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Towards a Novel Bioremediation System for Microplastic Contaminated Soils

A thesis submitted to the University of Kent for the degree of
Master of Research in Microbiology

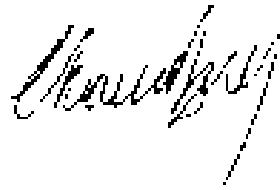
November 2020

Charlotte Bilsby

The School of Biosciences

DECLARATION

No part of this thesis has been submitted in support of an application any degree or qualification at the University of Kent or any other university or institute of learning. I can confirm all work submitted is of my own.

A handwritten signature in black ink, appearing to read 'Charlotte Bilby', written in a cursive style.

Charlotte Bilby

November 2020

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Firstly, I would like to acknowledge Dr Tobias von der Haar for all the support he has provided over a very difficult and unpredictable year, and for trusting in my scientific ability to establish this project within the Kent Fungal Group. I would like to thank my lab peers Noor Issa and Samantha Hobbs for keeping me sane. Furthermore, I would like to thank Dr Billy Ferrera and Dr Eleanor Thompson, at the University of Greenwich, for their time and resources. And finally, I would like to thank all of my family and friends in Reading for providing me with absolute unconditional support over the past year, despite not understanding a single thing I have been doing.

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ABBREVEATIONS

Abbreviation	Description
Amp	Ampicillin
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
BglI	A restriction enzyme
BHET	Bis(2-Hydroxyethyl) terephthalate
cAMP	cyclic-AMP
Cutinase	Cutinolytic esterase
Cyt	Cytoplasmic expression
<i>D. discoideum</i>	<i>Dictyostelium discoideum</i>
DNA	Deoxyribonucleic acid
EDL-DVLO	(Electrical Double Layer-Derjaguin-Landau-Verwey-Overbeek)
EG	Ethylene glycol
<i>I. sakaiensis</i>	<i>Ideonella sakaiensis</i>
<i>K. aerogenes</i>	<i>Klebsiella aerogenes</i>
LB	Lysogeny broth/ Luria broth
MHET	Mono-2-hydroxyethyl terephthalate
MHETase	Mono-2-hydroxyethyl terephthalate hydrolase
<i>P. pastoris</i>	<i>Pichia pastoris</i>
PCL	polycaprolactone
PEMRG	Plastic Europe's Markets Research and Statistics Group
PET	Poly(ethylene) terephthalate
PETase	Poly(ethylene) terephthalate Hydrolase
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
Sec	Secretory tag present
SorMC Buffer	Sorensen, Magnesium, Calcium containing buffer.
<i>T. fusca</i>	<i>Thermobifida fusca</i>

TPA	Terephthalic acid
UV	Ultra-violet

ABSTRACT

The proliferation of plastic, more specifically poly(ethylene) terephthalate (PET) as a durable, long-lasting material has also led to the polymer becoming an unprecedented environmental pollutant. Despite the technology that facilitated the growth of plastic production, the means of disposing the plastic has further negative impacts to the surrounding environment. Various researchers have explored the development potential of different bioremediation systems capable of removing different plastic polymers from the environment by biological degradation. Since the discovery of the PET hydrolysing enzyme, PETase in 2016, the bioremediation of PET is much more plausible. In this study we address a novel approach to remove microplastic pollution from terrestrial ecosystems. The model organism, *Dictyostelium discoideum*, was selected due to their ecological niche and fully established molecular tool kit. This study aimed to produce a model organism capable of secreting the PETase enzyme into their surrounding environment. The generation of a *D. discoideum* compatible vector plasmids containing the PETase gene was faced with a multitude of hurdles linked to the repeat content and A/T richness of the *D. discoideum* genome. Transformation of *D. discoideum* with the PETase gene was perceived as successful, and provided justification for further work towards the creation of a suitable bioremediation system.

Key words: Poly(ethylene) terephthalate (PET), *Dictyostelium discoideum*, plastic degradation, bioremediation, PETase, plastic pollution.

CHAPTER 1: INTRODUCTION

1.1 PLASTIC POLLUTION

Plastic polymers have risen to become an indispensable part of everyday life and are found to be significant contributors to the composition of many different consumer products. Synthetic polymers have rapidly replaced natural materials, across a large proportion of different industries. This has led to plastic production becoming an important player in the global economy (Thompson *et al.*, 2009) . Initially the increase in the global production of plastic was seen during the 1950s and each year since, production has grown exponentially. In 1950 only 2 million metric tonnes of plastic were produced, however, as of 2018 this has risen to approximately 380 million metric tonnes of plastic and is continuing to increase annually. The annually increasing production of plastic can be linked directly to the increase of consumer demand.

Plastic has proliferated to widespread global usage due to its low-cost to manufacture high longevity and durability. The specific plastic polymers that constitute over 80% of the European plastic production are shown in *Table 1*. It is often misconceived, by the general public, that all products containing plastic are made from the same plastic polymer and can be disposed of in a uniform way. Unfortunately, due to the differences in chemical structure some plastic materials are more readily recyclable than others. For example, in Europe, Poly(ethylene terephthalate) (PET) contributes only 7.7% of total market production but causes significantly higher damage to the environment than other plastic polymers (Webb *et al.*, 2013). This is directly linked to the high usage of the polymer with poor and inefficient means to dispose of it after use.

Table 1: Plastic demand and market production categorised by resin type and different use within industry. Data was obtained from the 2019 PEMRG research report.

Resin type	Market Production (%)	Use
Poly(ethylene terephthalate) (PET)	7.7	Plastic bottles, textiles and food packaging
Polyethylene low density (PE-LD)	17.5	Reusable bags, food packaging such as film, trays and containers
Polyethylene high density (PE-HD)	12.2	Toys, shampoo bottles, houseware and piping
Polypropylene (PP)	19.3	Food packaging, piping, bank notes and automotive parts
Poly(vinyl chloride) (PVC)	10	Window frames, floor and wall covering, garden hoses and inflatable pools
Polystyrene (PS)	6.4	Food packaging, building insulation, liners for a fridge.
Polyurethane (PUR)	7.9	Building insulation, pillows, mattresses
Other	19	Hub caps, optical fibres, touch screens, medical implants, surgical devices, membranes, protective coatings.

1.1.1 Poly(ethylene terephthalate) as a material

Despite Poly(ethylene terephthalate) (PET) contributing to a small percentage of European plastic production, it still one of the most commercially important plastic materials (Zhang *et al.*, 2017). Linked to its commercial importance and use throughout industry, PET is a significant contributor to pollution. To understand why it

is commonly used it is important to understand PET as a material. All plastics are an example of a man-made material, they are a linear hydrocarbon polymer composed of repeating units, monomers (*Figure 1a*). PET is composed of alternating units of ethylene glycol and terephthalic acid (*Figure 1b*). PET is a durable, strong, and flexible material. It is an example of a thermoplastic polymer meaning its structure can be heated and remoulded numerous times. These properties have led to its popularity as a consumer material and its plentiful applications. The global consumption of PET has increase significantly when compared to other synthetic materials polymers, with PET contributing over 50% of the total global synthetic fibre production (cPatel, 2010).

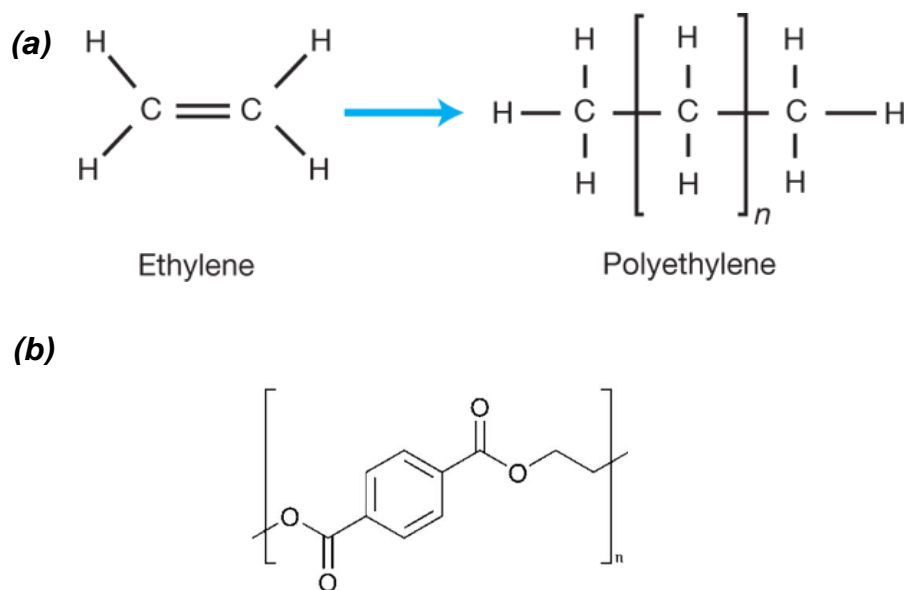


Figure 1: **(a)** Ethylene monomer structure links to form Polyethylene which constitutes the main structure of polyethylene plastic materials (Sharpe, 2015). **(b)** Chemical structure of PET (Nguyen and Trinh, 2019).

The resistance that plastics have to natural degradation, making them notoriously difficult to dispose of, is associated with their chemical and physical properties. The molecular weight of a plastic polymer significantly limits its susceptibility to natural degradation. PET has a molecular weight range of 30,000–80,000 g mol⁻¹, depending on its form. This is considerably low, when compared to other plastic polymers, but other properties of PET such as it's hydrophobic nature and frequent use of PET in a highly crystalline form limits its potential for natural degradation. The physical and chemical properties of PET are presented in *Table 2*.

Table 2: The physical and chemical properties of intrinsic PET (Awaja and Pavel, 2005)

Property	
Molecular Weight	30,000–80,000 g mol ⁻¹
Melting Point	250-265°C
Boiling Point	>350°C
Solubility (in H ₂ O)	Mostly Insoluble
Density	1.38g/cm ³

1.1.2 Poly(ethylene terephthalate) prevalence in the environment

Plastic pollution has evolved into an unprecedented threat to global ecology and is causing significant damage to the environment. Plastic has numerous pathways into the environment, however these can be summarised into two main routes; illegal dumping of domestic and industry waste or by inadvertent contamination due to poor disposal methods (Webb *et al.*, 2013). Most significant of the two is the incomplete or inefficient disposal of PET. The development of adequate disposal methods is hindered by the low cost associated with the production of new PET, meaning that PET is still continuously being produced and the used PET becomes waste. Once PET is present in an environment, they do not remain static. This means that plastics are able to travel between ecosystems and eventually land-based plastic pollutants may end up in marine or freshwater ecosystems, where they often accumulate. The accumulation of such pollutants within an environment results in adverse repercussions.

The accumulation rate of plastic in the environment is now over 25 million metric tonnes per year (Kaseem, Hamad and Deri, 2012). This figure is increasing annually, making plastic a notable and unignorable large threat to the environment. Once within an environment the plastic material will remain there indefinitely. Weathering and other environmental processes will cause the initial plastic product or material to degrade into smaller particles sizes. These are known as microplastics, nanoplastics and picoplastics. Microplastics are defined as particles of plastic less than 5mm in size. Microplastics can be further defined into different size classes. Particles that are less

than 1mm in size are defined as nanoplastics and those that are measured below the size of 1 μm are classed as picoplastics. The categorisation of plastic into micro, nano and pico, is important as the size of the plastic links to the extent of damage that it may cause to an ecosystem. Further to this their size categorisation will affect their ability to be transported through the environment and between ecosystems. However, at present, nanoplastics and picoplastics are rarely quantified accurately in terrestrial ecosystems.

The small size of a plastic particle and persistence within an environment can result in various organisms consuming them and therefore, the piece of plastic being carried through a food web (Hurley and Nizzetto, 2018). In recent years it has been recognised that microplastics act as a vector for pathogens, pesticides and heavy metals in an environment (Naik *et al.*, 2019). Despite the recognition of the impacts of plastic pollution long-term effects are still poorly understood.

Media coverage and global awareness of plastic pollution largely focuses on “idyllic” and economically important ecosystems such as marine and freshwater ecosystems. With other ecosystems often overlooked, for example non-farmland soil and terrestrial ecosystems. The interest in these “idyllic” ecosystems is reflected in publications. A literature review undertaken showed that on the Scopus database publications linked to microplastic pollution were not inclusive of all ecosystems (Qi *et al.*, 2020). Overall, 1331 publications were retrieved covering microplastic pollution between the years 2004 and 2019 of these publications 71% focused on marine, freshwater and other aquatic ecosystems. Whereas, only 5% of the publications were exclusively discussing the influence of microplastic pollution on terrestrial ecosystems (Qi *et al.*, 2020).

More recently it has been identified that plastic materials are entering soil and terrestrial ecosystems through plastic mulch films, atmospheric deposition, sewage, fertilizers and municipal waste (Qi *et al.*, 2020). As mentioned previously microplastics are not a static pollutant and it has been recently understood that soil microplastic pollution may contribute as a source of microplastics to aquatic ecosystems (*Figure 2*). The impact of microplastics on terrestrial ecosystem are often parallel to the impacts seen in aquatic ecosystems. It has been shown that microplastics are capable

of altering the soil's physical and chemical properties (Rillig, 2012), which in time may have adverse effects on microbiota and associated flora. Interest in research on soil plastic pollution is increasing as the pollutant is gaining recognition for its non-discriminatory behaviour and continuously damaging cycle of dispersal.

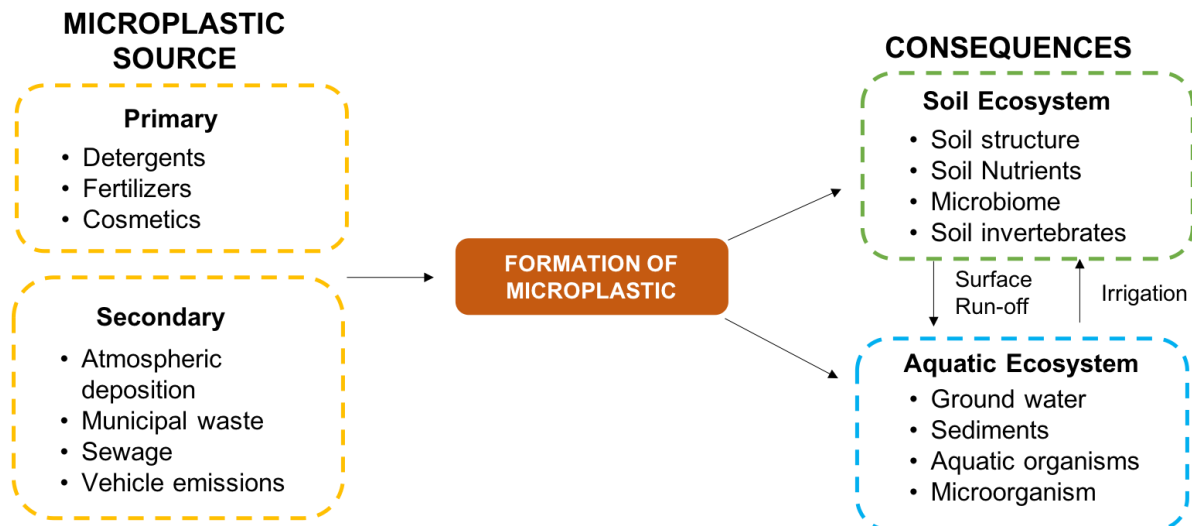


Figure 2: The most common sources of microplastic pollution and the subsequent consequence that these particles have on soil and aquatic ecosystems. It is presented that soil and ecosystem microplastic pollution is fluid.

1.1.3 The disposal and degradation of Poly(ethylene terephthalate)

It is recognised that plastics do not fully degrade when in a natural environment, hence remaining as a persistent environmental pollutant. Waste plastic that is sent to landfill or placed within a natural environment will eventually degrade into smaller pieces, rather than degrade entirely (Sigler, 2014). Degradation is the modification of the polymer chain including modifications to the main chain back bone, the side chain or side chain groups (Venkatachalam *et al.*, 2012). Their persistence is directly linked to their desirability as a strong and durable material (Koelmans *et al.*, 2017). Plastic will degrade through different mechanisms of degradation, however, the most common is photodegradation. Photodegradation is linked to other degradation processes such as thermal-oxidative degradation and biological degradation.

The process of photodegradation is initiated with UV radiation, harnessing energy from a solar source. This leads to oxygen atoms being incorporated into the plastic polymer, thus leading to the plastic becoming brittle and breaking into smaller pieces (Webb *et al.*, 2013). Specifically, in PET photodegradation of the polymer leads to the cleavage of the ester bond which forms a vinyl end group and carboxylic acid group (Gewert, Plassmann and Macleod, 2015) (*Figure 3*). Though a seemingly simple process this can take over 50 years to be fully complete and even then, plastics may still remain present within an environment as micro, nano or pico plastics.

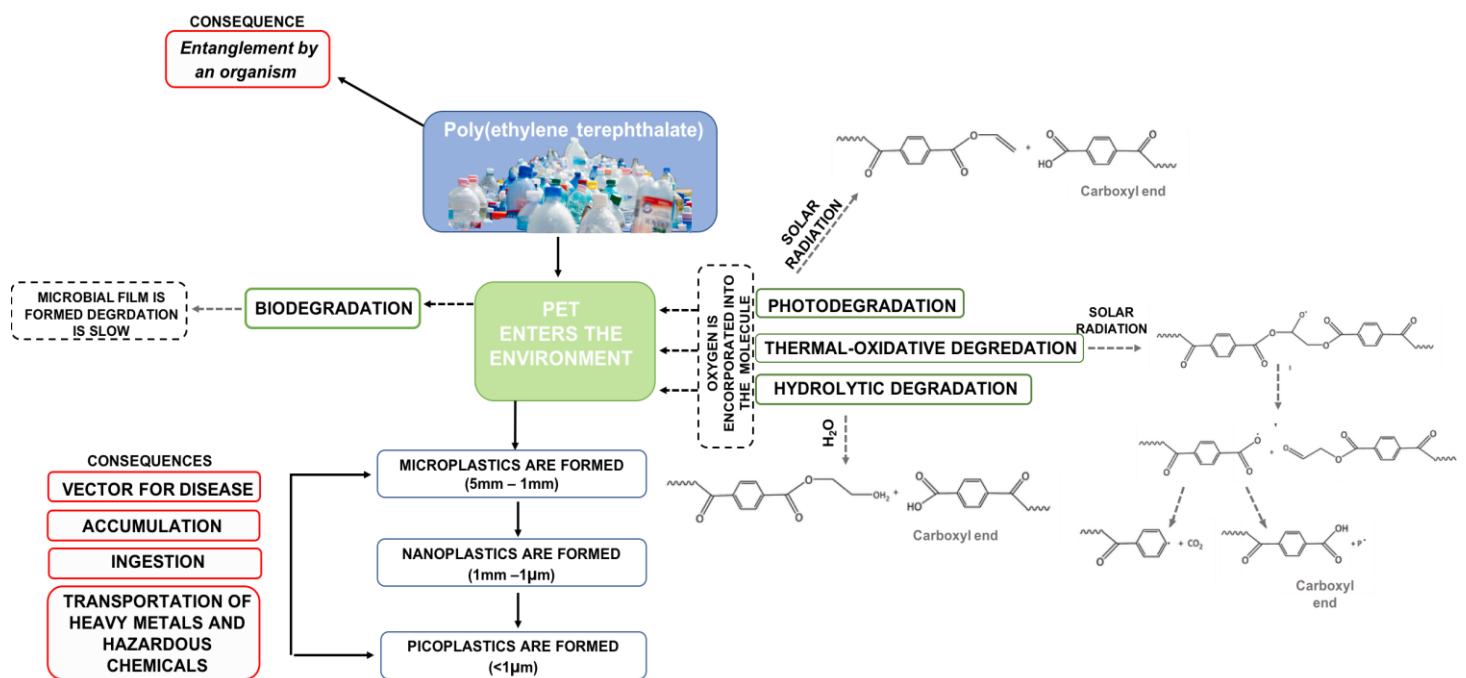


Figure 3: A schematic showing the degradation pathway of PET. The degradation pathways of PET from photodegradation. Thermal-oxidative degradation and hydrolytic degradation are presented with information adapted from (Venkatachalam *et al.*, 2012; Gewert, Plassmann and Macleod, 2015)

As previously mentioned in *Section 1.1.1* the physical and chemical properties of plastic limits its disposal and degradation potential. The molecular weight of a plastic limits the potential of microbial colonisation. Further linked to colony establishment is the surface structure of the plastic. The crystallinity, for example the density and spatial arrangement of individual fibres, of a plastic influences the attachment potential of a microbial community. Additives added to a plastic polymer, mainly examples of persistent organic pollutants, have been shown to cause further damage to an

environment (Hahladakis *et al.*, 2018). These additives, however, may also provide a starting point for microbial degradation due to their lower molecular weight in comparison to the rest of the polymer (Glaser, 2019). Furthermore, lower crystallinity will result in higher degradation rates.

1.1.4 *The Discovery of Biological Degraders*

The recycling of plastics into new plastic materials and the complete incineration of plastic waste, have proven ineffective and in most circumstances leads to further environmental damage. This has spurred an increase in interest in the utilisation of biological degraders to remove plastic with reduced environmental implications. Though this area of research is gaining increased media attention at present, the potential of plastic degrading microorganisms has been a topic of curiosity since the 1960's. During this time it was shown that multiple microorganisms consume paraffin as a form of carbon source, which can be utilised as a model for plastic polymer degradation (Iiyoshi, Tsutsumi and Nishida, 1998).

Since the threat that plastic poses to the environment was recognised, the design of plastic materials was re-approached to make plastic products more susceptible to microbial attack (Shah *et al.*, 2008). A biodegradable material is defined as a material which is capable of undergoing decomposition into non-hazardous compounds such as carbon dioxide, water or biomass capable of being degraded by microorganisms (Song *et al.*, 2009). The material degradation should occur over a short period of time and cause no negative impact on the surrounding environment. Plastic-like substitutes, that are biodegradable, are more commonly being employed due to increased attention on the negative footprint that plastic materials have left on the environment. Biodegradable materials are often derived from renewable and natural materials (*Figure 4*). Despite these alternatives being utilised and developed more frequently, degradation of these materials is still lengthily and may pose a threat to the environment.

BIO-BASED BIODEGRADABLE POLYMERS



Figure 4: Categorisation of popular bio-based biodegradable materials

PET has previously been demonstrated as non-biodegradable. However, in recent years significant progress has been made. It has been shown that bacterial cutinases, which are able to degrade cutin, a plant polyester, are also able to cleave ester bonds in PET making the plastic more susceptible to degradation (Roth *et al.*, 2014). This made the cutinases an extremely promising biological catalyst for the enhancement of biological degradation of PET products and provides a low environmental impact disposal method. Efficient production of cutinase was shown in *Thermobifida fusca* (Chen *et al.*, 2010). *T. fusca* is a thermophilic bacterium, meaning *T. fusca* has a higher thermostability than other bacterial species and have a greater tolerance to organic solvents making potentially invaluable to industrial processes associated with the modification of plastics, as well as their production (Roth *et al.*, 2014). However, the application of *T. fusca* to industrial disposal of plastic is still yet to happen.

Two species of fungi; *Fusarium oxysporum* and *Fusarium solani*, similar to *T. fusca*, have been found capable of degrading PET fibres, also by the secretion of cutinolytic esterase (cutinase) (Nimchua *et al.*, 2008). These fungal species were isolated through a screening process involving the identification of fungal species capable of growing on top of vegetation. Various studies have looked into enzymes associated with plants due to high levels of cutin and suberin being present in the vegetation. Cutin is similar to plastic polymers due to its high molecular weight, this suggests that it is thermoset polymer and has insoluble properties meaning biodegradation is restricted in ways similar to plastic materials (Heredia-Guerrero *et al.*, 2017). The isolates were placed onto selective media containing polycaprolactone (PCL), a biodegradable polyester. Both species presented significant clearance of PCL, which

was also observed with plates containing PET fibres (Nimchua *et al.*, 2008). The cutinase presented hydrolysis of the ester bonds in PET cloth material increasing the hydrophilicity of the fabric. This research can be used to inform current disposal approaches of PET containing materials.

It has been proven that there are various microorganisms capable of degrading PET. One of recent significant interest was the discovery of *Ideonella sakaiensis* by the Oda lab in 2016. *I. sakaiensis* is a novel bacterium capable of producing enzymes that can hydrolyse PET. The novel bacterium was discovered in 2016 at a plastic bottle recycling plant in Japan (Yoshida *et al.*, 2016). Over 250 PET contaminated samples were taken from the recycling plant. The samples were screened for microbes that utilised the PET as their major carbon source for growth (Yang, Yang and Jiang, 2016). From this isolation one culture produced a distinct microbial consortium that was able to form on top of the PET film, this led to the understanding of the secretion of the evolved enzymes: PETase and MHETase.

1.1.5 Metabolism of PET by *Ideonella sakaiensis*

Ideonella sakaiensis is a gram-negative aerobic bacterium. They're motile and are able to adhere onto the surface in PET. Once *I. sakaiensis* adheres onto the surface of the PET material, PET hydrolase (PETase) is secreted. The extracellular PETase initially hydrolyses PET to produce, monomeric mono-2-hydroxyethyl terephthalate (MHET) and bis(2-hydroxyethyl)-TPA (BHET), both heterodimers and Terephthalic acid (TPA), a monomer (*Figure 5*). A predicted lipoprotein, MHETase, hydrolyzes MHET to TPA and Ethylene Glycol (EG) (Austin *et al.*, 2018). The PETase enzyme from *I. sakaiensis* is thought to be the first of its kind, the enzyme functions by hydrolysing the ester bonds within PET with great accuracy and specificity. The Enzymatic pathway is presented in *Figure 5*.

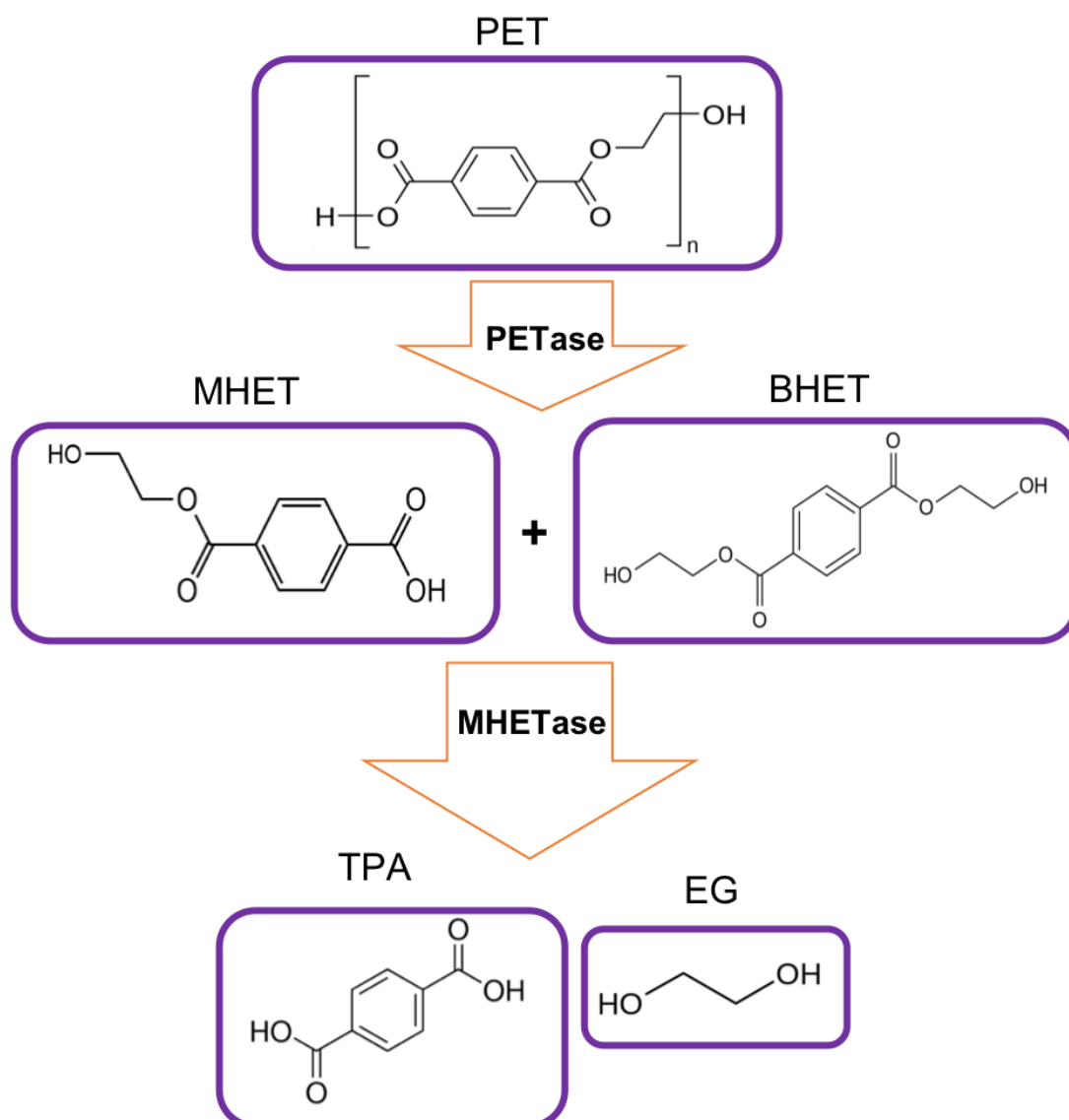


Figure 5: The PETase enzymatic pathway. The pathway presents the hydrolysis of Poly(ethylene terephthalate) to Terephthalic acid and Ethylene glycol (Yoshida *et al.*, 2016).

1.1.6 PETase Enzyme Structure and Function

The employment of enzymes to degrade PET into its original monomers is an environmentally friendly approach to reduce plastic waste and also provides a promising opportunity to recover petroleum-based materials; terephthalic acid and ethylene glycol (Chen *et al.*, 2018). Currently, it is understood that hydrolases, esterases and lipases that can modify and degrade biopolyesters, polymers found

naturally within the plant epidermis and periderm cell-wall macromolecules, such as cutin and suberin. This may also have significant effect on the modification and degradation of PET materials (Carvalho, Aires-Barros and Cabral, 1998). Bizarrely PET and cutin have little structural similarities other than one ester bond, this ester bond is 'attacked' and cleaved by the PETase (Chen et al., 2018).

At present, the differences between the structure of PETase and other PET-hydrolysing cutinases is largely unknown. Chen et al., (2018) reported the crystal structure of PETase and compared it to the previously described *T. fusca* cutinase, which shares a 47% sequence identity. PETase adopts a 'typical α/β -hydrolase fold, containing a twisted central β -sheet consisting of nine β -strands which is sandwiched by six α -helices' (Chen et al., 2018). The high sequencing identity suggests that the location of the enzymatic catalytic triad can be easily identify. The catalytic triad is a group of three amino acids that are commonly found in the active site of an enzyme. The triad is composed of the following residues: S131-H242-D177 (Chen et al., 2018). However, compared to other cutinases, the active site of the PETase enzyme is much wider, it has been suggested that therefore PETase is much more efficient at binding to PET.

A comprehensive understanding of the molecular mechanism of PETase is essential for further applications of the enzyme. The mechanism of PET degradation by PETase was described in the Chen et al., (2018) paper. Initially, PETase forms a cleft where the residue W156 can adapt various conformations. PET will then bind to the pocket formed, this is undertaken by hydrophobic interactions. The carbonyl group is positioned at the catalytic centre, with the O atom facing into the oxyanion hole, which is a pocket within the active site. A canonical hydrolytic reaction occurs, ensuing an intermediate acyl-enzyme formation takes place. A further nucleophilic attack by a water molecule happens, this cleaves the ester bond. The benzoic acid group that remains will form a broader planar shape, this formation is prone to π -stacking interaction with W156. Interestingly the conformational flexibility of W156 residue, unique to PETase, assists in the binding of the enzyme to the PET surface. Eventually, PET is rotated and released from the active site. PETase presents an astonishing

mechanism of hydrolysis of PET and presents hope for the further application to resolve plastic pollution and municipal waste disposal.

This study uses the PETase mutant (Austin *et al.*, 2018), the PETase mutant has a modified active site, creating more similarities to the previously mentioned *T. fusca*. At the active site a double mutant was produced which was aimed at narrowing the active site, and therefore increasing binding to PET (Austin *et al.*, 2018).

1.1.7 Applications of PETase as a biocatalyst

Since the discovery of PETase an increasing number of studies have analysed the potential of other model organisms to act as biocatalysts for PET. Each study selects a model organism to specifically target one aspect of PET pollution from systems to inform the biodegradation of PET in industry to specific environments such as marine or terrestrial.

Different microbial species have been investigated in their potential use in industry. A recent study analysed the ability to use the yeast species *Pichia pastoris* as a green alternative of PET recycling (Chen *et al.*, 2020). This study presented *P. pastoris* as the first example of a whole-cell biocatalyst capable of presenting PETase on the cell surface. From this study it is now understood that the stability of PETase can be increased when displayed on the cell surface. PETase pH and thermal stability is increased when cell surface expression occurs (Chen *et al.*, 2020). This study showed that PETase remained stable in a range of differing pH conditions up to a pH of 9, in native PETase expression systems such stability was not reflected (Yoshida *et al.*, 2016; Chen *et al.*, 2020). This presents promising applications of the enzyme as a biocatalyst and the stability of the enzyme on the cell surface in other expression systems should be researched in order to provide a green route for PET disposal.

Huang *et al.*, (2018) researched the capability of *B. subtilis* a gram-negative bacterium, found natively in soil ecosystems and the gut microbiome of both humans and animals, to be an efficient biological carrier of the PETase enzyme. The study investigated the influence of signal peptide on the secretion of PETase suggesting that

signal peptides are essential for sufficient secretion of PETase. Among this analysis they identified that some signal peptides are more efficient at directing secretion than others. Specific to *B. subtilis* it was shown that SP_{PETase} is the most efficient signal peptide. To test whether the PETase secreted by *B. subtilis* was able to actively degrade PET, supernatant from Δ Total Tat-SP_{PETase} was applied to PET. After 18 hours “pitting”, meaning abrasions on the surface of the PET, were observed, with larger “pits” forming after 48 hours. These results suggest that *B. subtilis* is a suitable organism capable of being engineered to biodegrade PET.

1.2 DICTYOSTELIUM DISCOIDEUM AS A MODEL ORGANISM FOR PLASTIC BIOREMEDIATION

1.2.1 Social Amoebas

Dictyostelium discoideum is a species of social amoeba, which were first described in 1869 (Bonner, 1944). *D. discoideum* was first classified and named in 1935 by Kenneth Raper (Raper, 1941). Since its identification *D. discoideum* has been a subject of extensive research. *D. discoideum* is a soil dwelling amoeba that undergoes three transitional lifecycles. *D. discoideum* is found within the Protista kingdom and within the Amoebozoa phylum. The name Amoebozoa is attributed to the amoeboid locomotion that all amoeboid cells exhibit during their life cycle. The full classification of *D. discoideum* is presented in *Table 3*. From the comprehensive understanding of *D. discoideum*'s evolutionary relationships, it is argued that the species itself bridges an important evolutionary gap. The *D. discoideum* species branched from the eukaryotic stem after the appearance of fungi.

Table 3: Classification of *Dictyostelium discoideum*

Classification	
Domain	Eukaryota
Kingdom	Protista
Phylum	Amoebozoa
Infraphylum	Mycetozoa
Class	Dictyostelia
Order	Dictyostelida
Family	Dictyostelidae
Genus	<i>Dictyostelium</i>
Species	<i>discoideum</i>

Research into the gene orthologs present in *D. discoideum* have been used to provide information that is relevant to higher metazoans. From sequencing analysis, it has been shown that *D. discoideum* is ancestrally closer to animals than plants and other fungi are, this is shown in *Figure 6* (Williams, 2010). At a molecular level *D. discoideum* presents certain mechanisms such as phagocytosis and chemotaxis that are conserved in human blood cells (Devreotes and Zigmond, 1988).). Similarities to higher metazoans are expressed at a cellular level. The organisation and structure of a *D. discoideum* ameboid cell, in the terms of the composition of lipid bilayer plasma membrane, cellular cytoskeleton and nuclear organisation reflect metazoan cells more so than other fungal species. In addition, it has been proved that the *D. discoideum* genome presents higher gene conservation with the human genome when compared to other fungal species (see *Section 1.2.6*).

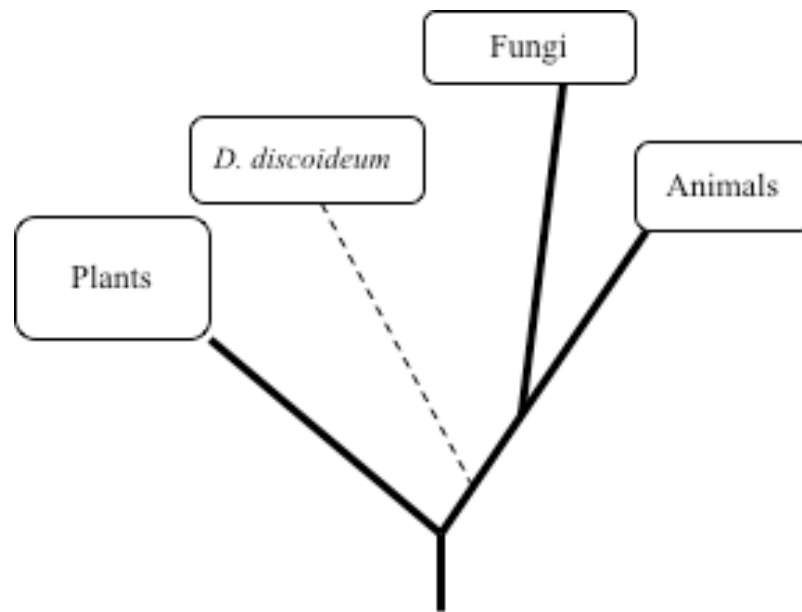


Figure 6: The evolutionary relationship between *Dictyostelium discoideum* and fungi, plants and animals. Adapted from Williams (2010)

1.2.2 The biology of Amoebozoa

To understand how *D. discoideum* may be implemented as a model organism for any study, it is imperative to understand Amoebozoans at a biological level. Amoebozoa characteristically have no defined shape and are constantly changing form by extending protrusions known as pseudopodia. Pseudopodia are actin-filled protrusions across the amoeboid cell surface that act as mediators of cellular movement (Bosgraaf and Van Haastert, 2009). Pseudopodia, alongside motility, can also be used to facilitate food discovery and consumption, these are known as phagocytosis pseudopodia (Mast and Doyle, 1933). Initially, cellular motility confounded scientists. This is due to cellular motility and migration being attributed to a complex set of interacting processes, not believed to be present within such a simple organism (King and Insall, 2009). This led to *D. discoideum* being a key model organism in understanding cellular motility within microorganisms.

D. discoideum presents a unique overlapping life-cycle with 3 different stages. These 3 stages can be divided into two different cell phases, unicellular growth and

multicellular developmental phases, in which the latter is initiated by starvation (Mathavarajah, Flores and Huber, 2017). Cellular motility is present in various life phases of *D. discoideum* (Figure 7). For the majority of their life, *D. discoideum* is found in a dormant-like state, where cells will divide periodically by mitosis and feed upon bacteria within the soil. When the food source is restricted a different life-phase is entered. This is divided into a social phase or sexual phase, which differ significantly. Within the social phase, the amoebae aggregate to form a mobile slug, which in turn will form a fruiting body. This is where efficient cell motility is significant to survival. During the sexual phase, the individual amoebae aggregate and within in this aggregation two cells of opposite 'sex' will fuse and then begin consuming other cells that are attracted. The victim cells will produce a cellulose wall to deter cannibalism, when completed however the diploid cell formed will undergo recombination and then meiosis (Flowers *et al.*, 2010).

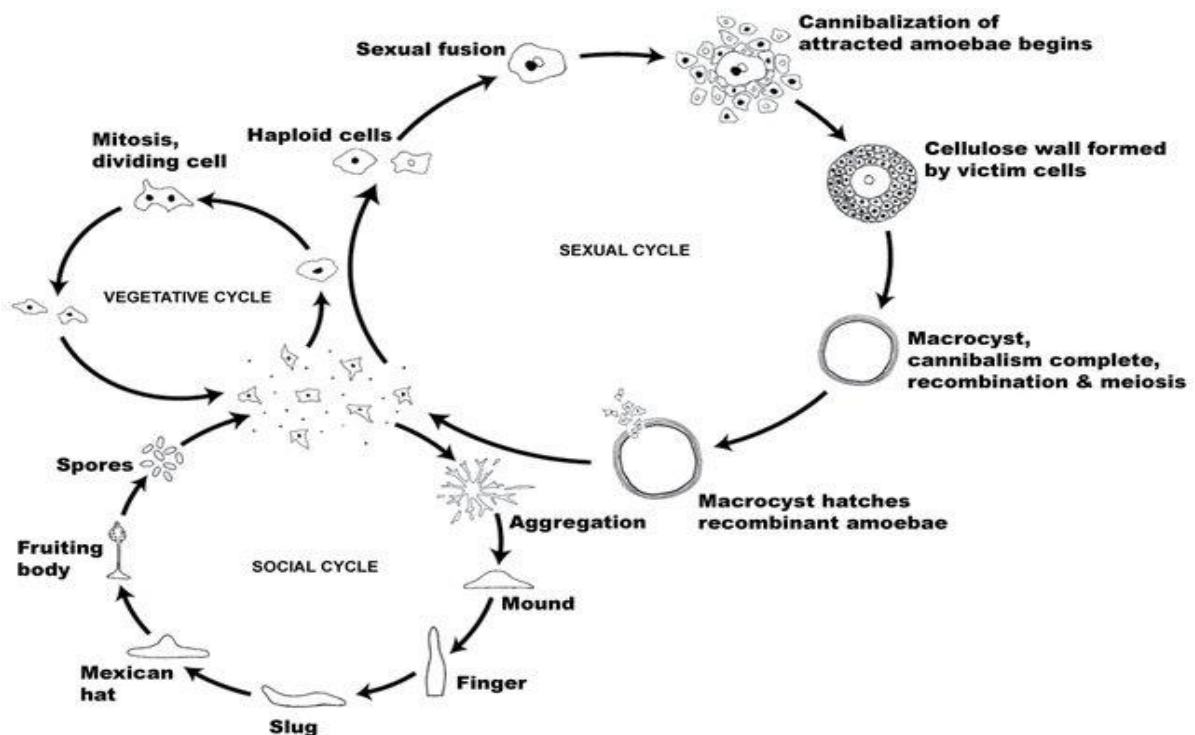


Figure 7: The life stages of *Dictyostelium discoideum* (Flowers *et al.*, 2010)

Interestingly, different strains of *D. discoideum* also present variations in colony morphology and life cycle. Primarily strains of *D. discoideum* can be divided into wild-type strains and axenic strains. This division is based upon the media in which they are grown, the life cycles they enter and the presence of food. The strains of *D.*

discoideum that are classified as wild-type will only present growth with the presence of complex food sources, such as bacteria. Mutant strains were developed from the extensively studied NC4 strain. This strain Ax2 can grow axenically without the addition of other organisms to the media as a food source. Axenic is defined as a culture of a species without the addition of any other organisms (Kayman and Clarke, 1983). Studies of *D. discoideum* will use different strains dependent on the experiments. Genetic and molecular studies will often use axenic strains whereas studies focusing on cellular motility and differentiation will use wild-type strains.

1.2.3 Chemotaxis in *Dictyostelium discoideum*

Chemotaxis is a complex process that incorporates cellular polarity, motility and the ability to understand and sense direction. Chemotaxis is an important mechanism in *D. discoideum* development and survival. When *D. discoideum* food supply is reduced a new life cycle is entered, this life cycle is initiated by a chain of transition phases controlled by the secretion of a chemoattractant. *D. discoideum* cells go from being a single amoeboid cell to a multicellular community, mediated by the secretion of cyclic-AMP (cAMP) (Cai *et al.*, 2011). The single amoeboid cells will aggregate in the areas in which the cAMP is being secreted. cAMP is secreted by differentiating cells in 6-minute cycles and amoeboid cells are equipped with cAMP receptors to respond to the cAMP secretion (Cai *et al.*, 2011). The response of the amoeboid cell to the cAMP secretion is a primitive example of chemotaxis.

1.2.4 Behavioural Ecology of *Dictyostelium discoideum*

Social cooperation between individuals is deep rooted in evolutionary history. Typically, when thinking of social organisms, common species such as primates or eusocial insects such as bees come to mind first. However, interestingly, *D. discoideum* has played a key role in our current understanding of social behaviour in eukaryotes. They have also been applied to broaden the understanding of the genetic basis that constitutes sociality (Flowers *et al.*, 2010). *D. discoideum* is considered a social organism due to their ability to form a multicellular structure when nutrient

conditions are reduced (Gaudet, Fey and Chisholm, 2008). Thus, the construction of their fruiting body life-stage results from cooperative behaviour.

A further point of interest relating to social capacity of *D. discoideum*, is their association to primitive farming. Primitive farming or primitive agriculture involves the utilisation of sophisticated behaviours such as recognition, communication and cooperation (Brock *et al.*, 2011). *D. discoideum* has shown the ability to harvest and disperse 'crops'. In simpler species this is known as husbandry. This is achieved by the species ceasing feeding early and incorporating the bacteria into their fruiting bodies, the bacteria is then carried through to spore stages in the vegetative life cycle. This an advantageous behavioural trait as if the new area they establish is lacking in food, it does not become a limitation (Brock *et al.*, 2011). This behaviour is expressed throughout various wild-strains of Dictyostelium; including NC4.

1.2.5 Dictyostelium discoideum as a model organism

The comprehensive research that has been done on the genome of *D. discoideum* has pushed for the increased use of the species as a model organism. There are multiple features of the genome that make it an ideal model organism. The genome of *D. discoideum* is 34 Mb long and is fully sequenced. Initial sequencing was faced with multiple challenges due its high repeat content and high A + T richness (Eichinger *et al.*, 2005). The genome is organised into six chromosomes, that overall yield 12,5000 protein-coding genes. *D. discoideum* has multiple homologous genes with humans, which was one of the largest motivations behind the sequencing of the *D. discoideum* genome and its use as a model organism.

The information provided from the sequenced genome is mostly used to facilitate the discovery of proteins that aid studies of orthologues in the human genome. Interestingly, the number of recorded orthologues of human disease in *D. discoideum* was much higher than other common model species such as *Saccharomyces cerevisiae* or *S. pombe* and comparative to *Caenorhabditis elegans* (Eichinger *et al.*, 2005). Thus, *D. discoideum* has been successfully employed to explore the molecular

basis of human disease, the mechanisms underpinning drug action and pathways that may result in resistance to such drugs and therapeutics (Gaudet, Fey and Chisholm, 2008). It has been used to overcome scientific hurdles and further understand biological phenomena. Specifically, *D. discoideum* finds its place as a model organism in studies focusing on cellular altruism, single cell gene expression, as a host of intracellular pathogens and allo-recognition (Paschke *et al.*, 2018). As well as this *D. discoideum* has been employed various times to inform research of higher vertebrates (Schilde and Schaap, 2013).

1.2.6 *Dictyostelium discoideum* and protein secretion

As an established model organism *D. discoideum* has been employed into studies as a mechanism of protein secretion. It has been identified that the *D. discoideum* chromosome contains a high density of genes encoding approximately 12,500 proteins, which is twice the amount that *S. cerevisiae* encodes (Karathia *et al.*, 2011; Gómez *et al.*, 2016). The proteome profile of *D. discoideum* has been analysed in depth. Bakthavatsalam and Gomer, (2010), identified 349 secreted proteins of the approximate 12,500 proteins encoded in the *D. discoideum* genome are secreted. During cell differentiation the proteins that *D. discoideum* cells secrete vary. At present the function of a large proportion of these proteins are unclear. Studies are working towards identifying the function of such proteins and have divided the 349 identified proteins into 8 potential areas of function; Protein metabolic processes, proteolysis, carbohydrate metabolic process, cell adhesion, negative regulation of proliferation, regulation of aggregation size, other and unknown (*Table 4*).

Table 4: Identified function of secreted proteins from *Dictyostelium discoideum*. Data taken from Bakthavatsalam and Gomer (2010).

Potential function	Number of proteins associated
Protein metabolic process	67
Proteolysis	29
Carbohydrate metabolic process	25
Cell adhesion	9
Negative regulation of proliferation	2
Regulation of aggregate size	3
Others	110
Unknown function	104

When compared to broader studies of recombinant protein expression *D. discoideum* is utilised less regularly than other common model organisms such as *S. cerevisiae*, *Bacillus megaterium* and other Methylophilic yeasts (Fernández and Vega, 2016). However, with increased research and the development of molecular tools *D. discoideum* has become a useful recombinant protein expression host and has proved the ability to secrete proteins efficiently. Key factors that have led to *D. discoideum* becoming an attractive expression host, especially in the production and expression of heterologous proteins, is linked to their ability to fold complex proteins, produce high secretion yields and form post-translational modifications (Gómez et al., 2016). All with relatively minimal laboratory costs associated. *D. discoideum* as an expression host has been employed into studies analysing growth factors that are secreted (Asgari et al., 2001; Chen et al., 2007) and the production of glycoproteins (Emslie et al., 1995; Griffiths et al., 1996). Further to this *D. discoideum* has been applied to the production of high quantities at a low cost of commercially important therapeutic proteins (Arya, Bhattacharya and Saini, 2008).

1.2.7 *Dictyostelium discoideum* genetic tool kit

The rise of *D. discoideum* as a model organism has been facilitated by the development of a comprehensive genetic tool kit. The *D. discoideum* 34 MB haploid genome is contained on 6 chromosomes. It is thought to encode approximately 12,500 proteins. Tools that have been developed include gene disruptions, replacements, expression of tagged genes and tools directed at facilitating genetic manipulations.

Specific vectors have been developed over time to contribute high efficiency transformations. Most *D. discoideum* vector plasmids are high copy and contain the strong and constitutive act15 promoter region. *D. discoideum* vector plasmids can be divided into 4 sections by unique restriction sites (*Figure 8*). *D. discoideum* vectors usually contain a region coding for resistance to selective markers such as G418, Blastidicin or Hygromycin. All vectors also contain a *E. coli* replication set. There are, at present 37 recognised and widely used *D. discoideum* expression vectors submitted to the Dictyostelium Stock Centre (<http://dictybase.org/StockCenter/StockCenter.html>) (Veltman *et al.*, 2009).

Alongside the consideration of specific vectors used with *D. discoideum* other factors must be considered when working with *D. discoideum*. Due to the high A+T concentration of the *D. discoideum* genome compared to most other species, genes must be codon optimised before being transformed into *D. discoideum*. Growth phases must be considered inducing with protein secretion. Protocols associated with the genetic transformation of *D. discoideum* with heterologous genes have been refined extensively, with protocols specific to Wild-type strains and axenic strains differing. Overall, such protocols can be compared to other model organisms in the terms of their simplicity and cost. DNA can be introduced by electroporation with expression being identified in 5-10 days.

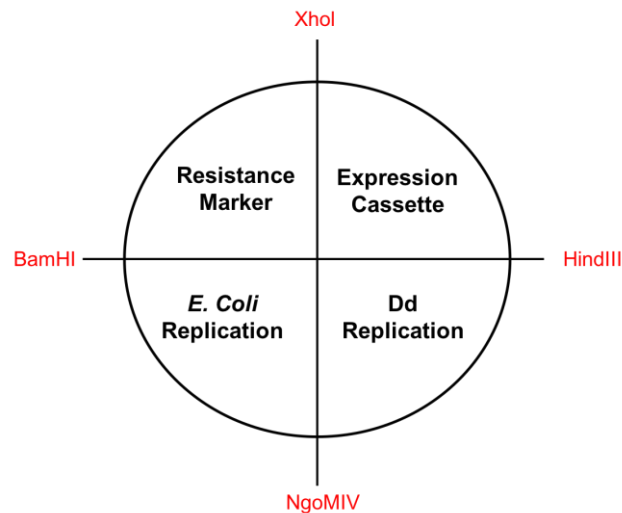


Figure 8 The basic layout of an example *Dictyostelium discoideum* vector plasmid. With unique restriction sites used to divide the plasmid highlighted in red.

1.2.8 *Dictyostelium discoideum* interaction with plastic materials

D. discoideum is motile during various stages in their life cycle. Movement is achieved by the extension and retraction of pseudopodia. Numerous studies have addressed *D. discoideum* substratum adhesion (Knecht, Fuller and Loomis, 1987; Schilde *et al.*, 2016; Kamprad *et al.*, 2018). These studies can be used to inform *D. discoideum* interaction with plastic and their potential to come into close enough contact with such plastics to be an efficient biological degrader. Adhesion to any substrate is entirely dependent on compatibility of the species to the surface chemistry of the substrate in question. As *D. discoideum* is present within a continuously varying environment, the species possess the ability to change their mechanism of adhesion (Tarantola *et al.*, 2014).

Unlike most microbial species *D. discoideum* lacks the genes to express extracellular matrix proteins (Knecht, Fuller and Loomis, 1987). Meaning they do not form focal adhesions. It is suggested that *D. discoideum* adhesion is achieved by various complex processes which presents them with increased flexibility and motility (Tarantola *et al.*, 2014). *D. discoideum* cells are able to move at a rapid pace, comparable to mammalian cells, on both hydrophobic and hydrophilic surfaces

(Loomis *et al.*, 2012).). *D. discoideum* can maintain adhesion in more challenging conditions, such as sheer stress and vertical orientations, compared to other cells. Studies have shown that *D. discoideum* adhesion is dependent on interactions linked to specialised adhesion molecules such as *sadA* and the glycocalyx membrane (Tarantola *et al.*, 2014) Further to contributions from the EDL-DVLO (Electrical Double Layer-Derjaguin–Landau–Verwey–Overbeek) force increases adhesion significantly (Kamprad *et al.*, 2018).

The EDL-DVLO force is responsible for initial adhesion and is influenced by van der Waals force and is dependent on polarizability and the cells repulsive electrostatics (Kamprad *et al.*, 2018). This is further dependent on the charges and ions of the solution in which *D. discoideum* is found. Interestingly, the adhesion of axenic *D. discoideum* to a substrate can be influence by the media in which they are grown in. HL-5 media which axenic *D. discoideum* is commonly grown in, contains high levels of glucose and amino acids which reduce adhesion. This is due to free amino acids and sugars disrupt not only the Van der Waals attraction within the glycocalyx membrane but also have been shown to disrupt hydrogen bonds formed between other molecules.

Aside from adhesion studies, interactions between *D. discoideum* and plastic polymers are, to my knowledge, sparse. This may be linked to the lack of awareness surrounding soil microplastic pollution, and the effect and influence it has on soil microbial communities. Often plastic polymers are used to study *in vitro* biological pathways, for example phagocytosis. Amoebozoans receive nutrients by phagocytosis. In short, the amoeba will surround the food particle, such as a bacterial cell, with their pseudopod then a phagosome is formed, and the cell is digested via specific enzymes. Often the phagocytotic substrates used in studies are often plastic particles such as latex beads, thus the understanding of the mechanism of phagocytosis and the studies surrounding the uptake of the latex beads can be directly applied to this study.

These studies can be used to inform this study regarding the likeliness of *D. discoideum* interacting, or ingesting plastic particles present within their environment. Relatively early studies showed that the uptake of latex beads in other social amoebas

such as *Acanthamoeba castellanii* happens at a rapid rate. Within 5 minutes the amoeba had bonded with the plastic polymer and by 10 minutes 80% of the plastic particles had associated with an amoeboid cell. This number continued to increase for a further 2 hours (Avery, Harwood and Lloyd, 1995). Recent studies have developed protocols in which *D. discoideum* phagocytosis is measured once again using $<1\mu\text{m}$ latex beads (Barak *et al.*, 2014; D'Souza *et al.*, 2016). The initiation of phagocytosis or the recognition mechanism is linked to 2 pathways; initially a lectin-like receptor mediates the binding of sugar, specifically glucose, derivatives found within the target cell. Furthermore, hydrophobic interactions from non-specific receptors guide the binding of such hydrophobic particles, such as bacterial species and in this case latex beads.

Earlier studies that aimed to identify biological degraders focused on the sourcing microorganisms capable of degrading natural polymers such as cellulose. Recently, it has been shown that *D. discoideum* are capable of secreting cellulose degrading enzymes and xylanase enzymes that can degrade xylan polymers, also associated to cellulose in plants. It is thought that these enzymes are used by *D. discoideum* to degrade plant tissue cells inadvertently taken up during feeding or bacteria containing high levels of cellulose. The potential of *D. discoideum* using these enzymes to degrade plastic is entirely unresearched at present but from previous research into cutinases or cellulose specific enzyme the proposal is not entirely implausible.

1.2 UNANSWERED QUESTIONS

There are various questions associated with this area of research that are important, remain under-researched and unanswered. Firstly, the extent of plastic pollution that is present directly within *D. discoideum* niche is entirely unresearched. Most studies that address microplastic pollution in terrestrial and ecosystem soil focus largely on agriculture due to the economic gain to reduce damage and risk to crops and livestock. No studies, to my knowledge, have directly and comprehensively addressed the direct and indirect impacts of microplastic pollution on microorganisms residing within the soil, most studies have focused on the effect of microplastics on macroorganisms such as soil invertebrates (Selonen *et al.*, 2020). Only recently studies have begun

addressing microplastic pollution in various terrestrial ecosystems, specifically those in which *D. discoideum* would be found.

Further to this there is still uncertainty in how to quantify the extent of microplastic pollution within soil samples to a level of accuracy in which can provide sound and reliable data. Often microplastics are measured using microscopy approaches. Measurements of nanoplastics and picoplastics is still not developed. There is consistent discrepancy present with definition of what a microplastic is, in the terms of size. This has led to misidentification and can lead to results being inaccurate, a standardised term for microplastic needs to be accepted globally (Helmberger, Tiemann and Grieshop, 2020).

Finally, the use of *D. discoideum* as a bioremediation system is yet to be explored. In Section 1.2.6 circumstances in which *D. discoideum* has been used to secrete proteins or as a “cell-factory” is outlined. However, there is no publications or studies that address the behaviour of *D. discoideum* as a “cell-factory” *insitu*. The influence that the secretion of a protein or the accumulation of the secreted protein within an environment is uncertain, models run parallel to this study would have to be undertaken to insure efficiency.

1.4 PROJECT AIMS

Working with *D. discoideum* as the model organism, this study aims to produce a new system for bioremediation of soil microplastic pollution. This will be achieved by producing an organism, *D. discoideum*, capable of secreting PETase into their surrounding environment in order to reduce soil plastic pollution. Techniques specific to *D. discoideum* must be understood, with plasmid DNA compatible with *D. discoideum* created. Upon the successful creation of plasmid DNA, *D. discoideum* will be transformed to secrete and intracellularly express PETase. A series of tests must be completed to investigate the extent in which PETase is expressed and secreted. Simulations of *D. discoideum*'s natural ecosystem can be created and tested to provide sound and further applicable results.

CHAPTER 2: MATERIALS AND METHODS

2.1 STRAINS UTILISED IN THIS STUDY

2.1.1 *Dictyostelium discoideum* strains

Three strains of *D. discoideum* were selected for this study (Table 5). One strain being an axenic derivative, Ax-2, the two other strains, NC4 and DdB, wild-type derivatives. See Section 1.2.2 for the definition of axenic growth. The Ax-2 strain was initially derived from the Ax-1 strain. The Ax-1 strain was first isolated by Sussman and Sussman, (1967) and the Ax-2 strain was first generated by Watts and Ashworth, (1970). Ax-1 and Ax-2 differ as the Ax-2 strain is not calf serum and liver extract in their growth media. NC4 is the parent strain to DbB. The NC4 strain was identified and established as a parent strain by Bonner, (1944). DdB differs from NC4 as it displays synchronised development and has overall reduced colony spread (Brackenbury and Sussman, 1975).

Table 5: Differences in *Dictyostelium discoideum* strains used within this study

Strain Name	Phenotype	Parental Strain	Reference	Source
Ax-2	axenic	DdB	Watts and Ashworth (1970)	Dictybase
NC4	wild	N/A	Raper, (1935).	Dr Elinor Thompson Laboratory, Greenwich University
DdB	wild	NC4	Brackenbury and Sussman., (1975)	Dr Elinor Thompson Laboratory, Greenwich University

2.1.2 *Klebsiella aerogenes* strain

The wild-type *K. aerogenes* strain used in this study was revived from the School of Biosciences (the University of Kent) bacterial culture collections. The strain was initially obtained from the American Type Culture Collection (ATCC, cat no.13048). The strain was first identified by Hormaeche and Edwards in 1960 (Tindall, Sutton and Garrity, 2017).

2.1.3 *Escherichia coli* strains

Two *E. coli* strains were used for recombinant DNA work; Top10 and NEB stable. These cells were utilised during the transformations of plasmids described in Section 2.4. The genotypes of the strains are presented in *Table 6*.

Table 6: Escherichia coli strains used in this study

Strain Name	Genotype
Top10	F ⁻ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK λ ⁻ rpsL(Str ^R) endA1 nupG
NEB Stable	F ⁺ proA+B+ lacI ^q Δ(lacZ)M15 zff::Tn10 (Tet ^R) Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14 ⁻ Φ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL (Str ^R) rph spoT1 Δ(mrr-hsdRMS- mcrBC)

2.2 GROWTH MEDIA

All media and buffers used in this study were autoclaved at 121°C for 10 minutes. Both liquid and solid media were stored at room temperature, 25°C, except media containing additional antibiotics, which were stored at 4°C. For long term storage

media and bacterial cultures were transferred to 4°C. H40 and H50 buffers were transferred to -20°C before use. Where possible media was made as and when it was required to insure freshness. All water used in the media and buffers were sterilised ddH₂O or MilliQ water. *Table 7* and *Table 8*, summarise the medias and buffers used within this study.

Table 7: Composition of Media used within this study. Components concentrations are presented in g/1000ml. Ampicillin and streptomycin is added when required at a 100µg/ml concentration from a 1000x stock.

Media	Purpose	Component	Concentration
SM Media	<i>K. aerogenes</i> growth	Glucose	10g/1000ml
		Bacto Peptone	10g/1000ml
		Yeast Extract	1g/1000ml
		MgSO ⁴ 7H ₂ O	2.0477g/1000ml
	<i>D. discoideum</i> growth	KH ₂ PO ₄	1.9g/1000ml
		K ₂ HPO ₄ (trihydrate)	0.992/1000ml
		ddH ₂ O	1000ml
		Agar	20g/1000ml
LB (+Ampicillin)	<i>E. coli</i> growth	Tryptone	10g/1000ml
		NaCl	10g/1000ml
	<i>K. aerogenes</i> growth	Yeast Extract	5g/1000ml
		ddH ₂ O	1000ml
		Ampicillin	100mg/1ml
		Agar	20g/1000ml
HL5 (+Ampicillin) (+Streptomycin)	Axenic <i>D. discoideum</i> growth	Difco Peptone	10g/1000ml
		Yeast Extract	5g/1000ml
		Na ₂ HPO ₄ -7H ₂ O	0.35g/1000ml
		KH ₂ PO ₄	0.35g/1000ml
		Streptomycin	100mg/1ml
		Ampicillin	100mg/1ml
FM Medium	Axenic <i>D. discoideum</i> growth	See Section 2.2.1	
SIH Medium	Axenic <i>D. discoideum</i> growth	See Section 2.2.1	

Table 8: Composition of buffers used within this study. Components concentration presented in g/1000ml excluding SorMC where a 1000x concentration is required and is then diluted to a 1x working solution.

Media	Component	Concentration
SorMC Buffer (1000x Concentration)	KH ² PO ₄	20.36g/100ml
	Na ₂ HPO ₄	5.47g/100ml
	ddH ₂ O	100ml
H40 Buffer	HEPES	9.52g/1000ml
	MgCl ₂	1000µl of a 1mM solution
	ddH ₂ O	1000ml
H50 Buffer	HEPES	4.76g/1000ml
	KCl	3.73g/1000ml
	NaCl	0.58g/1000ml
	MgSO ₄	0.12g/1000ml
	NaHCO ₃	0.42g/1000ml
	Na ₂ HPO ₄	0.156g/1000ml
	ddH ₂ O	1000ml

2.2.1 Selection of axenic media

The growth rate of Ax-2 was assessed in three different growth media. The synthetically defined minimal media, FM media, from ForMedium™ was assessed. FM media is composed of mineral salts, in micro and macro concentration, supplemented vitamins as well as amino acids. SIH, another synthetically defined medium purchased from ForMedium™ was also assessed. SIH has the same composition as FM media but additional Aspartic acid. Finally, HL5, the most common *D. discoideum* media, was also assessed. This media is used throughout most *D. discoideum* studies and is composed in house rather than being purchased pre-made. The differences in media composition are summarised in Table 9. All media used has the addition of ampicillin (100mg/ml) and streptomycin (300mg/ml) at x1000 concentration.

Differences in cell density between each media was determined using a haemocytometer, from these differences in growth could be assessed. Images were taken to assess cell health and contamination extent. All images were taken using the

Olympus IX81 inverted microscope. The light source for this microscope was the CoolLED pE4000 illumination system. All images in this study were captured with an Andor's Zyla 4.2 PLUS sCMOS camera. Cells were observed using the Olympus 100x objective lens.

Table 9: Composition differences between axenic media; FM, SIH and HL-5.

	HL-5	FM	SIH
Source	Franke and Kessin (1977)	Franke and Kessin (1977)	Han et al., (2003)
Component			
Glucose (mmol litre ⁻¹)	>56.0	56.0	56.0
Amino Acids (mmol litre ⁻¹)	81.7	63.7	70.8
Vitamins (mg litre ⁻¹)	35.7	1.7	1.7
Phosphate salts (mmol litre ⁻¹)	5.0	5.0	11.1
Salts (mmol litre ⁻¹)	14	3.72	1.72
Trace elements	20.8	26.78	26.78

2.3 DICTYOSTELIUM DISCOIDEUM CULTIVATION

K. aerogenes growth was initiated on SM-agar plates from long-term glycerol stocks, stored at -80°C. Plates were incubated at 30°C for 12 hours. 3ml of LB was inoculated with *K. aerogenes*. The liquid cultures were incubated at 37°C at 180rpm for a further 12 hours. Long-term stocks of liquid cultures of *K. aerogenes* can be stored at 4°C for 2-4 weeks. 400µl of *K. aerogenes* was inoculated with a single fruiting body from confluent *D. discoideum* plates. The initial *D. discoideum* plates used in this study were sourced from DictyBase and our collaborators Dr Billy Ferrera and Dr Eleanor Thompson, at the University of Greenwich. 200µl, 150µl and 50µl of the original 400µl of the *K. aerogenes*-*D. discoideum* suspension were plated onto SM-agar plates and grown for 48 hours at 22°C until a 4 x 10⁶ cells ml⁻¹ density was achieved. Cultures at this density were sub-cultured weekly to reduce risk of mutation.

2.3.2 Cultivation of axenic *Dictyostelium discoideum*

3ml of HL5 Media, supplemented with ampicillin and streptomycin (See Section 2.3.1) was deposited into each well of a 6-well plate. Each well was inoculated with a single Ax-2 fruiting body taken from fully developed *D. discoideum* cultures grown on a lawn of *K. aerogenes* on SM plates. The inoculated HL5 culture was incubated at 22°C for 48 hours in a static incubator. Cell density was calculated using a haemocytometer and wells containing a density of at least 5×10^4 cells ml⁻¹ were sub-cultured. 25ml of Fresh HL5(amp/strep) medium in a 15mm petri dish was inoculated with 250µl of the original culture, following a 1:100 dilution. Axenic cell cultures were grown until desired cell densities were reached.

2.3.3 Silica preservation of *Dictyostelium discoideum*

Silica preservation was identified as the most suitable method to create permanent long-term stocks of *D. discoideum*. 2ml glass vials were filled to one half with silica gel and baked at 180°C for 90 minutes. After baking the vials were cooled on ice for 30 minutes to reduce spore death. SM plates containing fruiting bodies, younger than one week, were harvested by being gently banged on a clean petri dish lid. Three to four plates were banged onto one lid to create a sufficient amount of stocks for preservation. Spores were resuspended in 400µl of chilled non-fat dairy milk solution. In each vial 400µl of the spore milk suspension was added. The vial was shaken for 7 seconds and placed back on ice for 5 minutes. Vials were then stored at -20°C.

2.3.4 Revival of silica preserved *Dictyostelium discoideum*

To reduce the chances of random mutation occurring within the strains, fresh cultures were made from the silica stocks every 2-4 weeks. 200µl of *K. aerogenes*, from a fresh overnight culture, was spread on a fresh SM plate. A few silica crystals were placed onto the surface and incubated at 22°C for 2 days.

2.3.5 Differences in growth rate between *Dictyostelium discoideum* strains

The differences between the growth rate of the *D. discoideum* strains used in this study were analysed using a basic growth rate analysis protocol. After the silica revival of each strain and cells were grown until fruiting bodies observed, 400µl of *K. aerogenes* was inoculated with a single fruiting body from each strain. The cell density of each strain was calculated using a haemocytometer and adjusted to ensure equal cell counts before growth was initiated. The adjustments to the cell density was undertaken by adding additional *K. aerogenes* culture to each sample. This was then placed on a fresh SM-agar plate and spread evenly. Growth was then monitored for 4 days. The diameter of the feeding zone (Figure 9) was measured twice a day for 4 days.

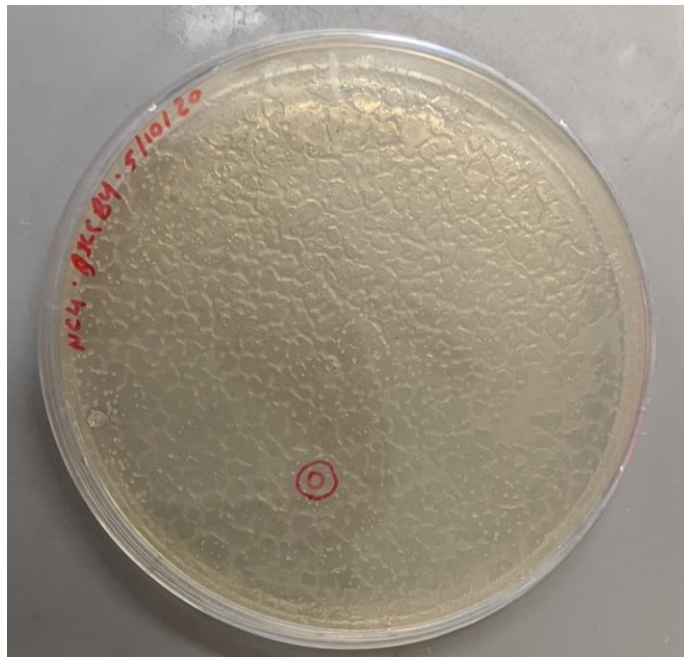


Figure 9: A confluent plate of *Dictyostelium discoideum* with the red rings showing the measurements of growth taken.

2.3.5 Genetic transformation of wild-type *Dictyostelium discoideum*

Transformations of the wild-type strains, NC4 and DdB, were attempted using the protocol outlined by Paschke et al., 2019. Initially, the bacterial food source was prepared. 1L of LB-medium was inoculated with a single colony of *Klebsiella aerogenes* and grown at 37°C shaking at 180rpm. Cells were spun down in two 500ml flasks at 6,000 x g for 20 minutes. Cells were washed once with 500ml of SorMC buffer for 20 minutes at 6,000 x g. Cell pellets were resuspended in 20ml of SorMC and the optical density was adjusted to $OD_{600} = 2$. 5ml. 100µl of the SorMC-*Klebsiella aerogenes* suspension was added to each well of a 6-well plate, in preparation for *D. discoideum* cultivation and transformation.

400µl of a 3ml *K. aerogenes* LB culture, grown at 37°C shaking at 180rpm for 12 hours was spread on a SM-agar plate. 400µl of a 3ml *K. aerogenes* LB culture, grown at 37°C shaking at 180rpm for 12 hours was spread on a SM-agar plate. The SM plate containing *K. aerogenes* was inoculated with NC4 and DdB cells, on independent plates. Plates were incubated at 22°C for 2-4 days ensuring that large and viable growth zones were present for transformations.

A 3cm² area of the growth zones on the *D. discoideum* culture plate was cleared using a sterile inoculation loop and was transferred to a 1.5 ml centrifuge tube containing 1ml of ice cold H50 buffer. Cells were washed in H50 buffer in a table top centrifuge for 2 min at 1,000 x g. Cells were resuspended in H50 buffer to a density of 4×10^7 . 100µl of the H50 *D. discoideum* suspension was applied to a chilled 2mm electroporation cuvette. 2µg of Plasmid DNA was also added and mixed gently. Cells were pulsed in a Bio-Rad Gene Pulser Xcell Electroporation system using the following settings: square wave, 350 V, 8ms, 2 pulses and 1 second pulse intervals.

100µl of electroporated cells were added per well to the earlier prepared 6-well plate containing the SorMC *Klebsiella aerogenes* suspension. Cells were left to recover for 5 hours. After the recovery period the selective marker, G418, was added to each well at a 20µg/ml concentration. 100µl of electroporated cells were added per well to the earlier prepared 6-well plate containing the SorMC *Klebsiella aerogenes* suspension.

Cells were allowed to recover for 5 hours. After the recovery period the selective marker, G418, was added to each well at a 20 μ g/ml. Cells were incubated at 22°C for 2 days. Successful transfectants were screened using the Olympus IX81 inverted microscope (see *Section 2.2.1*). Plasmid's containing the fluorescent tag were screened using GFP filters. Cells showing growth were sub-cultured into 10ml of SorMC containing *K. aerogenes* with additional G418 at the same concentration.

2.3.7 Genetic transformation of axenic Dictyostelium discoideum

Transformations of the axenic strain Ax-2 were attempted following a protocol provided by Dr David Traynor (MRC Laboratory of Molecular Biology, UK). 50ml of axenic culture at a 2-4 x 10⁶ cells per ml density was pelleted in a centrifuge at 500 x g for 3 minutes. Cells were resuspended in 500 μ l of ice-cold H50 buffer to a density of 3 x 10⁷ cells per ml. Resuspended cells and electroporation cuvettes were placed on ice for 5 minutes. 105 μ l of ice-cold cell suspension was placed into the electroporation cuvette, additionally 10 μ l of high concentration plasmid DNA was also added to the cell suspension and remained on ice. Plasmid DNA is discussed in *Section 2.4.3* and *Section 2.4.4*.

Each sample was electroporated in a Bio-Rad gene Pulser Xcell Electroporation system using the following settings: exponential decay pulse, a field strength of 0.74 kV, a capacitance of 25 μ and a 4.7 Ω resistance. Cells were electroporated in a 1mm cuvette with 2 pulses and with a 5 second interval. After the second pulse, 500 μ l of ice cold HL5 media with 200 μ g/ml of streptomycin was added to the cuvette, mixed by inversion and immediately placed on ice for a further 5 minutes. The contents of each cuvette were added to 14ml of HL5-streptomycin media. Dilutions of 1:10, 1:50, 1:100 and 1:200 were set up using 12ml of HL5-streptomycin media. Each dilution was loaded into a 96-well plate with 100 μ l per well. Plates were incubated at 22°C in a moist incubator.

After 24 hours 100 μ l of HL5-streptomycin and 20 μ g/ml of G418, the selective marker, were added to each plate. After 4 days of growth cells were sub-cultured by rapidly removing liquid by inversion and resuspended in 200 μ l of HL5-streptomycin and

10µg/ml of G418. Cells growth and health was checked using an inverted microscope. Successful transformants were cultured in larger quantities following steps outlined in *Section 2.3.2*.

2.4 PLASMIDS

Three different plasmids were selected for this study, two of which to act as a vector for the PETase gene and the third as a fluorescent control in genetic transformation procedures, to identify transformation efficiency (*Table 10*). All plasmids were selected based on *D. discoideum* compatibility and previous literature (see *Section 1.2.7*). Plasmid maps of the empty vector plasmids can be observed in *Figure 10*.

Table 10: Dictyostelium discoideum vector plasmid selection

Plasmid	Description	Reference
pDM1203	Extrachromosomal expression vector. Empty backbone plasmid. AmpR and Neo/KanR markers.	Paschke et al., 2018
pDM1208	N-terminal mCherry tagging. Extrachromosomal expression vector. AmpR and Neo/KanR markers.	Paschke et al., 2018
pDT29	Extrachromosomal expression vector. Empty backbone plasmid. GFP Tag. AmpR and Neo/KanR markers.	Manstein et al., 1995

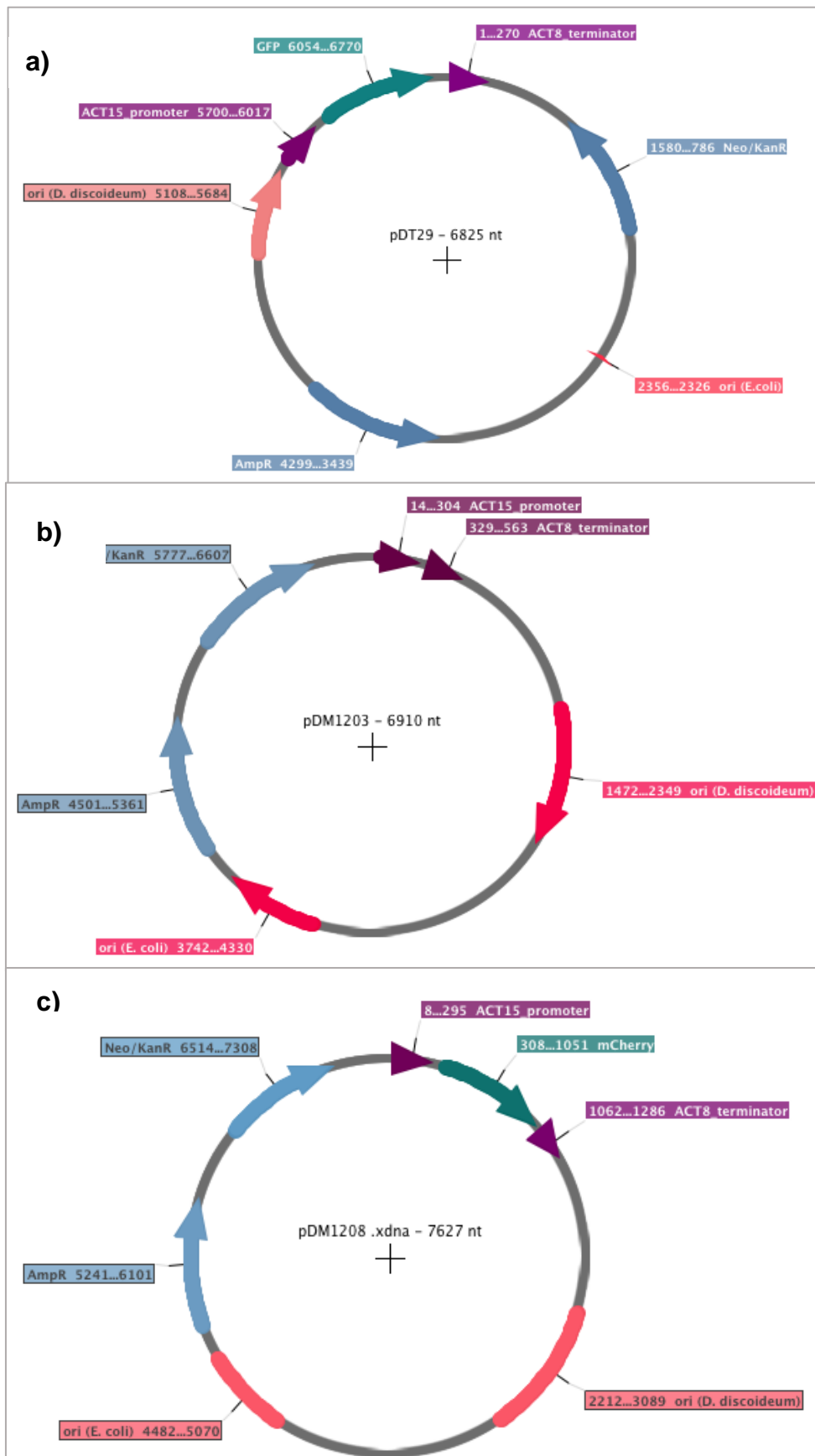


Figure 10: Backbone plasmid maps; a) pDT29 b) pDM1203 c) pDM1208

2.4.2 Design of *Dictyostelium discoideum* compatible PETase gene

A synthetic gene encoding PETase was designed to be compatible with *D. discoideum* codon usage. Preferred codons for each amino acid were identified using the Kazusa codon usage database (Nakamura, Gojobori and Ikemura, 2000), and a gene encoding the published PETase protein sequence (Yoshida et al., 2016) was constructed by using the preferred codon for each amino acid. The physical DNA of the codon optimised gene was ordered from Eurofins Genomics (Germany).

2.4.3 G+C richness analysis of the PETase gene

The G+C richness of the PETase_dicty gene, generated by codon optimisation was calculated using the Enmemo online G+C content calculator (<http://www.endmemo.com/bio/gc.php>). For comparison, the PETase gene was codon optimised for 2 other common model organisms; *E. coli* and *S. cerevisiae*. Codon optimisation was achieved using the steps outlined in Section 2.5.2. G+C richness of the codon optimised PETase gene was compared in *D. discoideum*, *E. coli*, *S. cerevisiae* and *I. sakaiensis*.

2.4.4 Cloning of PETase into vector plasmids

Primers were designed to allow for the insertion of the PETase gene between the *act15* promoter and *act8* terminator on pDT29 and pDM1203. Primer sequences were designed combining the sequences at the beginning and the end of the PETase gene with adjacent sequences in the plasmid. 3 primers were designed, 2 forward primers one promoting PETase secretion and one sequencing for cytoplasmic expression of PETase for each vector plasmid (Table 11; Table 12)

Table 11: Primers designed for the insertion of the PETase gene into the pDM1203 vector plasmid

Primer ID	Sequence
pDM1203_secretoryPETase_forward	TAAATTAATAAAAAATAAAAATCAGATCTAAAATGAA ATTTCAACATACATTTATTGC
pDM1203_PETase_reverse	ATTAAATAATTTATTTATTTAACTAGTACTAGATCTTTA TTATGCATAATCTGGAACATCATATGG
pDM1203_cytoplasmicPETase_forward	TAAATTAATAAAAAATAAAAATCAGATCTAAAATGAA TTTTCCAAGAGCATCAAGATTAATGC

Table 12: Primers designed for the insertion of the PETase gene into the pDT29 vector plasmid

Primer Design	Sequence
pDT29_secretoryPETase_forward	CCCAAGCTGTACCGAGCTCGGATCCAAAATGAAATT TCAACATACATTTATTGC
pDT29_cytoplasmicPETase_forward	CCCAAGCTGTACCGAGCTCGGATCCAAAATGAATTTT CCAAGAGCATCAAGATTAATGC
pDT29_PETase_reverse	TGGTAAACTTGAATTGATCCTCTAGATTATTATGCAT AATCTGGAACATCATATG

PCR was performed using GoTaq Green Master Mix (Promega UK) according to the manufacturer's instructions. The PCR reaction included 25µl of master mix, 1µl of PETase DNA construct, 1µl of forward primer, 1µl of reverse primer, 22µl of MilliQ H₂O. The reaction involved 30 cycles with an annealing temperature of 52°C with a 1-

minute extension step. The PCR reaction was confirmed using a 1% agarose gel. Bands were extracted and purified using the QIAquick Gel Extraction Kit.

Insertion of the PETase gene into the vector plasmid was attempted by a Gibson assembly reaction. An overview of the reaction can be observed in *Figure 11*. The Gibson assembly reaction was transformed into the Top10 strain competent cells. 3.5µl of the BglIII-digested plasmid and 1.5µL of the subsequent PCR product was mixed with 15µl of Gibson Master mix. The 1.5µl of PCR product was substituted with 1.5µl of ddH₂O to act as a control. The reaction was incubated at 50°C for 1 hour.

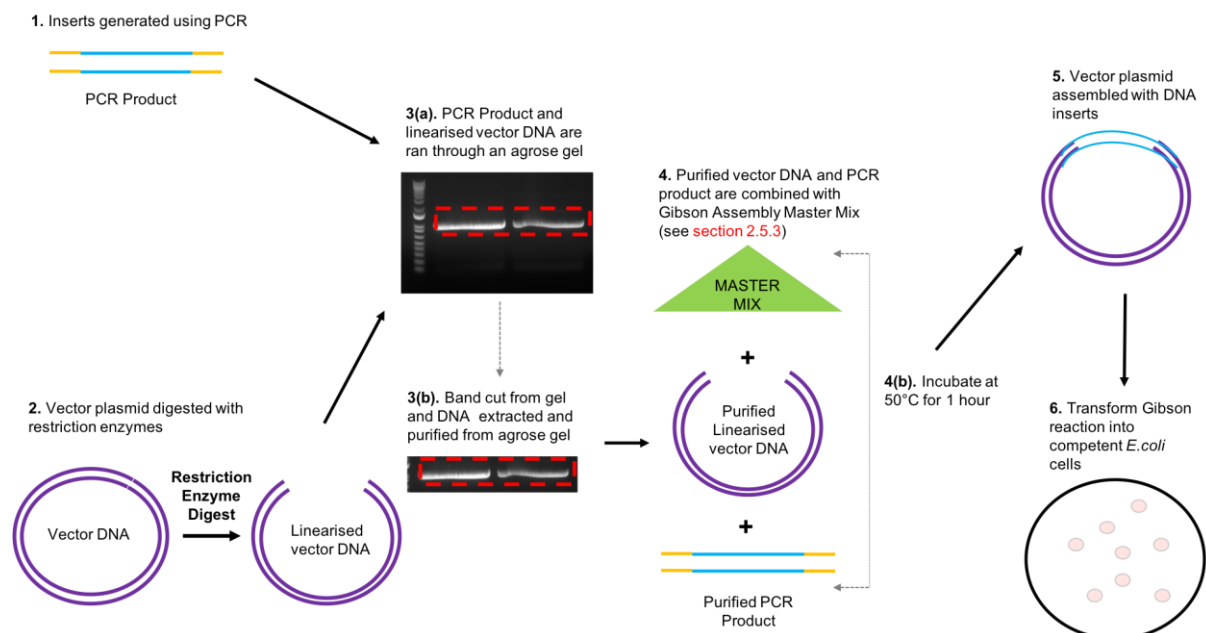


Figure 11: Procedure outline for the Gibson assembly reaction.

10µl of the Gibson assembly reaction was combined with 100µl of Top10 competent cells and placed on ice for 20 minutes. The cell-DNA mixture was heat shocked at 42°C for 45 seconds. Subsequently, 250µl of LB was added to the reaction and was then incubated at 37°C for 45 minutes shaking at 180rpm. The reaction was then centrifuged at 3,000 x g for 5 minutes, the pellet was then resuspended in 250µl LB. Each reaction was inoculated on LB(amp) plates and incubated for 12 hours at 37°C. 4 colonies from each plate were selected from each plate and 3ml of LB was inoculated and grown for 12 hours at 37°C shaking 180rpm.

DNA was extracted from the LB cultures using the Qiagen Hispeed plasmid miniprep kit following manufacturers guidelines. Success of cloning was analysed using a DNA 1% Agrose Gel. The gel was composed of purified agrose (purchased from ThermoFisher Scientific™) and dissolved in 1x TAE buffer (0.04M Tris-acetic acid, 10mM EDTA). Once dissolved the 1% agrose was cooled to 60°C and poured into silica gel mould with an 8-tooth comb. 4µl of 10mg/ml ethidium bromide solution was added to the mould. Once set the 8-tooth comb was removed. The gel was placed into an electrophoresis tank and submerged in 1x TAE buffer.

The extracted plasmid DNA was digested using selected restriction enzymes using the following reaction; 8µl of plasmid DNA, 2µl of NEBuffer™ 3.1, 1µl of restriction enzyme, 9µl sterilised milliQ water. Reactions were incubated at 37°C for 1 hour. 4µl of digested plasmid was added to 1µl of loading dye. The DNA gel was run at 75volts for 50 minuets. The DNA gel was visualised using the UV transilluminator and imaged using a GelDoc imaging system.

Clones that were perceived as successful were sequenced using Sanger Sequencing (Eurofins Genomics, Germany. 5µl of extracted plasmid DNA was combined with the forward primer which had been diluted in MilliQ water 1:30 from its original concentration of 100 uM. Clones that were identified as successful by sequencing analysis were once more transformed into *E. coli* in order to obtain higher concentrations of plasmid DNA. The Qiagen HiSpeed plasmid maxi-prep kit was used in order to ensure that the higher DNA concentration required for *D. discoideum* transformation was achieved (Paschke *et al.*, 2018).

2.4.5 Analysis of tandem repeats in PETase generated plasmids

Full sequences of each vector plasmid containing the PETase gene was generated using the homology cloning feature on Serial Cloner 2-6-1. The sequence was uploaded to Benson, (1999) Tandem Repeat Finder to analyse the number of tandem repeats and the location within the plasmid of each repeat. This was completed for each vector plasmid and annotations were added to each plasmid sequence on Serial Cloner 2-6-1.

2.4.6 Analysis of G+C richness PETase generated plasmids

The G+C richness of each vector plasmid containing the PETase gene was analysed using the Enmemo online G+C content calculator (<http://www.endmemo.com/bio/gc.php>).

CHAPTER 3: RESULTS

3.1 DIFFERENCES IN GROWTH BETWEEN *Dictyostelium discoideum* STRAINS

3.1.1 Influence of axenic media on cell health

The strain Ax-2 was grown in three different axenic media; HL5, FM, SIH. After 4 days of growth images were taken (*Figure 13*). From these images is clear to see that growth was significantly higher in the SIH media (*Figure 13c*), however, more contamination was present when compared to FM, which presented moderate growth (*Figure 13b*). Significant contamination and reduced cell growth were observed in the HL5 medium (*Figure 13a*).

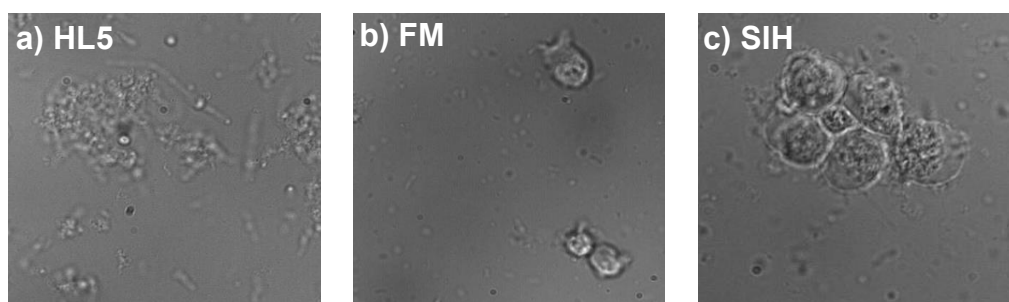


Figure 12: Axenic Dictyostelium discoideum, the Ax-2 strain, grown in different axenic media; a) HL5 b) FM c) SIH

3.1.2 Differences in growth between wild-type strains

Differences in growth rate between *D. discoideum* strains; DdB and NC4 were measured. All strains were grown using wild-type protocols outlined in *Section 2.3.1*. The diameter of the cleared zones was measured over a 4-day period. Stable growth was presented for the first 2 days with the DdB strain presenting faster initial clearing (Table 13). On day 2 DdB showed a confluent plate with no distinct clearing zone being measured, whereas NC4 presented one further extension of clearing in the same area (Figure

Table 13: Wild-type *Dictyostelium discoideum* growth over a period of 4 days. Cells that are shaded represent insufficient measurement of growth. All measurements presented in cm.

Strain	Day 1	Day 2	Day 3	Day 4
NC4	0.2	0.6		
DdB	0.3			

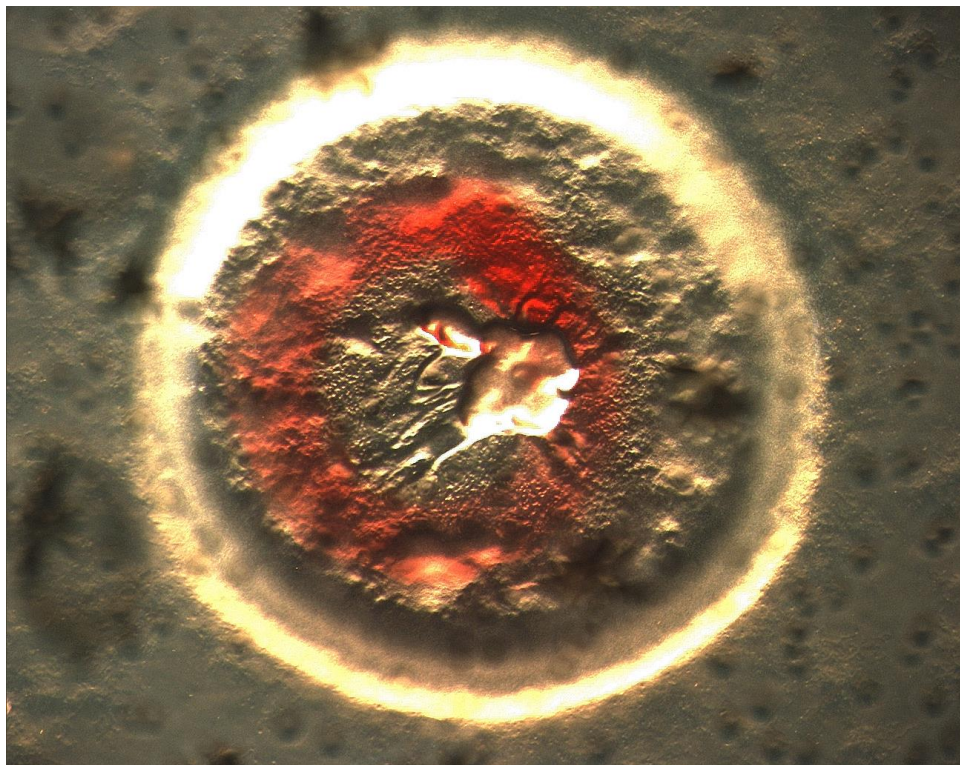


Figure 13: Presentation of clearing zone in NC4 with the red circle being the Day 1 measurement of growth. The 'halo' being the newly cleared zone of growth.

3.2 PLASMID GENERATION

3.2.1 Codon optimisation of the PETase gene

The PETase gene was codon optimised for insertion into *D. discoideum* expression vector plasmids. The optimisation protocol is outlined in Section 2.4.2. The protein sequences for the *D. discoideum* optimised gene is presented in Table 14. The sequence alignment between the *D. discoideum* optimised PETase and the original nucleotide sequence for the PETase gene from *I. sakaiensis* can be observed in Figure 14.

Table 14: Protein sequence of the codon optimised PETase gene for *Dictyostelium discoideum*

	Protein Sequence
<i>D. discoideum</i>	MKFQHTFIALLSLLTYANANFPRASRLMQAAVLGGLMAV SAAATAQTNPYARGPNPTAASLEASAGPFTVRSFTVSRP SGYGAGTVYYPTNAGGTVGAIIVPGYTARQSSIKWWG PRLASHGFVVITIDNSTLDQPSSRSSQQMAALRQVASL NGTSSSPIYGKVDTARMGVMGWSMGGGSLISAANNP SLKAAAPQAPWDSSTNFSSVTVPTLIFACENDSIAPVNS SALPIYDSMSRNAKQFLEINGGSHSCANSNGNSNQALIGK KGVAVWMKRFMDNDTRYSTFACENPNSTRVSDFRTANC SYPYDVPDYA

Score	Expect	Identities	Gaps	Strand
141 bits(155)	5e-37	515/804(64%)	2/804(0%)	Plus/Plus
Query 82	ATGCAAGCAGCAGTTTTAGGTGGTTTAAATGGCAGTTTCAGCAGCAGCAACAGCACAAACA	141		
Sbjct 28G..C..C...C..C..C..GC.G.....C..G..G..C..C..C..C..C..G..C	87		
Query 142	AATCCATATGCAAGAGGTCCAAATCCAACAGCAGCATCATTAGAAGCATCAGCAGGTCCA	201		
Sbjct 88	..C..C..C..CC.C..C..G..C..G.....C..C...C.C.....CAGC..C..C..G	147		
Query 202	TTTACAGTTAGATCATTACAGTTTCAAGACCATCAGGTTATGGTGCAGGTACAGTTTAT	261		
Sbjct 148	..C..C..GC.C..G..C..C..GAGCC.C..GAGC..C..C..C..C..C..C..G..C	207		
Query 262	TATCCAACAAATGCAGGTGGTACAGTTGGTGCAAATTGCAATTGTTCCAGGTATACAGCA	321		
Sbjct 208	..C..C..C..C..C..C..C..C..G..C..C..C..C..C..G..G..C..C..C..G	267		
Query 322	AGACAATCATCAATTAATGGTGGGGTCCAAGATTAGCATCACATGGTTTTGTTGTTATT	381		
Sbjct 268	C..C..G..GAGC..C.....C..GC.CC.G..C..G..C..C..C..G..C..C	327		
Query 382	ACAATTGATACAAATTC AACATTAGATCAACCATCATCAAGATCATCACAACAAATGGCA	441		
Sbjct 328	..C..C..C..C..C..C..GC.C..C..G..G..CAGCC.C..G..G..G..G.....C	387		
Query 442	GCATTAAGACAAGTTGCATCATTAAATGGTA-CATCATCATCACCAATTTATGGTAAAGT	500		
Sbjct 388	..GC.GC.C..G..G..C..GC.C..C..C.C..G..G..G..-..G..C..C..C..G..	446		
Query 501	TGATACAGCAAGAATGGGTGTTATGGGTTGGTCAATGGGTGGTGGTTCATTAAATTC	560		
Sbjct 447	C..C..C..CC.C.....C..G.....C.....G.....C.....C.....C..GC.G..C..	506		
Query 561	AGCAGCAATAATCCATCATTAAAAGCAGCAGCACCACAAGCACCATGGGATTCATCAAC	620		
Sbjct 507	G..G..C..C..C..G..GC.G.....C..G..G..G..G..C..G.....CAGC..G..	566		
Query 621	AAATTTTTCATCAGTTACAGTTCCAACATTAATTTTGCATGTGAAAATGATTC AATTGC	680		
Sbjct 567	C..C..C..G..G..C..C..C..GC.G..C..C..C..C..C..G..C..CAGC..C..	626		
Query 681	ACCAGTTAATTCATCAGCATTACCAATTTATGATTCAATGTCAAGAAATGCAAAACAATT	740		
Sbjct 627	C..G..C..C..G..C..CC.G..G..C..C..CAGC.....GC.C.....G..G..G..	686		
Query 741	TTTAGAAATTAATGGTGGTTCACATTCATGTGCAAATTCAGGTAATTC AATCAAGCATT	800		
Sbjct 687	CC.C..G..C..C.....C..G..C..C..C..C..CAGC..C..CAGC..C..G..GC.	746		
Query 801	AATTGGTAAAAAGGTGTTGCATGGATGAAAAGATTTATGGATAATGATAC AAGATATTC	860		
Sbjct 747	G..C..C..G..G..C..G..C.....GC.C..C.....C..C..C..GC.C..C..	806		
Query 861	AACATTTGCATGTGAAAATCCAAA	884		
Sbjct 807	C..C..C..C..C..G..C..G..	830		

Figure 14: Sequence alignment using the NCBI BLASTn platform (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The query sequence being the PETase Codon optimised gene for *Dictyostelium discoideum* and the subject sequence being the reference sequence from *Ideonella sakaiensis*. The "." represents aligned identities.

3.2.2 Cloning of pDM1203 with the PETase gene

In order to recombine the PETase gene with the *Dictyostelium* expression vector pDM1203, we designed a Gibson-assembly based strategy which relied on linearization of the vector using a unique restriction enzyme site, and on the introduction of homologous ends into the PETase gene using PCR.

The PETase gene was inserted into pDM1203 by amplifying the gene using pDM1203_sec_f and pDM1203_cyt_f primers. The pDM1203_sec primer amplifies both the signal sequence and the PETase core sequence of the synthetic construct, thereby allowing for secretion of the expressed protein, whilst the pDM1203_cyt primer amplifies only the core sequence therefore leading to cytoplasmic expression. The PCR product generated (*Figure 15*), using the previously stated primers, was inserted into the BglIII-digested pDM1203 plasmid using a Gibson assembly reaction.

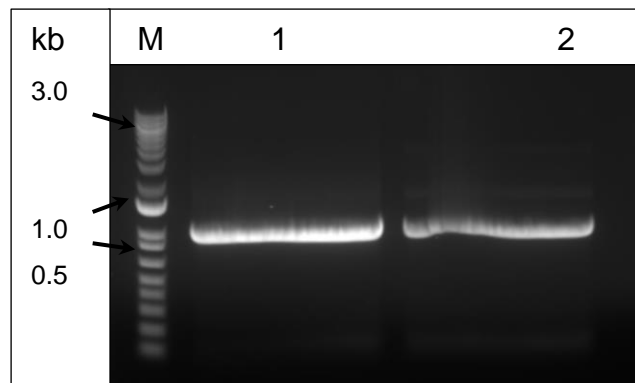
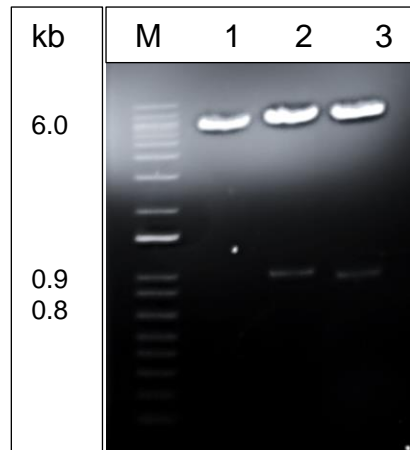


Figure 15: DNA agarose gel showing the PCR reaction for the pDM1203 plasmid

The product from the Gibson assembly reaction was transformed into Top10 competent cells. After growth was present 4 colonies per LB(Amp) plate were selected and 3ml of LB(Amp) was inoculated with each colony and grown for 12 hours at 37°C, shaking at 180rpm. DNA was extracted using the Qiagen HiSpeed filter Mini-prep kit. Success of the cloning was assessed using a DNA agarose gel after the plasmid was digested with BglIII, which is expected to cut on either side of the PETase gene. The linearized vector is clearly seen at 6.0kb (*Figure 16*). The PETase gene with the intracellular expression is present at 0.9kb and the PETase gene with cytoplasmic expression is shown at 0.8kb (*Figure 16*). *Figure 16* show that the observed results matched the predicted banding pattern suggesting that generation of pDM1203 with the PETase gene was successful. This was further confirmed by sequencing analysis.



*Figure 16: A DNA agarose gel presenting the successful cloning of the pDM1203 with the PETase gene. The 1% agarose gel was run with the NEB 1kb plus DNA ladder (**M**). **Lane 1** shows the empty BglI-digested pDM1203 plasmid; **Lane 2** shows the BglI-digested pDM1203 plasmid containing the secretory tagged PETase gene and **Lane 3** shows the BglI-digested pDM1203 plasmid containing the PETase gene containing no secretory tag.*

Plasmid maps presenting the location of the PETase gene in pDM1203, were generated using the recombinant cloning function on Serial Cloner 2-6-1, for secretory and cytoplasmic expression (Figure 17).

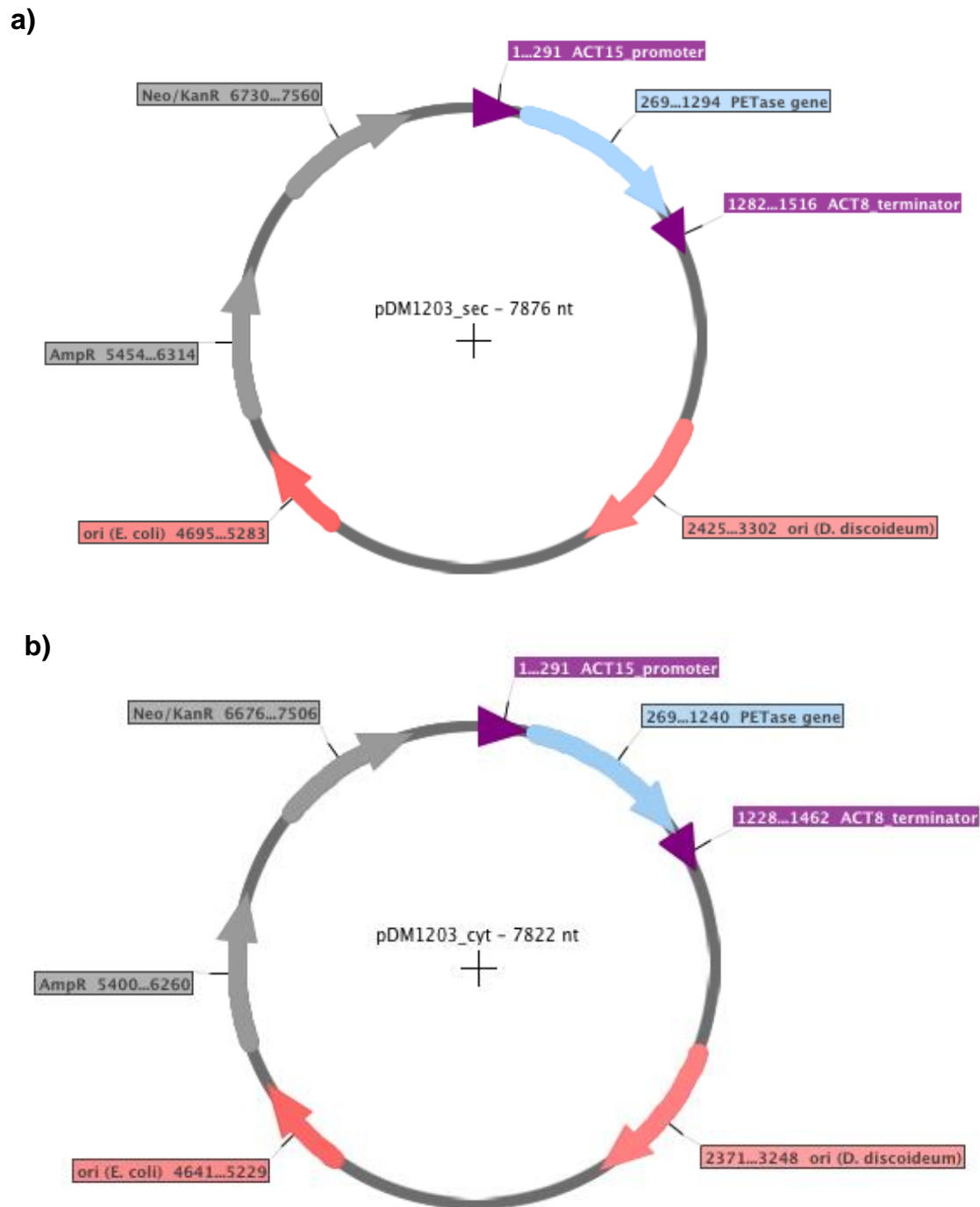


Figure 17: Plasmid maps generated by Serial Cloner 2-6-1 a) the location of the PETase gene within the vector plasmid pDM1203 with the secretory gene present b) The location of the PETase gene within the vector plasmid pDM1203 without the secretory tag, to act as a negative control.

3.2.3 Obtaining higher concentrations of pDM1203

Successful clones of pDM1203_sec and pDM1203_cyt were re-transformed into Top10 competent cells and a Qiagen Hi-speed Maxi-prep kit was used to obtain higher concentrations of DNA. The higher concentration DNA was further concentrated by DNA precipitation. The high concentration DNA was BglIII-digested and checked on a 1% agarose gel. All agarose gels upon the re-transformation of the confirmed pDM1203 clones into Top10 competent cells presented unexpected multiple banding patterns (Figure 18). This differed from the banding pattern observed during the clone analyses (Figure 16).

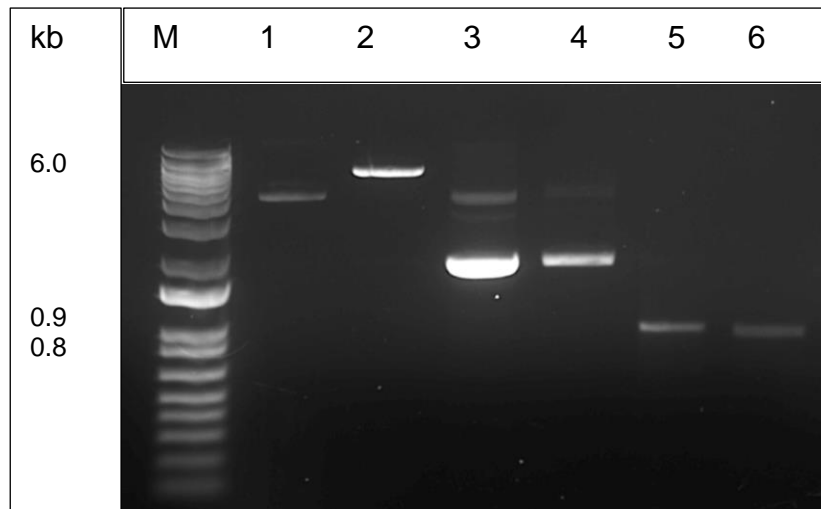


Figure 18: A DNA agarose gel used to assess the stability of the clones after a higher yield of DNA was extracted and precipitated. The agarose gel was run with the NEB 1kb plus DNA ladder (M); Lanes 1-3: pDM1203_sec; Lanes 4-6: pDM1203_cyt

3.2.4 Influence of competent cell strain on multiple banding patterns expressed in pDM1203 clones

We hypothesized that the multiple banding pattern following amplification of the plasmid could be explained if the plasmid we generated was prone to recombination when expressed in *E. coli*. This could be due to the fact that genes codon optimized for *D. discoideum* are very AT-rich, whereas native *E. coli* genes are usually more GC-rich. The strain of competent cell used when generating higher DNA concentrations of

pDM1203, containing the PETase gene, was investigated. Plasmid DNA which was sequenced confirmed was transformed into the new competent cell strain, NEB stable. Colonies were re-grown and DNA was once again extracted using the Qiagen Hispeed Miniprep kit and BglIII-digested. Success of cloning and the transformation was assessed using a 1% agrose gel. Multiple banding patterns were observed once more (*Figure 19*).

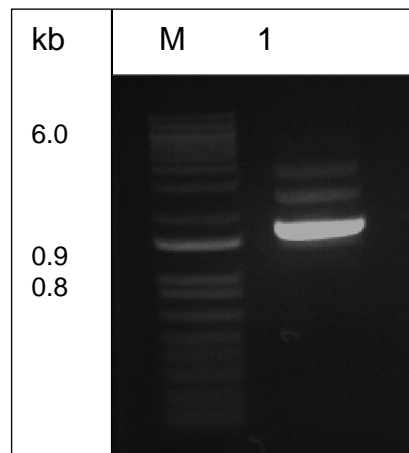


Figure 19: A DNA agarose gel used to assess the success of the NEB stable cells in the E.coli transformation with the pDM1203_sec plasmid. The gel was run with the NEB 1kb plus DNA ladder (M). Lane 1: BglIII-digested pDM1203_sec.

3.2.5 G+C richness within the PETase gene

Multiple banding patterns were consistently expressed throughout changes of competent cell strain. This suggests that presentation of multiple banding could be attributed to the codon optimised PETase gene. The G+C richness of the PETase gene was analysed. Differences in G+C richness of the codon optimised *D. discoideum* gene was assessed using the G+C content calculator platform on Enmemo (see Section 2.4.3). This was compared with three other model organisms expressing PETase, two of which were codon optimised and the third expressing the naturally evolved sequence for PETase found in *I. sakaiensis* (Figure 20). The novel species *I. sakaiensis* has a G+C richness of 50.97%, whereas *E. coli* has the highest G+C richness with 57.75%. Comparatively, the *D. discoideum* optimised gene has the lowest G+C richness with 34.17%.

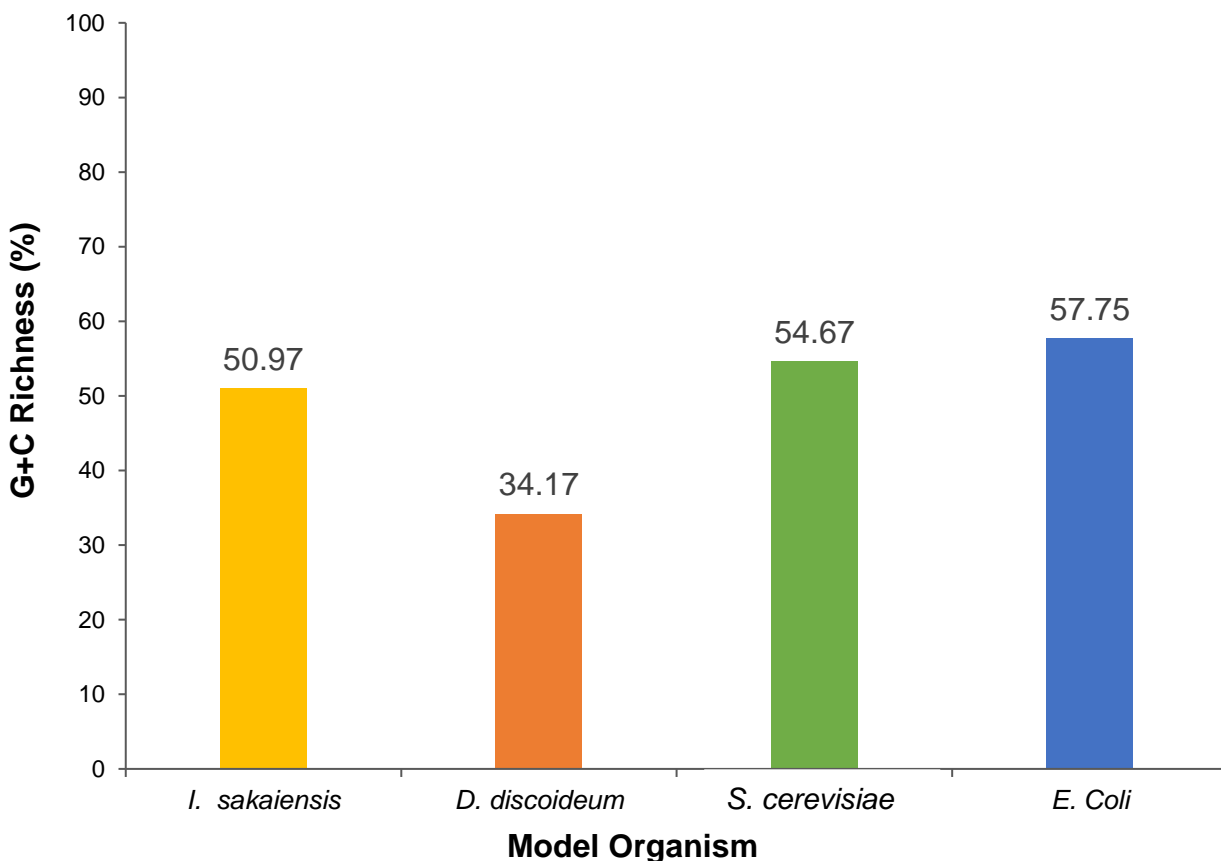


Figure 20: G+C richness (%) of the codon optimised PETase genes for *I. sakaiensis*, *D. discoideum*, *S. cerevisiae* and *E. coli*.

3.2.5 Cloning of pDT29 with the PETase gene

Due to multiple banding patterns being presented numerous times when cloning pDM1203 we decided to re-clone the PETase sequence into a more commonly used expression vector, pDT29. Once again to generate pDT29 with the PETase construct integrated, a Gibson assembly approach was undertaken. To linearize pDT29 in preparation for the insertion of the PETase gene using the Gibson assembly approach relied on the selection of two suitable restriction sites, XbaI and BamHI. Homologous ends, to facilitate Gibson assembly, were introduced to the *D. discoideum* optimised PETase gene by PCR.

The PETase gene was once again inserted into the pDT29 by amplifying the gene using pDT29_sec_f and pDT29_cyt_f, with a respective pDT29 reverse primer (see Section 2.4.4). Once more, the pDT29_sec primer allows for the intracellular expression of the PETase enzyme whilst plasmids using the pDT29_cyt primer will only present cytoplasmic expression, once again acting as the negative control. The PCR product generated, using the stated primers was inserted into the digested plasmid by a Gibson assembly.

On the completion of the Gibson Assembly, the reaction was transformed into the NEB Stable strain of *E. coli*. After incubation 4 colonies were selected and 3ml of LB(Amp) was inoculated and grown for 12 hours at 37°C, shaking at 180rpm. The DNA was extracted initially using the Qiagen Hispeed filter Mini-prep kit. Successful cloning was analysed using a DNA agarose gel, the XbaI and BamHI digest pDT29 was ran on the gel for 45 mins at 75v. The successful generation of pDT29 with the PETase construct is observed in *Figure 22*. The linearized vector plasmid, pDT29, is expressed at 6.0kb, with the PETase gene with intracellular expression at 0.9kb and the PETase gene with cytoplasmic expression at 0.8kb (*Figure 21*). The banding pattern was predicted previous to generation, suggesting that generation of pDT29 was successful. Plasmid maps presenting the location of the PETase gene in pDT29, were generated using the recombinant cloning function on Serial Cloner 2-6-1, for secretory and cytoplasmic expression (*Figure 22*).

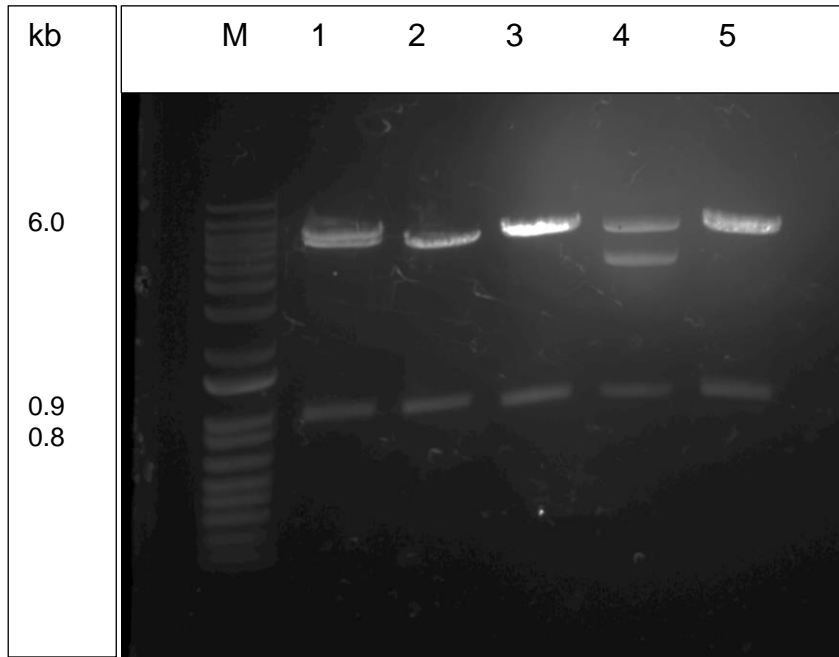


Figure 21: A DNA agarose gel used to assess successful generation of pDT29 with the PETase gene. The gel was run with the NEB 1kb plus DNA ladder (M). Lane 1-2: XbaI and BamHI digested pDT29_sec. Lane 3-5: XbaI and BamHI digested pDT29_cyt.

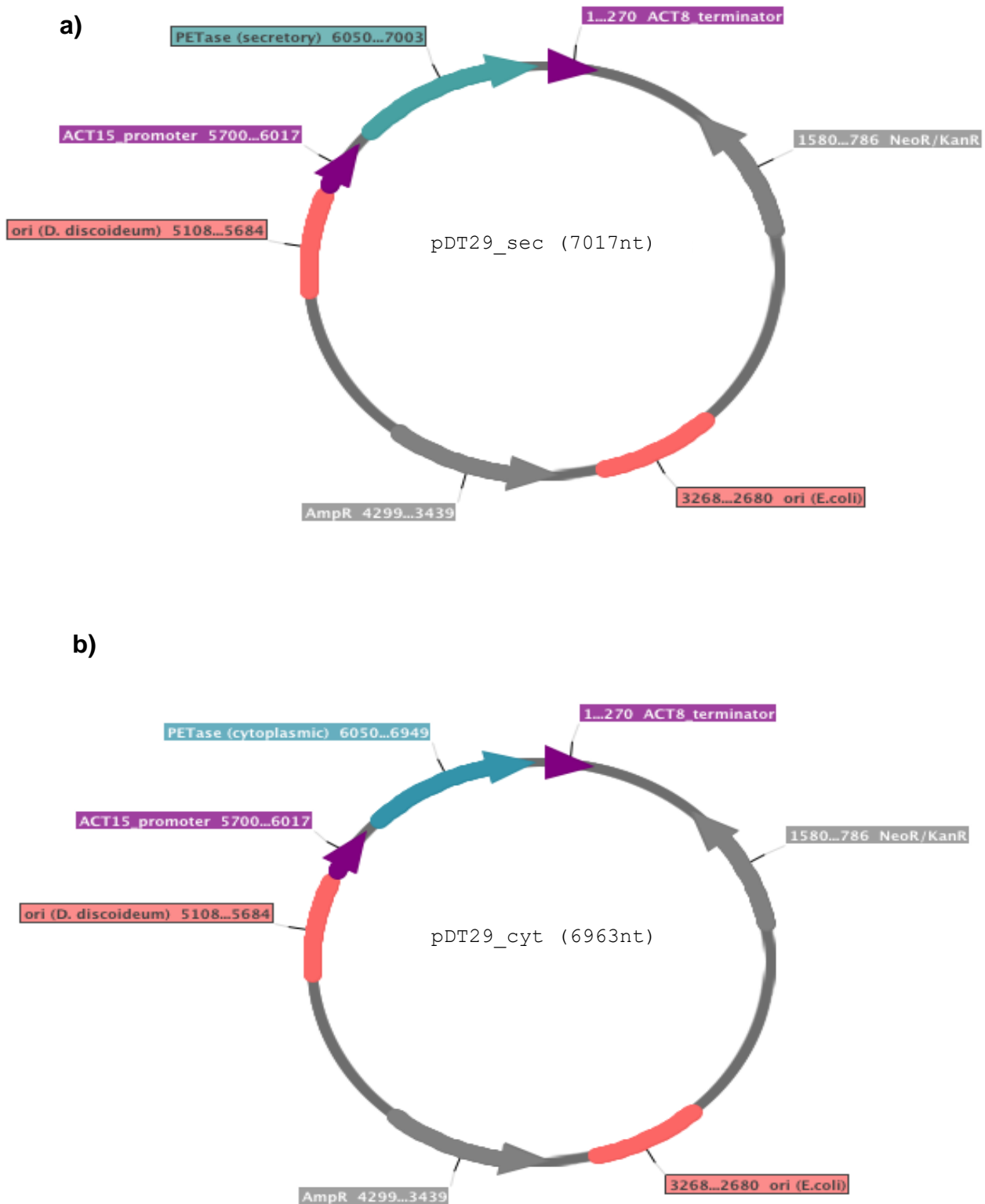


Figure 22: Plasmid maps generated by Serial Cloner 2-6-1. a) the location of the PETase gene within the vector plasmid pDT29 with the secretory tag; b) the location of the PETase gene within the vector plasmid pDM1203 without a secretory tag present, therefore acting as negative control.

3.2.6 Analysis of multiple banding in secondary *E.coli* transformation

We re-transformed the plasmid as described in *Section 2.4.4* and re-isolated the DNA. The DNA agrose gel did not exhibit a multiple banding pattern on any of the subsequent transformations. This suggested that the multiple banding pattern present in pDM1203 can be attributed to the plasmid itself. Further analysis was undertaken (*Section 3.2.8*).

3.2.7 Obtaining increased concentrations of pDT29

After confirmation of the stability of the pDT29 plasmid containing the PETase gene, the plasmid was once again transformed into *E. coli*, the NEB strain. A Qiagen Hi-speed Maxi-prep kit was used to obtain higher concentrations of DNA. The concentration of DNA was analysed using a microplate reader (*Table 15*).

Table 15: The DNA concentration of the pDT29 plasmid containing the PETase gene after the use of the Qiagen Hispeed Maxi-prep kit.

Plasmid	DNA Concentration ($\mu\text{g/ml}$)
pDT29_sec	135
pDT29_cyt	610

The high concentration DNA was XbaI and BamHI digested and checked on a DNA agrose gel. The agrose gel further presented successful generation of pDT29 with the PETase gene, with no unexpected banding patterns (*Figure 23*). Once more, the linearized pDT29 is observed at 6.0kb, with the PETase gene with intracellular expression at 0.9kb and the PETase gene with cytoplasmic expression at 0.8kb (*Figure 23*).

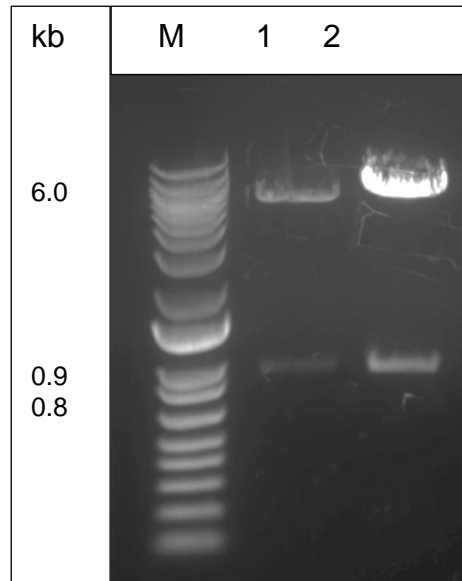


Figure 23: A DNA agarose gel used to assess the success of yielding higher concentrations of pDT29 with the PETase gene. The gel was run with the NEB 1kb plus DNA ladder (**M**). **Lane 1**: XbaI-BamHI-digested pDT29_sec; **Lane 2**: XbaI-BamHI-digested pDT29_cyt.

3.2.8 Analysis of tandem repeats in *Dictyostelium discoideum* vector plasmids

In order to investigate the occurrence of suspected recombination events in the pDM1203 plasmid, a repeat analysis was undertaken. This was done to identify the differences in the quantity and location of tandem repeat regions in all *D. discoideum* vector plasmids containing the PETase gene used in this study. The online repeat finder platform developed Benson (1999) at the University of Boston was used to identify the location of the repeat regions in each plasmid. No repeat sequences were identified in the PETase gene, as expected, so each of the same plasmid contained the same number of repeating regions. Meaning, the pDT29_sec and pDT29_cyt both contained 7 repeat regions identified in the same locations (Figure 24).

The pDM1203 plasmid, both pDM1203_sec and pDM1203_cyt, contained 21 repeat regions (Figure 25). Evidently, this is much higher than the pDT29 plasmid, thus a key

difference in the two vector plasmids and a suggestion of the causation of recombination occurring in the pDM1203 plasmid. To further understand the differences in these repeating regions the Benson (1999) platform provides further information about each repeat region. Once more, the results are identical for pDT29_sec and pDT29_cyt and are presented in *Table 16*. This also applies to pDM1203_sec and pDM1203_cyt which are presented in *Table 17*.

In pDM1203 plasmid, it was identified that 5 repeat regions had a consensus sequence with a period size of 1 (*Table 17*). Whereas, in the pDT29 plasmid 2 repeat regions contained a period size of 1 (*Table 16*). A further significant difference between the two plasmids was the size of repeat sequences. In the pDM1203 plasmid the largest repeat sequences was 121 nucleotides long Found in repeat region 8 (*Table 17*). In the pDT29 plasmid, the longest period size for a repeat was only 22 nucleotides long (*Table 16*).

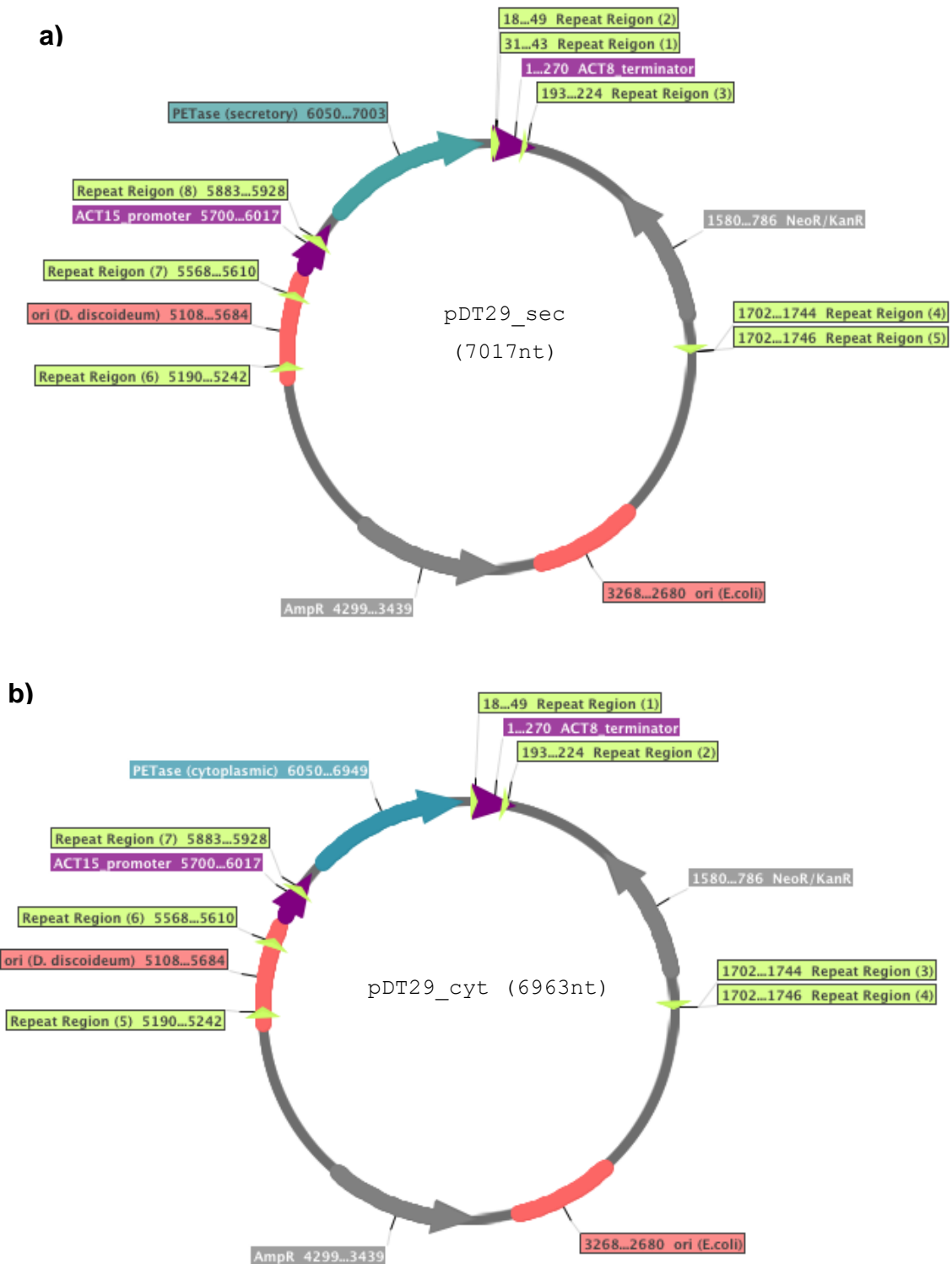


Figure 24: Plasmid map generated by Serial Cloner2-6-1. *The repeat regions identified using the Benson (1999) tandem repeat finder online platform, were added to the plasmid map. a) pDT29_sec b) pDT29_cyt.*

Table 16: pDT29 tandem repeat analysis for both pDT29_sec and pDT29_cyt. Results taken from the tandem repeat analysis (Benson, 1999). The indices refer directly to sequence used in the associated plasmid map (Figure 24).

Indices	Repeat Region	Consensus Sequence	Consensus Size	Period Size	Copy Number	Match Percentage (%)	Indel Percentage (%)	Alignment Score	Entropy (0-2)
18-49	1	AAAATAAAA AAAA	13	13	2.4	94	5	55	0.64
193-224	2	T	1	1	32.0	100	0	64	0.00
1702-1744	3	AAAAATAAA AAAAATAA	17	17	2.6	89	7	63	0.52
1702-1746	4	AAAAAATA AAAAATAT	16	16	2.9	81	18	60	0.57
5190-5242	5	AAAAAAAAA GTGTCATGA CAAA	22	22	2.4	100	0	106	1.35
5568-5610	6	TGTCATGAC AAAAAAAAA AAAG	22	22	2.0	100	0	86	1.48
5883-5928	7	T	1	1	46.0	100	0	92	0.00

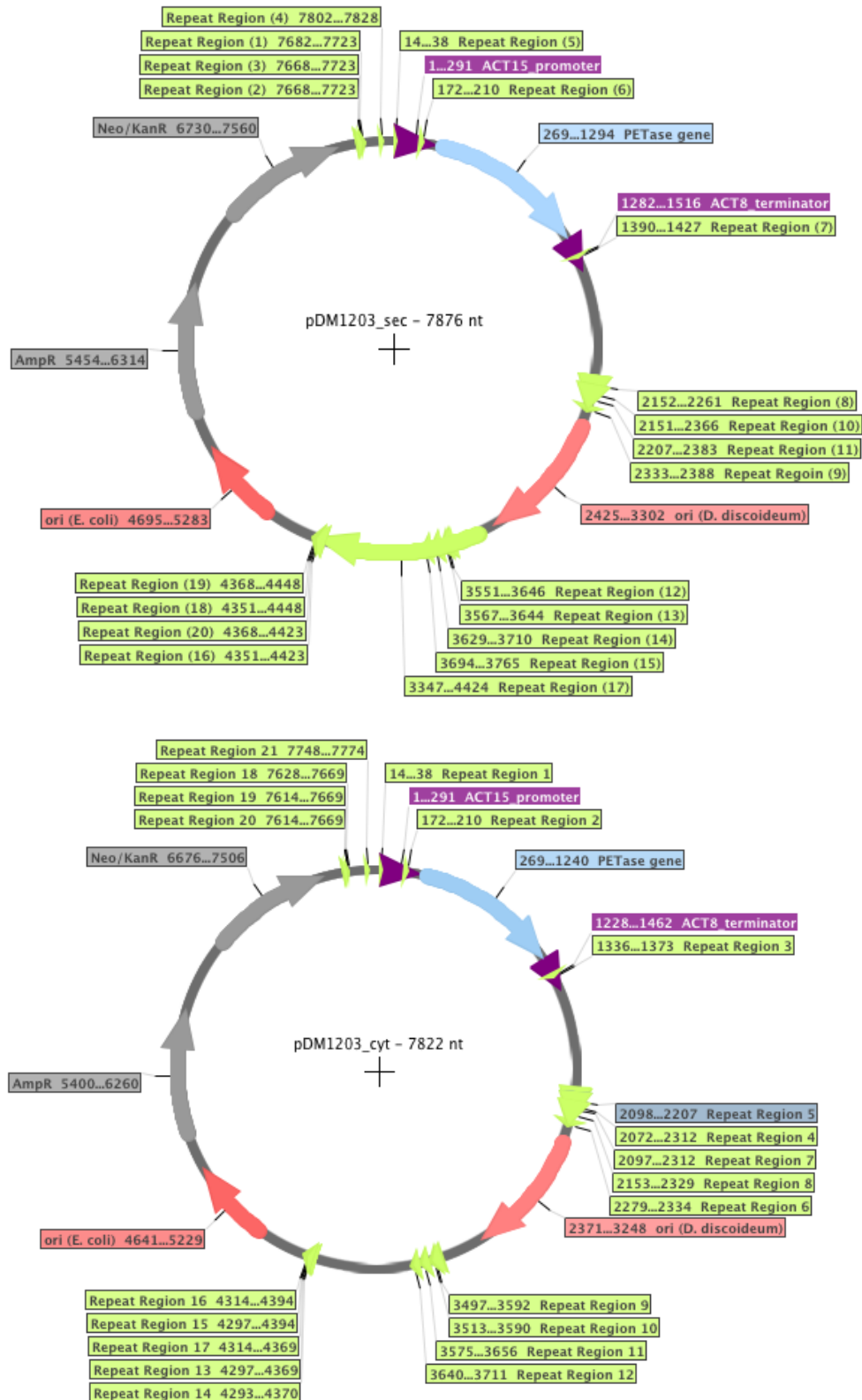


Figure 25: Plasmid map generated by Serial Cloner 2-6-1 for pDM1203. The repeat regions identified using the Benson (1999) tandem repeat finder online platform, were added to the plasmid map. **a)** pDM1203_sec **b)** pDM1203_cyt

Table 17: pD1203 tandem repeat analysis for both pD1203_sec and pD1203_cyt. Results taken from the tandem repeat analysis (Benson, 1999). The indices refer directly to sequence

Indices	Repeat Region	Consensus Sequence	Consensus Size	Period Size	Copy Number	Match Percentage (%)	Indel Percentage (%)	Alignment Score	Entropy (0-2)
953-994	1	A	1	1	42.0	95	0	66	0.28
939-994	2	AAAAAAAAAATTA AAA	17	17	3.4	87	7	71	0.62
939-994	3	AAAAAAAAAATTA AAAAA	19	19	3.1	87	5	71	0.62
1073-1099	4	A	1	1	27.0	100	0	54	0.00
1161-1185	5	ATTTTTATTT	10	10	2.5	100	0	50	0.72
1319-1357	6	T	1	1	39.0	100	0	78	0.00
2537-2574	7	T	1	1	38.0	100	0	76	0.00
3273-3513	8	AAAAATTATTA TTATTATGTAATA AAGCTATTAATA ATTATAAAAAA ATAACAAA ATTTAAAAATATA TTTGTAAGAAAGTA TTAAAAATATTATG AAAAAAAAATTA	119	121	2.0	80	12	269	1.31
3299-3408	9	AAAAATTATTA	12	12	9.1	71	16	68	1.29
3480-3535	10	AAAAAGTATCTAA AAAATTA	24	26	2.3	81	12	60	1.14
3298-3513	11	TAAAAAGCTATTA AAATAATATGAAA AAAAAATACAAAA ATTTAAAAATATT TTTTG	61	60	3.6	78	13	216	1.32
3354-3530	12	TTTGTAAGAAAGCT ATTAAATAATTAT GAAAAAAATTTT TAAAAATTA TTGTT	61	60	2.9	82	7	220	1.31
4698-4793	13	TCTTTT	6	6	15.5	71	14	52	1.18
4714-4791	14	TTCTTTATCTTCT TCTT	18	18	4.3	75	14	63	1.19
4776-4857	15	TCTCTTCTTCTTC TTCCGATACGTGCG GTGTT	33	33	2.5	86	4	103	1.58
4841-4912	16	TTC	3	3	24.0	94	0	126	1.10
5498-5570	17	AAAAAAAAAAAT TTAAAAA	21	21	3.6	89	7	107	0.56
5494-5571	18	TTTTAAAAA AAAAAAAAA AAAAAAAAA	38	38	2.0	90	2	120	0.72
5498-5595	19	AAAAAAAAA ATAAAA	20	21	4.6	80	14	90	0.73
5515-5595	20	AAAAAT	7	6	11.7	80	16	57	0.65
5515-5570	21	A	1	1	5.6	96	0	76	0.37

3.2.10 G+C content differences between pDM1203 and pDT29

The G+C richness (%) of each *D. discoideum* vector plasmid generated with the PETase sequence was calculated using the Enmemo platform (see Section 2.4.3). Both pDM1203 plasmid's have a lower G+C richness when compared to the pDT29 plasmid's (Figure 26). In both pDM1203 and pDT29 plasmids those generated with the PETase gene containing the secretory tag had marginally reduced G+C richness (Figure 26). The higher prevalence of repeats, and the even lower GC content, may explain why the PETase gene was more prone to recombination in pDM1203 than pDT29.

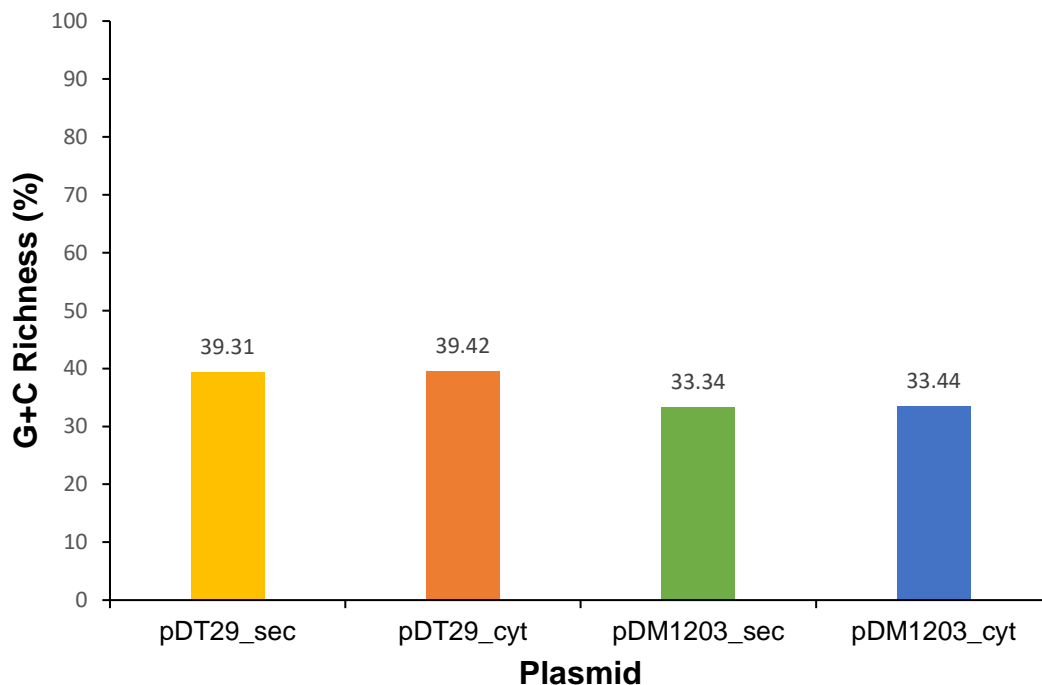


Figure 26: G+C richness (%) of each *D. discoideum* vector plasmids.

3.3 TRANSFORMATION OF *DICTYOSTELIUM DISCOIDEUM*

3.3.1 Wild-type transformation of *Dictyostelium discoideum*

Multiple attempts were made at transforming wild-type *D. discoideum* using the protocol outlined in Section 2.3.5. Cell growth was only observed in a small proportion of transformed cells. In the final transformation procedure cell density was measured

after 4 days of growth. 2 out of 8 cultures contained viable cells after 4 days of growth, when the presence of the antibiotic is expected to have killed all non-resistant cells. Their cell density was measured. One petri dish containing transformed NC4 cells generated with the pDT29_sec plasmid had a density of 1.6×10^6 . The second petri dish containing transformed NC4 cells generated with pDT29_cyt had a density of 0.7×10^6 . Images of the *D. discoideum* cells were taken, the presentation of viable cells indicates that the transformations were successful (Figure 27). A western blot to confirm success was unable to be performed due to reduced cell densities and time constraints.

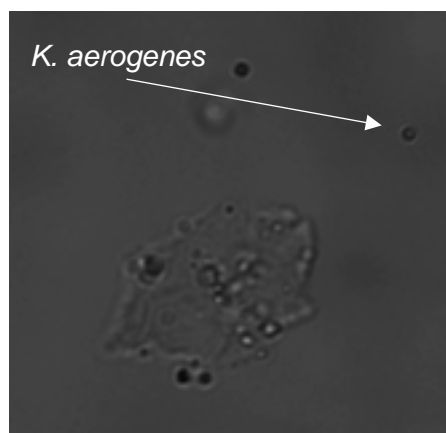


Figure 27: Image of transformed *D. discoideum* cell. *K. aerogenes* has been labelled for clarity. Image taken using Olympus IX81 inverted microscope.

3.3.2 Axenic transformation of *Dictyostelium discoideum*

Axenic transformations were attempted at Greenwich University as a part of collaborative work with Dr Billy Ferra and Dr Elinor Thompson. All axenic transformations were attempted using the pDM1203 and pDM1208 plasmid. It was reported that cell growth was observed early on, however after 48 hours no successful transformants were present.

CHAPTER 4: DISCUSSION

This study explored the use of novel microbial hosts as delivery chassis for enzymes in environmental bioremediation applications. It has also brought to light the difficulties associated with introducing model organisms to a research group. Interestingly, issues associated to the generation of a suitable secretor, ready for the implementation of bioremediation practices, are widely known and studied. The genome composition of A/T and G/C nucleotides has significant influence on cloning the PETase gene into *D. discoideum* vector plasmids. Finally, the location and presence of repetitive sequences differs between plasmids utilised within this study, suggesting the plasmid used influences the consistent multiple banding patterns observed when working with the pDM1203 plasmid.

4.1 SUCCESS IN *DICTYOSTELIUM DISCOIDEUM* TRANSFORMATION INFLUENCED BY VECTOR PLASMID AND STRAIN

4.1.1 Transformation efficiency is linked to transformation approach

Though the transformation of *D. discoideum* was not fully confirmed by a western blot, cell growth was observed in the presence of a selective antibiotic (*Figure 27*). This provides preliminary evidence that the pDT29-based plasmid could be successfully transformed into *D. discoideum* cells. A newly developed transformation protocol for non-axenic cells growing on bacterial food, which has not yet been widely used was employed in this study (Paschke *et al.*, 2018). Other approaches using axenic strains of *D. discoideum* are more readily employed. However, approaches undertaken with the non-axenic strain, NC4, were selected due to better growth of the NC4 strain (See *Section 4.3.1*). As well as the reduced complexity associated with the transformation procedure and application value when working with wild-type cells.

After 48 hours of incubation at 22°C growth was seen in *D. discoideum* transformed with both the PETase containing pDT29 plasmid in the presence or absence of a signal sequence. Whereas no growth was exhibited in cells transformed with the pDT29

plasmid containing the GFP fluorescent tag instead of the PETase gene. This could be linked to the lower DNA concentration of the pDT29 plasmid in the earlier preparation stages. Background fluorescence was exhibited in the bacterial food source, *K. aerogenes* making analysis and identification of cells successfully transformed difficult. Despite success of transformation being identified visually, western blot analysis was not possible due to the number of cells grown and time constraints. However, with further work and transformation protocols being optimised it is now known that transforming *D. discoideum* with such plasmids is possible.

4.1.2 Wider success in axenic *Dictyostelium discoideum* transformation

Despite the fact that in this study success of transformation using the axenic strain Ax2 was unsuccessful, other research studies have presented high levels of success with the strain and transformation procedure developed. Since the development of the Ax2 strain in 1970 (Watts and Ashworth, 1970). It has been a favoured strain due to its growth without bacterium and ease of maintenance. Further to this with the Ax2 strain there is more control of when *D. discoideum* enters specific lifecycles, when compared to other wild-type strains such as NC4 (Watts and Ashworth, 1970).). Therefore, studies targeting specific points of the *D. discoideum* lifecycle will use axenic strains preferentially.

In *Sections 1.2.5 and 1.2.6* it was described how *D. discoideum* has been employed as an expression host. It is fitting to look at further ways this organism has been employed successfully. As mentioned in *Section 4.1.2* both *D. discoideum* and the unicellular protozoan *P. falciparum* share genetic similarity, with their rich A/T content throughout their genome and homologous genes. Thus, *D. discoideum* proves an important expression system for the secretion of proteins associated with *P. falciparum* and has been employed to do so various times (Fasel *et al.*, 1992; Naudé *et al.*, 2005). In this case, *D. discoideum* employed as an expression host has proved pivotal in leading research into the development of malaria vaccinations and furthering our understanding of the effect of Chloroquine resistance associated with the PfCRT transporter. This unique transporter is found within the parasitic protozoan, *P.*

falciparum as well as *D. discoideum*. This further supports the implementation of *D. discoideum* as an expression host.

In recent years, the use of axenic strains of *D. discoideum* has become more common than the use of wild-type strains. It has been mentioned that *D. discoideum* is often employed to further our understanding of pathogenesis. In a study undertaken in 2000, *D. discoideum* cells were used to study the intracellular pathogenesis of the *Legionella* bacterium, which can result in severe Pneumonia in humans. The study justifies their use of *D. discoideum* and the Ax2 by comparing *D. discoideum* with other amoeboid species (Hägele *et al.*, 2000). It was shown that infection rate of *D. discoideum* cells with *Legionella* species was increased compared to the other species of amoeba, *A. castellanii*. The ease of genetic manipulation associated with the Ax2 strain and the availability of cellular markers and cell signalling knowledge facilitated the implantation of the species. Furthermore, it was understood that only amoeboid single cells were susceptible to infection by *Legionella* (Hägele *et al.*, 2000). This therefore links back to the benefits of using Ax2 when needing to work with the species at specific point in their life-cycle due to an element of control.

D. discoideum has been used to study autophagy, cell communication and aggregation for years (Cosson and Soldati, 2008; Li and Purugganan, 2011). However, its homologous proteins associated with the human genome has recently led to the species being utilised as a recombinant protein expression system. A recent example of such is the employment of *D. discoideum* as a model organism to study the secretion of peroxidase A, to further our understanding of its antimicrobial influences in mammalian immune responses (Nicolussi *et al.*, 2018). This study showed that secretion of the enzyme varied throughout the *D. discoideum* lifecycle and presented that peroxidase A in *D. discoideum* influences bacterial contamination of fruiting bodies, it also allowed for a further understanding of the influences of post-translation modification, where the architecture of the active site of peroxidase A in *D. discoideum* was significantly altered (Nicolussi *et al.*, 2018).

4.2 *DICTYOSTELIUM DISCOIDEUM* AS AN EXPRESSION SYSTEM FOR THE SECRETION OF RECOMBINANT PROTEINS

4.2.1. Importance of *Dictyostelium discoideum* strain

D. discoideum can be grown in two different ways, in a nutrient broth (axenically) or on the surface of nutrient agar plates with a bacterial food source provided. Those grown on agar-bacterial lawns have much faster generation time of 3 hours, when compared to those grown in liquid media which have a generation time of 9 hours (Muñoz-Braceras, Mesquita and Escalante, 2013). All strains of *D. discoideum* are capable of growth with an external food source however, not all strains are able to survive without. Only axenic strains are capable of survival without an external food source.

Overtime, *D. discoideum* has been used as an expression host to produce recombinant proteins for various areas of research. *D. discoideum* has been used numerous times to expression proteins important to furthering the production of therapeutics (Arya, Bhattacharya and Saini, 2008). Protein production for therapeutics is often undertaken in *D. discoideum* due to post-translational modification being a necessity for the activity of the therapeutic (Rai and Padh, 2001). Further to this the Ax2 strain is often used preferentially, due to the removal of bacteria as a food sources, which would abide regulations set within the pharmaceutical industry (Lu *et al.*, 2004). *D. discoideum* has been used as a eukaryotic expression system for studies investigating vaccination development associated with the Rotavirus (Williams, Emslie and Slade, 1995), the expression of human antithrombin III (Glöckner *et al.*, 2001) and and the production of gonadotropins human choriogonadotropin to understand human endocrine function (Linskens *et al.*, 1999). All the previously stated studies worked with the transformation of axenic strains with human proteins.

D. discoideum is a desirable host for the expression of heterologous proteins due to its ease of growth and similarities of plasmids found in other eukaryotic nuclei. Furthermore, most studies applying *D. discoideum* as their host expression system tend to do so because of the package of complex features the species houses. For

example, chemotaxis and glycosylation both of which are important functions throughout mammalian cell lines (Lu *et al.*, 2004). The broad scope in which the Ax2 strain shows that the implantation of such strain to studying the expression of homologous proteins is important. This study we have undertaken could further benefit with such the Ax2 strain being used.

We undertook analysis specifically looking at difference in growth between strains in order to identify the most suitable strain for this study. Literature largely focuses on the use of axenic strains when undertaking genetic modification (see *Section 4.2.2*), however with the equipment and application of the genetically modified *D. discoideum* wild-type strains were selected.

From performing a growth analysis where clearing zone diameter was measured, the NC4 strain showed the most applicable growth. This strain presented distinct and predictable clearing zones when diameter was taken over the first two days. The predictability of the growth can be advantageous when applying the species as a bioremediation system. However, the growth assay was unable to be completed as by day 3 growth was less predictable for both strains (*Table 13*). However, growth was observed to be significantly faster in the NC4 strain. Often faster growth is correlated with good cell health. Furthermore, faster growth in other model organisms also directly correlates with good recombinant protein production.

4.2.2 The pitfalls associated with Dictyostelium discoideum as an expression host

For the most part, *D. discoideum* is an example of a robust expression system. It has been mentioned numerous times the ways in which the species has been employed to investigate differing areas of scientific interest. Numerous studies have employed the species to act as a recombinant protein expression host, however this where the protein was extracted and not often employed in the sense we intended with this study. There are various aspects of *D. discoideum* that reduce its desirability as a model organism this is linked to their low cell density maximum, $1-2 \times 10^7 \text{ ml}^{-1}$, especially

when grown axenically (Chen *et al.*, 2007). Furthermore, when grown axenically, growth is relatively slow. Both attributes link to relatively low yields in protein production, thus reducing the utilisation of the species.

4.2.3 *Dictyostelium discoideum* as a terrestrial bioremediation system

A bioremediation system is a biological system that is put in place to reduce and resolve environmental contaminants, often by degradation. Genetically modified organisms (GMOs) are often involved in bioremediation processes where microbes found within an environment are no longer effective. In *Section 1.1.4* we discuss natural plastic biodegrading microbes that are already residing within in the terrestrial ecosystem. However, it has been established that these naturally occurring microbes are not efficient or effective enough to combat the large scale of plastic being released into soil ecosystems.

Bioremediation of plastic within the environment or within a landfill site have been approached numerous times (Sattlewal *et al.*, 2008; Caruso, 2015; Yang *et al.*, 2018). Some studies will identify native microbial communities found within plastic contaminated soils and from there genetically modifying compatible microbes so little risk is associated with application (Sattlewal *et al.*, 2008). I have termed this approach a bottom-up strategy, where bioremediation is attempted by looking at the microbial consortium in situ and applying genetic techniques to increase efficiency.

In this study we implement a top-down strategy, where based upon literature, we selected our model organism to apply as a bioremediation system. We decided to approach a potential bioremediation system for microplastic contaminated soils with the model organism *D. discoideum*. This organism was mainly chosen based upon its ecology, being found natively within in soil, and their differing life phases.

4.2.4 Restrictions of GMO associated work

When working towards creating an efficient bioremediation system it is important to identify the restrictions associated with the creation of such. In this study, the bioremediation system would be comprised of genetically modified *D. discoideum* to secrete the PETase protein in order to degrade and reduce plastic present in the soil ecosystem.

When releasing a GMO, the environment in which it intends to be released in should be understood. The natural environment is assembled of complex interacting processes home to various communities of differing organisms. Various considerations must be made before the release of a GMO into an environment to insure there no unanticipated repercussions. The idea of genetically modified organisms being implemented into an environment was first introduced in the 1980's, even then the act of doing so seemed far-fetched (OLSON REFERENCE). Since then genetically modified organisms (GMOs) have been employed in various settings, from medical to agricultural. In more recent times GMOs have been used as novel bioremediation systems.

Despite our hypothesised approach of bioremediation being informed and an applicable system, considerations must be undertaken before their release into the environment is even possible. Numerous controls have been put in place for the release of a GMO into the environment for research purposes, these restrictions are defined by various authorities across the UK. In England, the authority responsible for the release of any GMO linked to the environment is the Department for Environment, Food and Rural Affairs (Defra).

Initially, a GMO will undergo numerous contained and controlled tests outside of the environment before release is even considered (Prakash *et al.*, 2011). Those involved in the decision and testing of a GMO range from geneticists to ecologists, and though within different countries risk assessments vary, a common framework of assessment can be broadly observed (Hill, 2005). Frameworks for risk assessment may differ internationally, but the steps associated broadly remains the same (*Figure 28*).



Figure 28: Common risk assessment criteria for the release of GMOs into an environment.

The steps outlined in *Figure 28* are all undertaken before release of a GMO is possible, which also requires further permission from governments. This presents a key issue in the development of a GMO that has a direct environmental application. This process, start to finish, can take years for all the correct approvals to be gained. Therefore, despite promising results presented in the study, especially with success in the transformation of *D. discoideum* with the PETase containing plasmid, application of a such bioremediation system is in the distant future.

4.3 THE OCCURANCE OF RANDOM RECOMBINATION IS LINKED TO SELECTION OF VECTOR PLASMID

4.3.1 A/T richness as a cause of random recombination

D. discoideum possess 6 gene dense chromosomes, which encodes approximately 13.600 proteins (Bakthavatsalam and Gomer, 2010). The genome of *D. discoideum* is fully sequenced, however, this was not achieved with ease. The high A/T content, contributing to 87% of the genome, and the presence of repeats made bacterial clones incredibly unstable when taking a Bacterial Artificial Chromosome sequencing approach (Loomis, 2006). The *D. discoideum* genome hosts multiple long-stretches of poly(A/T) sequences, these sequences produce issues when attempting PCR and cloning large inserts.

The high percentage of A/T nucleotides also has significant influence on codon usage. We codon optimised the PETase gene to ensure that the gene could achieve efficient levels of secretion when transformed into *D. discoideum* (Section 2.4.2). When comparing the A/T content of the newly codon optimised PETase gene to the novel PETase gene from *I. sakaiensis* and codon optimised genes for *E. coli* and *S. cerevisiae* (Figure 20). It is evident that the G+C richness for *D. discoideum* is much lower than the three other species analysed. *D. discoideum* possesses a 34.17% G+C content, compared to 50.97% in *I. sakaiensis*, 54.67% in *S. cerevisiae* and 57.75% in *E. coli*. This remains consistent with the high A/T requirements of *D. discoideum*.

D. discoideum promoter, terminator and intron sequences can harbour over an 85% A/T content (Eichinger *et al.*, 2005). This has been shown to result in deleterious deletion events occurring in *E. coli* when attempting plasmid-based cloning (Mukai, Ichiraku and Horikawa, 2016). When using circular plasmids with a high A/T content in *E. coli* instability is presented. This is attributed to issues with secondary structure formation and interference within the transcription and replication systems of the *E. coli* (Mukai, Ichiraku and Horikawa, 2016). Furthermore, it has been shown that A/T rich regions may mimic plasmid replication sites within *E. coli* and cause instability

(Godiska *et al.*, 2009). Nonspecific regions that have a high A/T content in *E. coli* are important for replication, additional A/T may influence such negatively. Thus, it can be concluded that a significant parameter contributing to the instability of *D. discoideum* plasmids when transformed into *E. coli* is the rich A/T content which leads to frequent recombination events.

4.3.2 Overcoming issues associated with high A/T content

The high A/T content of the *D. discoideum* genome has presented significant issues in numerous studies. Regions of DNA that exceed an A/T content of 75% present significant difficulties when cloning *D. discoideum* plasmids in to *E. coli* (Mukai, Ichiraku and Horikawa, 2016). Studies have acknowledged such hurdles and have developed different approaches to overcome issues associated working with high A/T content plasmid DNA.

A study which addresses issues associated with the high A/T content in *Plasmodium falciparum* presents differing approaches to overcome such barrier faced (Pan *et al.*, 1999). The *msh-1* gene, yielding a 74% A/T content presented low stability when cloning in *E. coli*. It was identified that the rich A/T content prevented stable cloning and resulted in alteration to the function of the *msh-1* gene (Pan *et al.*, 1999). Issues were resolved by altering the A/T content of the gene, by back-translating the amino acid sequence and optimising the DNA with human codon usage. This reduced the A/T content to 55%.

Furthermore, the gene itself was modified at specific points that may present issues during expression or cloning (Pan *et al.*, 1999). In this present study the PETase gene A/T was analysed. In *Figure 20* it is clearly seen that the A/T content of the *D. discoideum* optimised gene was significantly lower than the other identified optimised genes. However, when analysing the repeat content present throughout the plasmids used in this study, a significant cause of instability, no repeats were identified. However, altering the gene sequence accordingly may increase overall stability.

Other issues which could have been addressed to facilitate success the cloning of the PETase into pDM1203 are linked to technicalities. When undertaking the PCR steps, associated to the Gibson Assembly (*Figure 11*), the temperature should be monitored and optimised carefully. When working with high A/T content DNA PCR temperatures should not exceed 60°C. This linked to A/T rich sequences being amplified less efficiently at higher temperatures (Pan *et al.*, 1999). Extraction of DNA from agarose gels after completion of PCR must be done with care restricting which light is transmitted, for example avoiding usage of UV light, as well as not using chemical elution kits. This is due to chemical approaches can lead to higher chances of A/T rich fragments being denatured (Převorovský and Půta, 2003). Such mediation was undertaken in this study and are not guaranteed to resolve the issues presented but may increase the efficiency of cloning.

4.3.3 Influence of repeats on random recombination occurrence in pDM1203

The analysis undertaken in *Section 3.2.8* primarily identified tandem repeats within each vector plasmid. However, interpreted correctly the results can provide additional information on the occurrence of interspersed repeats. The data provided by the tandem repeat analysis provides each sequence and the point of which it occurs. When looking at this data in further detail a reference sequences is provided and the variations of this sequences in tandem. Identity of the sequences to the reference sequence are not entirely 100%, therefore interspersed repeats are able to be identified. For example, in both plasmids' poly(A/T) sequences were identified at different positions within the plasmid. When looking at these sequences in further detail they are not entirely poly(A/T) and occur throughout the plasmid. Therefore, they're not exclusively a tandem repeating sequence.

As mentioned in both plasmids' poly(A/T) stretches are identified. It is known that an increased A/T richness can result in long stretches of homopolymer runs, therefore influencing the number of repeating sequences identified. From further analysis most repeating sequences identified have an extremely high A/T content. In pDM1203 out of the 21 repeats 5 are either (poly)T or (poly)A. With stretches of single nucleotides having a copy number of 42 (*Table 17*). Whereas, in pDT29 out of the 7 repeats

identified only 2 are homopolymer sequences. Despite the differences observed in the count of tandem repeats similarities can be observed between the two plasmids, pDT29 and pDM1203. All plasmids formed contain repeating sequences in the *Actin 8 (Act8)* and in the *Actin 15 (Act15)* genes. These promoter and terminator sequences contain high A/T contents (Hori and Firtel, 1994).

Repetitive poly(T) stretches are important in promoter and terminator regions. For example, it has been shown that when a poly(T) stretch is deleted from the promoter region the expression level of the inserted gene is decreased in *D. discoideum* (Hori and Firtel, 1994). Furthermore, the influence of such high poly(T) sections have previously been observed to increase recombination and rearrangement once transformed into *E. coli* (Hori and Firtel, 1994). Therefore, further supporting the instability of the pDM1203 plasmid in *E. coli*.

Overcoming such issues present at *E. coli* transformation stage linked to repeat content is complex, many studies suggest on employing either new vector plasmids or using a more stable strain of *E. coli*. Both alternative approaches were undertaken with evident success in the latter. pDM1203 is a relatively novel plasmid only used in few studies (Paschke *et al.*, 2018). The comparative increased stability found in the pDT29 plasmid is reflected in its wide usage and recommendation by our collaborators at the University of Greenwich (Nuttall, Hettema and Watts, 2012).). It is evident that pDT29 is a better suited vector plasmid for this study due to its continued stability and all instability observed in the pDM1203 plasmid may be linked directly to the different quantity of repeating sequences identified.

4.3.4 Strain of competent cell has no influence on plasmid stability

One step taken to reduce issues presented in this study linked to plasmid stability was to change the strain of *E. coli* used. Recombinase-deficient *E. coli* strains were developed specifically to increase stability in plasmids containing high A/T contents and large quantities of repetitive sequences (Mukai, Ichiraku and Horikawa, 2016). In this study, we switched from the commonly used competent cell strain Top10 to the

NEB stable strain. However, when re-transforming cells into the new strain of *E. coli* no change was seen, and once more multiple banding patterns were exhibited (Figure 21). It should be noted that colonies were present in all transformation procedures, the employment of the new strain was to combat issues presented at the final stages when the plasmid DNA was analysed on an agrose gel to check for successful cloning.

4.4 FURTHER WORK

This study addresses key aspects in the development of a novel bioremediation system applicable to microplastic contaminated soils. However, with the time constraints associated with a MSc-R projected and the additional disruption caused by COVID-19, various areas were not investigated.

There are numerous steps needed to be taken to ensure the further success of this project. Initially, identifying the interactions of the expression species, *D. discoideum*, with PET is critical. Assays involving the species ability to grow on or even near plastic would be a important step to understand the ways in which the system could applied. Further to this, working with soil samples taken from the environment in which *D. discoideum* is found and identifying whether there are high levels of plastic material would be important.

It is also important to mention the development of sound methods of quantifying microplastics within soils is required. Various assays have been developed but no standardized approach has yet been presented. Further to this quantifying and analysing the effect of the by-products made by the PETase enzyme, ethylene glycol and terephthalic acid, on soil microbiomes and the wider ecosystem is extremely important and will allow for steps to be taken for this bioremediation system to be implemented.

Further successful transformations, confirmed by western blots analysis, will allow for secretion of the PETase protein to be optimised. In the regards of quantity of secreted protein and efficiency of degradation. Alongside this, understating the change in secretion of the PETase protein throughout its differing life-stages is imperative to its

application. Once the interaction of *D. discoideum* and PET is understood the degradation rate can be identified. Further work undertaken by leading researchers in the PETase field can be applied to the gene used in this study increase degradation efficiency. All of which will work towards *D. discoideum* being a suitable bioremediation system.

4.5 SUMMARY

This study has presented important steps towards to the development of a novel bioremediation system for microplastic contaminated soils. Initially, when working with a new model organism, difficulties are expected to be encountered. This study presented multiple hurdles linked directly to *D. discoideum* being used as the expression system. The theory of the application of *D. discoideum* to act as a bioremediation system is well backed with literature. The difficulties presented within this study are linked to complexity associated with the high A/T and repeat content of the *D. discoideum* genome. Which was analysed and compared to other model organisms, from this analysis it was evident that the differences expressed in the PETase gene in *D. discoideum* could have significant influence on the suspected recombination events occurring throughout our analysis. Moreover, when comparing the different plasmids used within this study, pDM1203 and pDT29, the repeat content has definitive influence on the instability presented when working with the model organism and transforming *D. discoideum* optimised plasmid DNA into *E. coli*.

Despite the hurdles presented, we were able to show competence when working with *D. discoideum* and success when transforming the cells with the pDT29 plasmid containing the PETase gene. This presents an exciting revenue for further research into applying *D. discoideum* back into their native environment to resolve the unprecedented effect of plastic pollution. Since the discovery of the PETase enzyme in 2016, many studies have approached the application of the novel enzyme from industrial approaches (Chen *et al.*, 2020) to the marine ecosystem (Moog *et al.*, 2019). However, as of yet, no system has been developed targeting soil ecosystems, where we believe *D. discoideum*, due to its ecology and expansive molecular tool kit is extremely fitting. We hope that future research on this topic will allow the soil dwelling

amoeba to contribute to the resolution and remediation of the ever-growing plastic pollution.

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