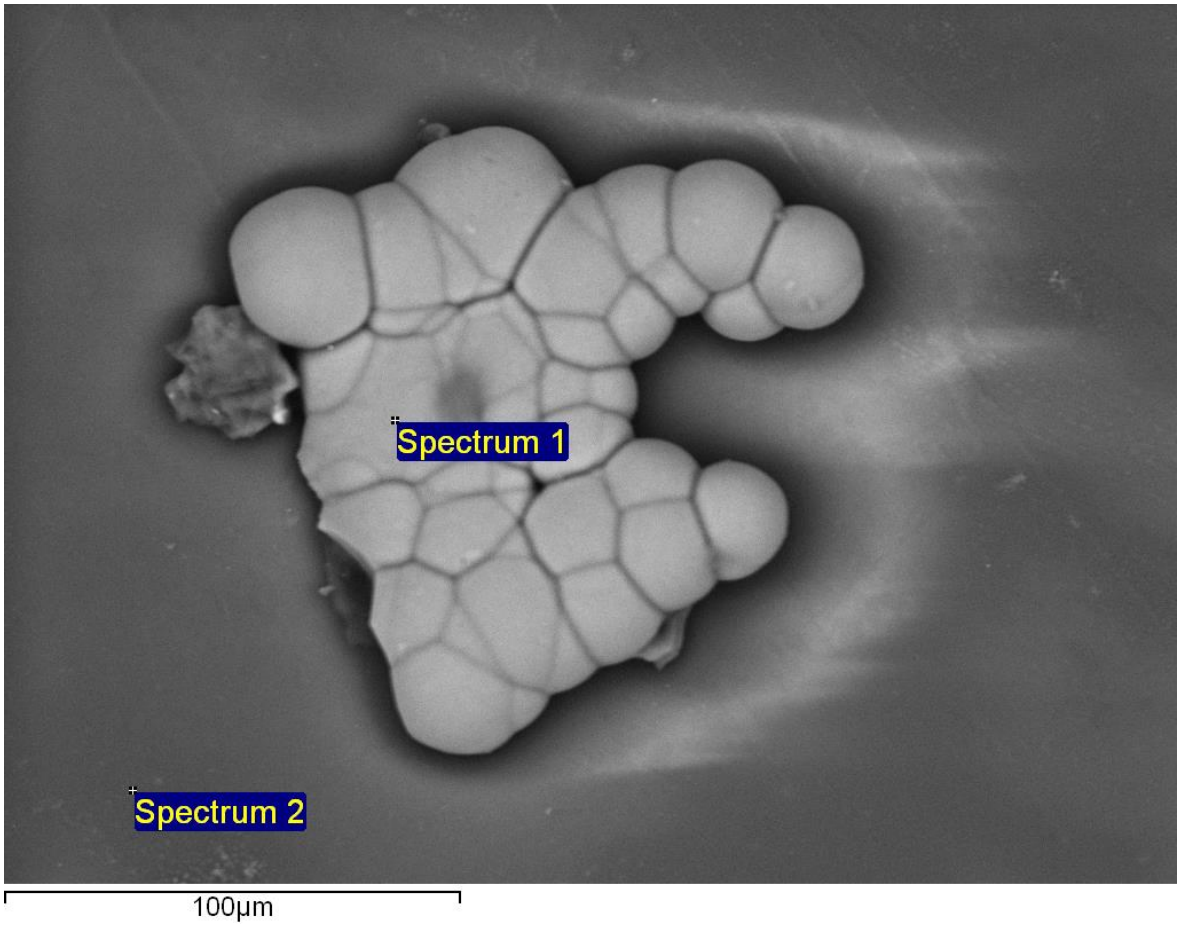


Figure 32: Shows a PET fragment treated with surfactant and incubated with PETase.

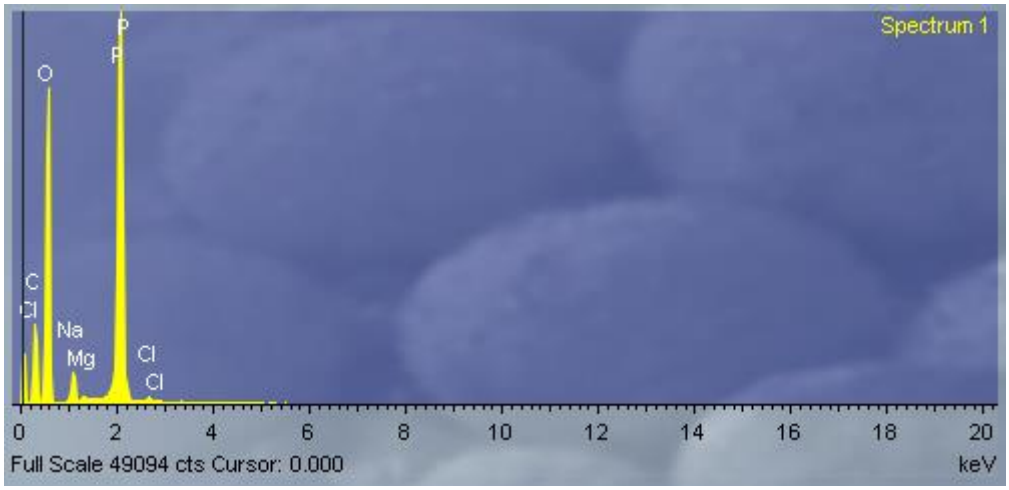


Figure 33: Shows an EDX spectrum for the area on figure 32 labelled Spectrum 1.

This spectrum shows the elements present within the pits, in this case these elements correspond to those found in the buffer used to extract recombinant PETase as well as those expected in the enzyme. In comparison to the spectrum taken for the non-treated PET, the element Sulphur is not shown.

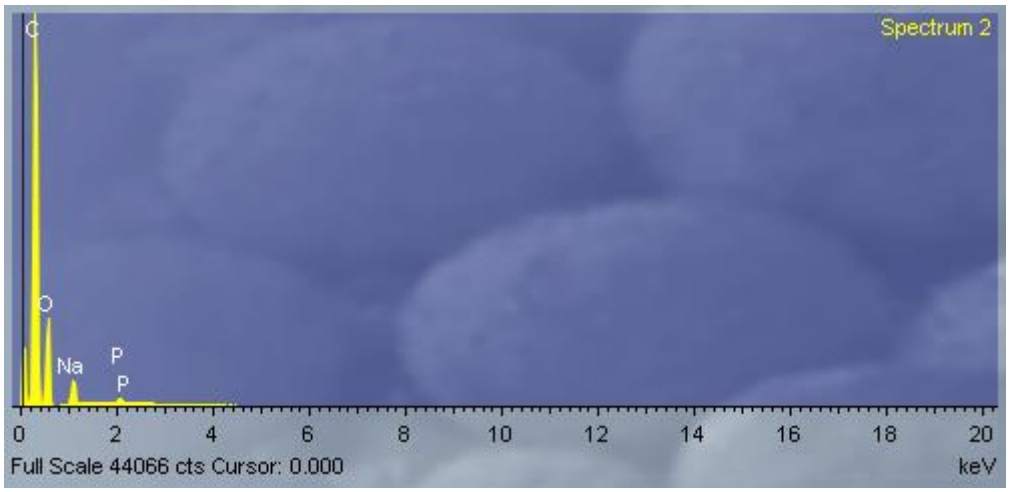


Figure 34: Shows an EDX spectrum for the area on figure 32 labelled Spectrum 2. Spectrum 2 shows the elements found outside of the pitting area, this shows fewer elements, which suggests that there has not been any enzyme activity within this area.

Overall, the recombinant PETase enzyme showed to have capabilities of degrading plastic as the characterised “pitting” behaviour was seen throughout the samples. However, unlike the study suggested, the addition of 0.025% SDS as a surfactant did not show any improvement to the degradation of PET by PETase, instead few and smaller pits were seen. This was further confirmed by the lack of sulphur in the elemental spectra in figure 34, as the enzyme forms two disulphide bridges, which could suggest that the surfactant used denatured PETase.

5. Discussion:

The prevalence of plastic pollution within the environment and its consequent impact on society and the entire global ecosystems has led to a great scientific and environmental drive to find solutions to tackle this ever-growing issue. Previous work within the scientific community allowed the discovery of several enzymes which could be used as novel bioremediation tools for the plastic pollution problem. This was then further improved after the discovery of the novel bacterium *Ideonella sakaiensis,* which employs two enzymes PETase and MHETase to degrade the commonly used plastic, PET, into its original monomers, whilst also using TPA as a carbon source of energy.

Since the publication of the research by Yoshida et al., several groups have explored more efficient ways of utilising this enzyme for novel methods of the bioremediation of plastic waste. Which has been the aim of this project. We decided to use *Saccharomyces cerevisiae* as a host organism for the recombinant production of the mutant PETase enzyme, which was developed by (Austin et al. 2018). This organism was chosen for its proven versatility as well as its efficacy in the production of various heterologous protein production from various organisms as it is a eukaryotic cell capable of post-translational modifications, which are vital for several proteins' functions.

In this project we decided to use two laboratory strains of *S. cerevisiae,* this was due to the observed differences in the level of secretion of recombinant protein by the two different strains.

5.1 Expression of PETase in *Saccharomyces cerevisiae*

The first step of this project involved the generation of an optimised form of the PETase gene, to suit the transcription activity of *Saccharomyces cerevisiae* as the original sequence of PETase originated in a bacterium. Following this we decided to use the constitutive promoter TDH3 which codes for the glycolytic enzyme glyceraldehyde-3- phosphate dehydrogenase. When comparing the various types of promoters that can be used in engineering, a constitutive promoter was favoured to an inducible promoter. This was due to the fact that with a constitutive promoter,

stable levels of expression of heterologous proteins are maintained without the need for changes in environmental stimuli, whereas inducible promoters, such as GAL10 would require the need to change carbon sources to drive transcription. Additionally, galactose is an expensive carbon source which would not be ideal to use in industry. This change of carbon source may then have secondary effects on secretion of the heterologous PETase enzyme. The TDH3 promoter has also been utilised in several heterologous protein expression studies, which has proven its efficacy. (Peng et al. 2015)

As a first step to transforming the two strains of *S. cerevisiae* we decided to use a single copy centromeric plasmid (pTH644_mPETase) which already contained the optimised PETase gene. Alongside this, the synthetic leader peptide combining sequences from the yeast OST1 and MAT-alpha genes was also present, which would allow the expressed PETase to be co-translationally translocated out of the cell.

After western blot analysis, we were able to observe the expression of PETase within the cells of both the SK1 and BY4741 strains. On one occasion this showed that there was low level secretion in the SK1 cells, but no secretion was observed in the BY4741 cells. This was also apparent by the differences in the signal intensity shown in the blots. As the intracellular products of the SK1 cells was lower than that of BY4741.

One interesting thing to note was that two bands were seen for the secreted PETase enzyme in SK1, one was observed to be higher than the expected 29kDA of the PETase, this may be due to N-glycosylation of the secreted protein prior to export This is not unusual to see in secreted proteins, as most homologous proteins which are secreted by *Saccharomyces cerevisiae* are destined for the cell wall or cell surface which are N-glycosylated, whereby sugar groups are added to asparagine residues. (Kim, Yoo, and Kang 2015) Alternatively, this could be processing intermediates in the secretion pathway, as the pre- and pro-sequences are cleaved off the product sequentially.

Although some secreted products were seen in the supernatant of SK1, it seems that a large proportion of expressed protein was remaining in the cell. Furthermore, as SK1 has been shown to be a better secretor than BY474. This was demonstrated

by independent results from our lab when expressing other recombinant proteins. However, for *Saccharomyces cerevisiae* to be used as an industrial solution to the removal of plastic waste by the production and secretion of the PETase enzyme, the secretory aspect of this system will need to be improved.

Following this observation, a different single copy centromeric plasmid (pTH798) containing a different leader peptide sequence was cloned using Gibson assembly and the optimised PETase gene was inserted to form a new plasmid (pTH798_secPETase). This new plasmid had the leader peptide sequence from the SUC2 gene which encodes the yeast invertase enzyme, this naturally uses the co-translocation translation pathway. This was then used to transform BY4741 and SK1. When cell extracts and supernatant samples were analysed it was found that only the cell extracts of the transformants contained the PETase protein, no secretion was seen.

A frequently used leader is MAT-α, however this utilises a post-translational pathway for translocation into the ER. The OST1-AT leader was engineered to combine the efficiency of the MAT peptide with co-translational translocation. SUC2 is thought to use co-translational translocation naturally. (Carlson et al. 1983)

One of the drawbacks of using centromeric plasmids is that, when transformants are not grown on minimal media, the strains easily lose the expression plasmid. However, use of complex media often increases secretion yields and such media are also cheaper to produce for biotech applications. From this we decided to use an integrative plasmid which could be inserted into the genome of both yeast strains to overcome this and potentially have the ability to observe secreted protein. To compare the two methods of transforming the yeast strains.

The pRS306 integrative plasmid was selected from those produced by Robert S. Sikorski and Philip Hieter (Sikorski and Hieter 1989), which incorporates the pBLUESCRIPT backbone from more highly efficient plasmids usually used in bacterial cloning vectors as it contains several useful features for DNA cloning, including ssDNA production, high plasmid DNA yields, an extensive polylinker, unidirectional deletion formation, and simplified cloning. The pRS306 also contains the URA3 selective marker to allow for screening of transformed cells. This plasmid is integrated into the yeast genome through homologous recombination following

deletion of the segment of a gene locus and then the insertion of the desired gene, which is mediated by the extensive polylinker in the pRS backbone.

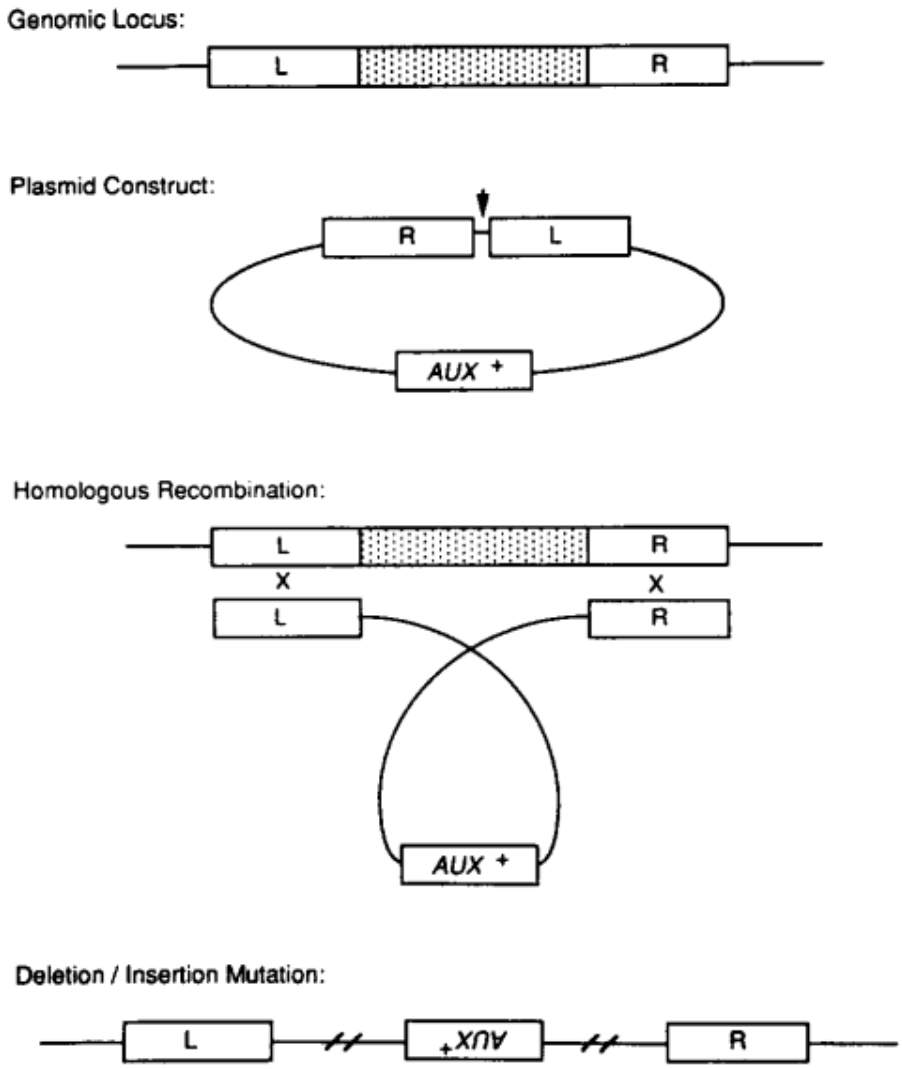


Figure 35: Shows γ-transformation of yeast by the site directed deletion of a gene locus and the replacement with the desired gene. The AUX segment represents the selective marker gene, which in our study was URA3. DNA segments flanking the segment that is to be deleted are cloned into a pRS YIP in tandem but reverse order. After linearization at the site designated with an arrow and transformation, the construct recombines as shown. The overall result is a deletion of the chosen DNA segment and an insertion of the entire plasmid. ((Sikorski and Hieter 1989))

Using this integrative plasmid, the PETase gene was amplified by PCR from pTH644_mPETase and then inserted into the vector by Gibson assembly to form the new pRS306_secPETase plasmid. Following transformation into the BY4741

and SK1, the transformed cells were screened for the intracellular expression and secreted heterologous protein production by a Western blot. This also showed no secretion in either strain, but intracellular expression was observed.

One method which could be researched is the suppression of the quality control mediator Vsp10 which is responsible for targeting non-native protein and results in the accumulation of proteins in vacuoles, due to them displaying different conformations than those of the native proteins.(Rakestraw et al. 2009) In the study conducted by Fitzgerald et al, it was found that the suppression of vsp10 a sorting receptor in the secretory pathway after the Golgi, resulted in increased levels of secretion of the heterologous protein. This suppression was performed by either the deletion of the vsp10 gene or truncating the sorting receptor. By truncating the receptor, they were able to avoid aggregation of the protein in vacuoles whilst also allowing vacuolar hydrolases to be sorted, something which was disrupted when Vsp10 was completely deleted.

Alternatively, a different method of transforming the cells could be used, where instead of using plasmid dependent engineering, the CRISPR-cas9 method is used. In this method the gene of interest is inserted after a site directed double strand break in the host's genomic DNA.

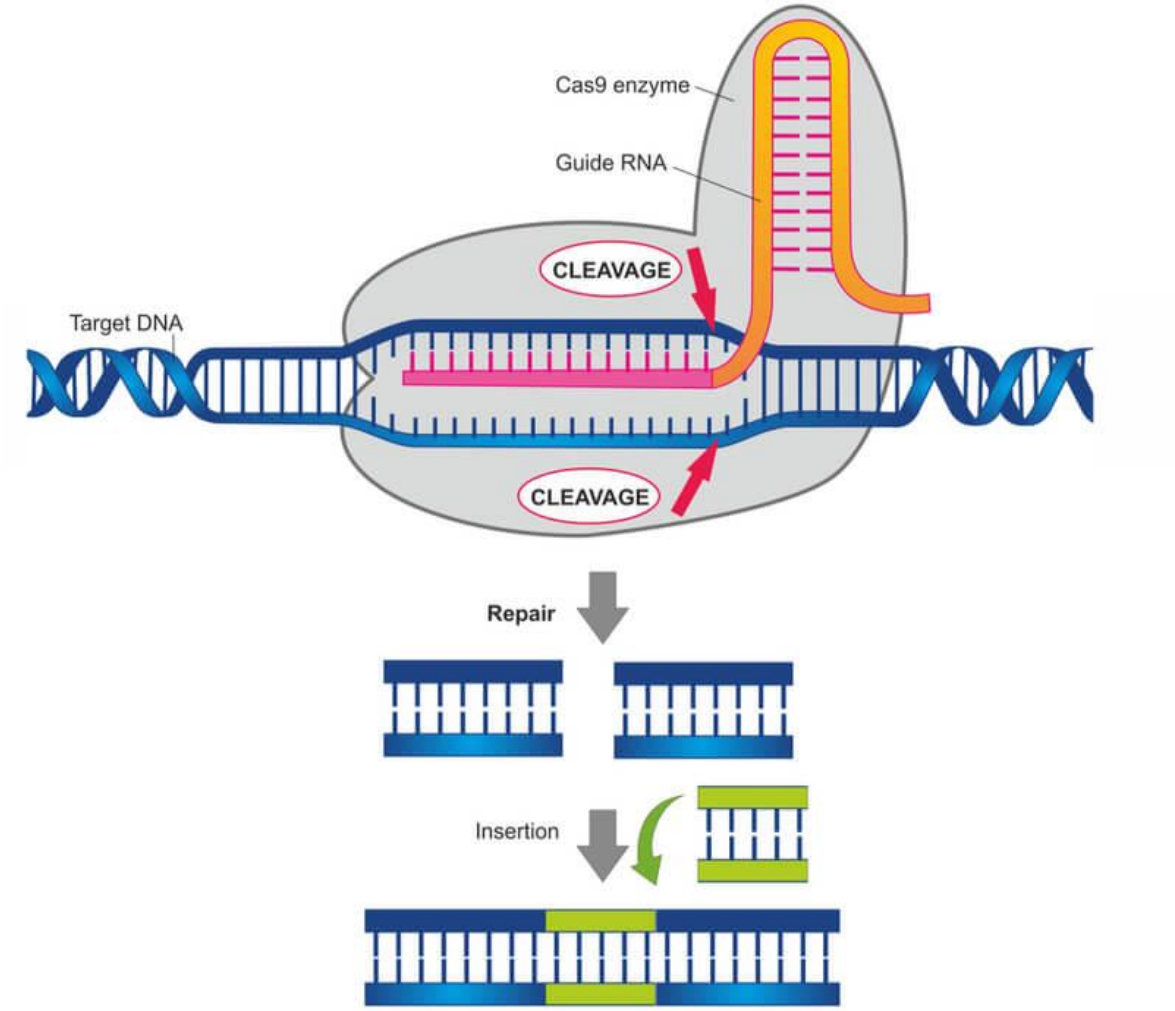


Figure 36: showing a schematic diagram of how a gene is inserted into the host genome after site direction by a guide RNA and the cas9 enzyme. The gene is then used in a repair mechanism.(Pickar-Oliver and Gersbach 2019)

Since the discovery of CRISPR technology several studies have shown its success in effectively editing and engineering a multitude of organisms for the production of heterologous proteins. This is due to the fact that this method requires no need for the selection of promoters, leader peptide sequences, or the use of other cloning methods. It has also been shown to increase production of proteins which researchers previously found difficult to synthesise. ((Pickar-Oliver and Gersbach 2019))(Reider Apel et al. 2017)

5.2 Activity of heterologous production of PETase

During this project we also decided to test the activity of the intracellularly produced PETase enzyme, to assess whether its production in *S. cerevisiae* would impact the enzyme's function. This was done by harvesting the protein from the intracellular extract of BY4741 cells that had been transformed with the pTH644_mPETase plasmid and applying the extract directly onto sterile PET fragments obtained from an Innocent™ Smoothie bottle. This is was done differently to other studies which use a lower crystallinity model; hence this provides a more realistic image of the efficiency of the PETase enzyme on waste plastic.

The activity of the enzyme was measured qualitatively by imaging the PET samples following 72 hours of incubation with PETase-containing cell extracts, this was then viewed by EM microscopy. Similar to other studies, the PET fragments showed some pitting in a similar fashion to those observed in other studies. However, this was not as prominent for our results. This may have been the result of using waste PET samples in comparison to lower crystallinity PET used in other studies. Furthermore, there was no increase in degradation seen in the fragments of PET that had been treated with SDS as observed in ((Furukawa et al. 2018). Again, this may have been due to different concentrations being used for the study.

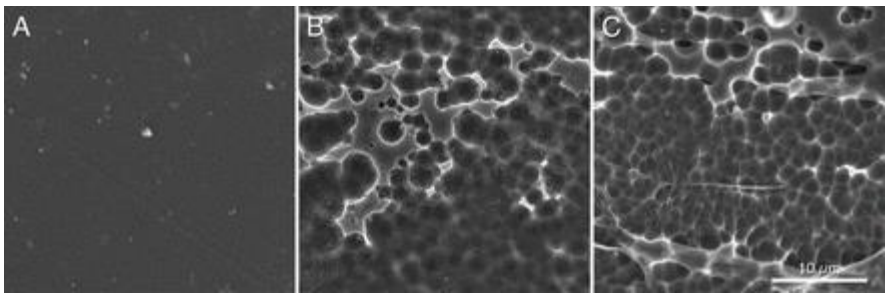


Figure 37: An image taken from (Austin et al. 2018). A, shows a control PET fragment used, B is after treatment with the wild type PETase, C is after treatment with the double mutant PETase.

Figure 37 shows PET samples with more significant pitting in comparison to the pitting seen for this study.

Although some degradation was observed in the SEM images, this experiment could be further improved by purifying the enzyme out of the YPER reagent to obtain a standardised concentration of the enzyme. Furthermore, to obtain quantitative results for the degradation of the PET by the hydrolase enzyme, quantification of the by-products of the degradation (MHET, BHET, TPA, and EG) could be measured by HPLC in a similar fashion to that conducted by ((Moog et al. 2019)) to further show the activity of recombinant PETase from *S. cerevisiae.* Finally, these results were obtained from intracellularly produced PETase, secreted PETase may have different activity levels, and will need to be further investigated.

5.3 *Saccharomyces cerevisiae* biofilm formation on PET

For the industrial application of using *S. cerevisiae* as a tool to remediate plastic pollution, we decided to investigate the adherence of the cells to PET fragments identical to those used in the experiments described in section 5.2. From these we were able to see the attachment of both BY4741 and SK1 cells in the presence of plastic. After visualising the PET fragments some cells were seen to have formed clusters and attached to each other and the plastic. Although the attachment shown is promising the clusters observed were minimal and in an industrial setting this would not be enough cells to sustain a significant amount of degradation.

After having read the literature it was shown that several factors can impact biofilm formation in *S. cerevisiae,* which in turn regulates Flo11 which is responsible for flocculation and adhesion of the cells to an abiotic surface, in this case PET. In the study ((Verstrepen and Klis 2006)) it was found that generally BY4741 has reduced Flo11 expression, which may explain why smaller clusters were seen for this strain in comparison to SK1. SK1 is also known to aggregate more than BY4741, which may be due to its increased ability to sporulate. ((Paulissen and Huang 2016)) Hence, upregulating the Flo11 gene could increase the levels of adhesion of the cells to the plastic surface. This was observed in (Purevdorj-Gage et al. 2007) where FLO11 upregulation resulted in increased biofilm formation of BY4741 as well as 50% additional coverage of a hydrophobic surface.

Lastly studies showed that the Flo11 gene was also regulated by Tup1p and Cyc8p. These regulators are involved in the biofilm formation mediated by glucose availability. When glucose concentrations increase Cyc8p is upregulated and biofilm formation is suppressed. On the other hand, in decreased glucose concentrations Tup1p induces biofilm formation. These two regulators work synergistically and can both be targeted to improve biofilm formation in *S. cerevisiae.* (Van Nguyen et al. 2020)

One other method of increasing cell adhesion to an abiotic surface is to change environmental conditions to allow the biofilm production response to be mediated without the need for additional genetic engineering. Several factors are responsible for biofilm formation, including energy source depletion, changes in pH, or changes in ethanol concentration. This elucidates a stress response in the yeast cells, however causing stress in the cells could have detrimental impacts on expression and secretion of recombinant proteins, thus CO_2 concentration changes have also been studied. In one study it was shown that increases in CO_2 concentration upregulated cell-cell interactions and mitotic growth in *S. cerevisiae*. This could then also be applied to test for changes in biofilm formation. These conditions were also observed to increase biofilm formation in the pathogenic yeast *Candida albicans.* (Volodyaev, Krasilnikova, and Ivanovsky 2013)

5.4 Further work

The results from this project have shown promising results for the development of *Saccharomyces cerevisiae* into a potential organism to be used to tackle the current plastic pollution crisis. We were overall able to confirm that a recombinantly produced PETase enzyme had the potential to degrade PET, as well as demonstrate that through the adherence of the yeast cells to the hydrophobic PET surface a sustainable system could be further developed in the bioremediation of plastic pollution.

Although we were able to show these results, an improved secretory system will have to be developed to allow *S. cerevisiae* PETase efficiency to be calculated without the need for intracellular extraction. Secondly, an improved and quantitative

method should be developed to allow for better measurement of the activity of any secreted PETase product. Having worked with two different strains in this project, it is not very clear which would be best to use as a host organism, as more experiments will need to be conducted to determine this, however it does seem that SK1 may be a better candidate, as this was the only strain where any secretion was observed, as well as showing a slightly better cell-adhesion properties. Lastly, it will be vital to improve biofilm formation and adherence to the hydrophobic PET surface to allow for the creation of a self-sustainable system of PET degradation.

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