Assessment Of Plastic Degrading Abilities Of Autotransporter Domains Containing Esterase On Synthetic Polyesters

Master Project in Biotechnology

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Popular science summary

A new kind of plastic degrading enzyme can assist plastic waste management.

Scientists are trying to improve current methods of plastic recycling. Now a new kind of enzyme might contribute to the transition to greener recycling methods.

A new candidate for the club of plastic degrading enzymes has emerged. It is a group of enzymes called “autotransporter domain containing esterase” that has been drawing attention. The group of enzymes are believed to have the abilities to break bonds in plastic molecules and degrade the molecules into smaller fragments that can be reused to produce new plastic products. If the enzymes have a high degradation rate they might be of good use in plastic waste management and simplify the transition to a more eco-friendly recycling.

Plastic pollution has become a global threat with plastics in every part of the planet, including the Mariana Trench (Gibbens, 2022). And every minute, enough plastic enters the oceans to fill up two garbage trucks (OCEANA, n.d.). This is a threat to our whole ecosystem and animals take a lot of the damage, for example sea turtles are mistaking plastic bags in the ocean for jellyfish and 22% of sea turtles that ingest plastic bags die (WWF, 2019). Another problem with plastic pollution is that plastics degrade into microplastics and enter the food chain and accumulate at the top. Since humans are at the top of the food chain we eat the amount of plastic equivalent to the weight of a credit card each week (Cillizza, 2022). The effect of microplastic on human health has not yet been reported and it might be very bad news for all of us. These are all reasons to improve plastic recycling and one way can be the use of plastic degrading enzymes that require less chemicals and the recycling conditions are less inhospitable compared to conventional plastic recycling (www.nrel.gov, n.d.).

The research that has been made on the area has led to some contradictory results with the indication that the enzymes have potential in degrading plastics such as PET, that is used in beverage bottles. However, the ability to degrade plastic seems to be limited and that might lead to difficulties in drawing an apparent conclusions and more research is needed before these enzymes can be used in plastic waste recycling.

In summary, plastic pollution is a big problem that we need to solve. One solution, can be the use of plastic degrading enzymes that makes plastic recycling more eco-friendly. The enzyme group “autotransporter domain containing esterase” can be an interesting candidate for industrial use in plastic recycling as there are indications that it has plastic degrading abilities. Nonetheless, before these enzymes can be used industrially more apparent research is needed.

Abstract

In today's world, plastic pollution has become a widespread problem with plastics in oceans, rivers and forests. The use of plastic has increased rapidly since it first was developed and that is because of its advantageous abilities. This scientific report focuses on the evaluation of two enzymes known as "autotransporter domains containing esterase" for its ability to degrade a variety of plastics such as polyethylene terephthalate (PET). In this report the ability to degrade plastics with enzymes has been tested by introducing two different autotransporter domains containing esterase to different kinds of plastic and letting them react under different conditions to determine the plastic degrading abilities in the enzymes. The results indicated that both enzymes had an ability to degrade different plastics to some extent, but the extent of degradation varied depending on the experimental conditions. However, the results were not entirely conclusive as some of the results were contradictory. The biggest problem was replication of the results, this can be due to several issues such as no expression of enzyme, problem with the column and storage time of the enzyme. Despite the inconclusive results, the study provides valuable insights into the potential of this kind of enzyme for plastic degradation. It is suggested that further studies are needed to confirm the effectiveness of the enzymes. The study highlights the importance of continuing to explore biological solutions for plastic waste management and the challenges that come with developing new solutions in this field.

Preface

This report describes a master degree project conducted during the spring term of 2023 at the Division of Biotechnology at Lund University. The aim of the project was to study the plastic degrading capabilities of two autotransporter Domains Containing Esterase, with the goal of finding a suitable enzyme that can be used to degrade plastic at reasonable operation conditions in plastic recycling. The project was carried out under the supervision of the Division of Biotechnology's faculty members and especially Javier Linares-Pastén, who provided invaluable assistance and support throughout the research process. I hope that this report provides a useful contribution to the field of plastic waste management and inspires future research in this critical area.

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1. Introduction

Plastic pollution has become a major environmental concern around the world, with the widespread use of plastic in various industries. The amount of plastic waste generated has significantly increased in recent years and the durability of plastic makes it resistant to degradation and has led to its accumulation in the environment, where it can persist for hundreds of years. Plastic pollution poses a significant threat to wildlife, as it can cause entanglement, ingestion and other forms of harm that can result in death (Parker, 2019). Additionally, plastic pollution can also impact human health, as microplastics have been found in both food and water (Pironti et al., 2021). Given the significant negative impact of plastic pollution on both the environment and human health, there is a growing need for effective strategies to reduce plastic waste and improve plastic recycling.

Plastic degrading enzymes have recently been on the rise as a promising solution to the issue of plastic pollution. These enzymes are naturally occurring proteins that can break down various types of plastic, including polyethylene terephthalate (PET), one of the most commonly used plastics in the world (Olson-Sawyer and Madel, 2020). The discovery of plastic degrading enzymes offers a potential solution to the growing problem of plastic pollution, as it provides a way to break down plastic waste into smaller, more manageable components that can be recycled or safely disposed of (Kaushal, Khatri and Arya, 2021). Unlike traditional plastic degradation methods, which often involve high temperature or toxic chemicals, plastic degrading enzymes offer a more sustainable and environmentally friendly approach to plastic waste management (Dell, 2022). Research in this area is ongoing and scientists are working to better understand the mechanisms of plastic degradation and to develop more efficient and effective enzymes that can be used on an industrial scale (Kaushal, Khatri and Arya, 2021).

However, the efficiency of different enzymes in degrading plastics is variable and often dependent on specific conditions, including pH, temperature and substrate type (Sutipatanasomboon, 2021). Therefore, the aim of this study is to evaluate the plastic-degrading capabilities of two membrane bound enzymes under different conditions in order to optimize their efficiency. This will be accomplished through a multifaceted approach. Firstly, in silico assays will be performed by modeling and docking to identify the most effective enzymes for plastic degradation. Afterwards, the selected enzymes will be recombinantly expressed and purified to enable their use in the assays. The enzymes will then be used to catalyze the degradation of various types of plastics and the enzymatic activity will be determined by monitoring the rate of terephthalic acid (TPA) released from plastic degradation. Finally, the reactions will be evaluated to identify the optimal conditions for plastic degradation and then these conditions will be used in a microwave reactor to compare conventional heating to microwave heating, to determine if it is more beneficial for these types of reactions.

2. Aim

The aim of this master thesis is to analyze and evaluate the plastic degrading abilities of two different autotransporter domains containing esterase on various synthetic polyesters and to optimize the enzymatic reaction to enhance the breakdown of synthetic polyesters into more manageable compounds.

3. Scientific background

3.1 Plastics

Plastics are synthetic polymer materials that are composed of long chains of repeating molecular units, called monomers, known for their high strength and tolerability. The first plastic material was first invented in the early 20th century and was initially used for industrial and military applications. However, after the second world war the production of plastics increased dramatically and it became an important material in our everyday life due to its versatility and low cost (Ritchie and Roser, 2018). Plastics are used in a wide variety of products including packaging, building materials, medical devices, toys, electronics and textiles. The widespread use and disposal of plastic have led to environmental concerns, including pollution of oceans, landfills and wildlife. Plastics are not biodegradable, meaning they do not break down naturally in the environment. Instead the plastic waste accumulates in nature and can persist for hundreds of years, releasing harmful chemicals and microplastics into the ecosystem (Rodriguez, 2018).

Additionally, the production of plastic requires fossil fuels such as petroleum and natural gas, which are non-renewable resources. The production process also generates greenhouse gasses and other pollutants, contributing to climate change and air pollution (OECD, 2019). Efforts to address the environmental impacts of plastic have included the development of biodegradable and compostable plastics, recycling programs and regulations to reduce plastic waste. However, these efforts have faced challenges, including the high cost of biodegradable plastics and the limited infrastructure for recycling and waste management (Moshood et al., 2022). Overall, plastic is a versatile and valuable material that has transformed modern life. Yet, its widespread use and disposal have led to significant environmental concerns, highlighting the need for sustainable solutions to manage plastic waste and reduce its impact on the planet (Rodriguez, 2018).

3.1.1 Plastic recycling

Plastic recycling is the process of collecting plastic waste and transforming it, in a variety of ways, into new products and it reduces the amount of plastic waste that ends up in landfills, oceans and other natural environments and conserves natural resources by reducing the need for

production of new monomers (Hopewell, Dvorak and Kosior, 2009). There are several methods of plastic recycling, including mechanical recycling, chemical recycling and thermal recycling. Mechanical recycling is the most common form of plastic recycling and involves melting and remolding plastic waste into new products. Chemical recycling involves breaking down plastic waste into its chemical components and using them to create new plastic products (Plastics Europe, n.d.). Thermal recycling involves burning plastic waste to generate energy (Schwarz et al., 2021). Despite the benefits of plastic recycling, there are several challenges associated with the processes, such as sorting and high cost. One challenge is the lack of standardized recycling methods and infrastructure, which makes it difficult to recycle certain types of plastic. Another challenge is contamination, as even a small amount of non-recyclable material can ruin a batch of recycled plastic. Efforts are being made to improve plastic recycling through the development of new technologies and the promotion of sustainable practices. These include the use of biodegradable plastics, increased investment in recycling infrastructure and education campaigns to promote responsible plastic use and disposal (Hopewell, Dvorak and Kosior, 2009).

3.1.2 Enzymatic degradation of plastic

Enzymatic degradation of plastic refers to the process of breaking down plastic waste using enzymes, which are biological molecules that catalyze chemical reactions. This method of plastic degradation has gained interest as a potential solution to the problem of plastic waste, as it offers a more sustainable and environmentally friendly alternative to traditional methods, such as incineration, landfills and chemical recycling (Kaushal, Khatri and Arya, 2021). Enzymes can degrade plastic waste by breaking the chemical bonds that hold the polymer chains together, for example, the enzymatic reaction degrades the plastic molecule PET into TPA and ethylene glycol (EG), figure 1. The produced building blocks such as TPA and EG can then be reused to create new plastic products or be degraded into smaller compounds and be used in other areas. There are several types of enzymes that have been identified as potentially useful for plastic degradation, including lipases, esterases and proteases. These enzymes are capable of breaking down various types of polyesters, including PET and polyethylene (PE) (Temporiti et al., 2022). One of the challenges of enzymatic plastic degradation is the need to identify and optimize the specific enzymes and conditions required for each type of plastic. Enzymes may also require additional assistance, such as the use of surfactants or solvents, to effectively break down plastic waste (Kaushal, Khatri and Arya, 2021). Another factor in enzymatic degradation of plastics is the structures of the plastics, how crystalline or amorphous the plastic molecule is can have a substantial effect on how suitable the plastic is for enzymatic degradation. An amorphous molecule lacks a long-range order and makes the molecule more susceptible to enzymatic degradation. The disorder of the structure makes the molecule more accessible for degrading enzymes, it is also more accessible for moisture and oxygen which can increase the degradation further. Crystalline structures are in opposite to amorphous molecules, well structured which makes it harder to reach for both degrading units such as enzymes but also for moisture and oxygen leading to a low degradation rate (Pantani and Sorrentino, 2013).

Despite these challenges, there have been several promising developments in the field of enzymatic plastic degradation. For example, researchers have identified several enzymes that are effective at breaking down PET, which is commonly used in single-use plastic bottles (Zhu et al., 2022). In addition, some researchers are exploring the use of genetically modified enzymes to increase their effectiveness and specificity for plastic degradation (Bosch, 2022). Enzymatic plastic degradation offers a potential solution to the problem of plastic waste by providing a more sustainable and environmentally friendly alternative to traditional methods of disposal, such as incineration and storage at landfills. Further research and development are needed to optimize this approach and make it commercially viable for large-scale use (Kaushal, Khatri and Arya, 2021).

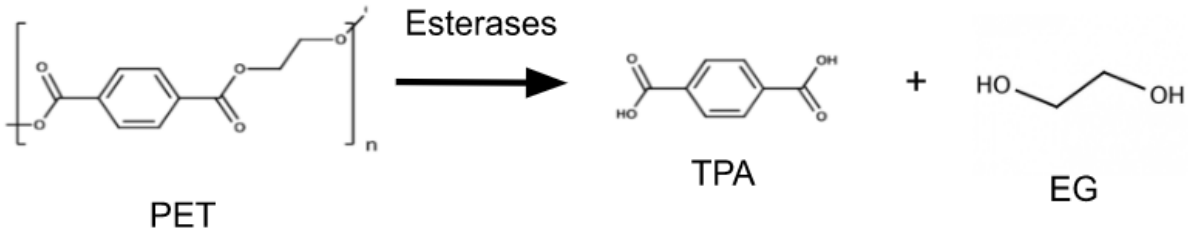

Figure 1. Enzymatic breakdown of a PET polymer into terephthalic acid (TPA) and ethylene glycol (EG).

3.1.3 Plastics used in this report

Akestra^{TM} 90 and Akestra^{TM} 110 , figure 2, are a new kind of plastic that can replace polystyrene with its high heat resistance. Akestra^{TM} is an amorphous molecule, with a high strength and other properties that makes it a good candidate for food packaging (Perstorp, n.d. A)(Perstorp, n.d. B). The numbers 90 and 110 presented in the names indicate a different composition of the molecules that causes different glass transition temperatures, which is when the amorphous regions of the molecules start to lose their rigidity and become more flexible (Anshuman Shrivastava, 2018, pp.1–16).

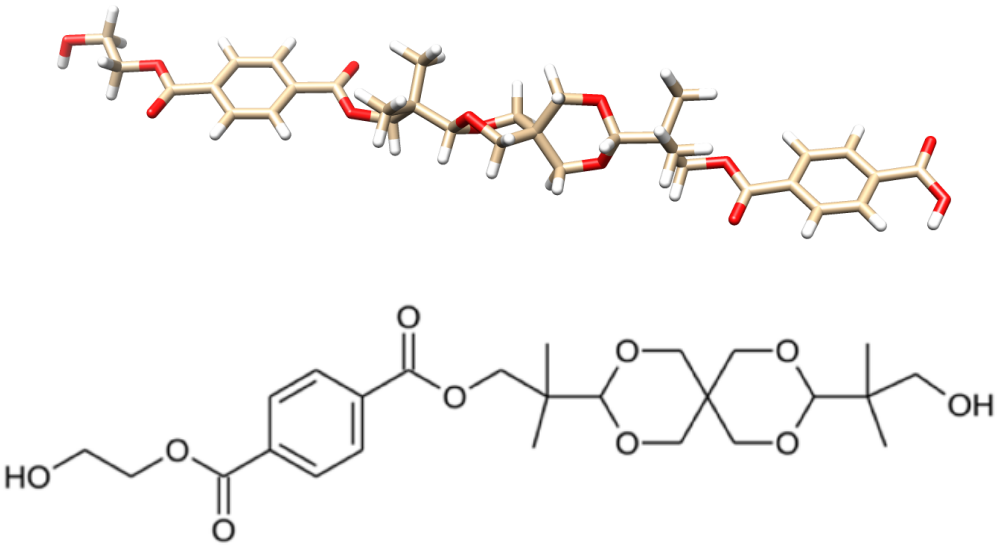

Figure 2. Computational 3D model of a fragment of an Akestra™ molecule built using Avogadro software and 2D structure of Akestra™.

Polyethylene terephthalate (PET) is a polyester composed of repeating units of ethylene glycol and terephthalic acid, with a semi-crystalline structure, figure 3. It is a widely used polymer in the production of beverage bottles, food packaging and other consumer goods. PET is characterized by its strength, lightweight and good barrier properties against moisture and oxygen (Guarino and Ambrosio, 2018, pp.37–69).

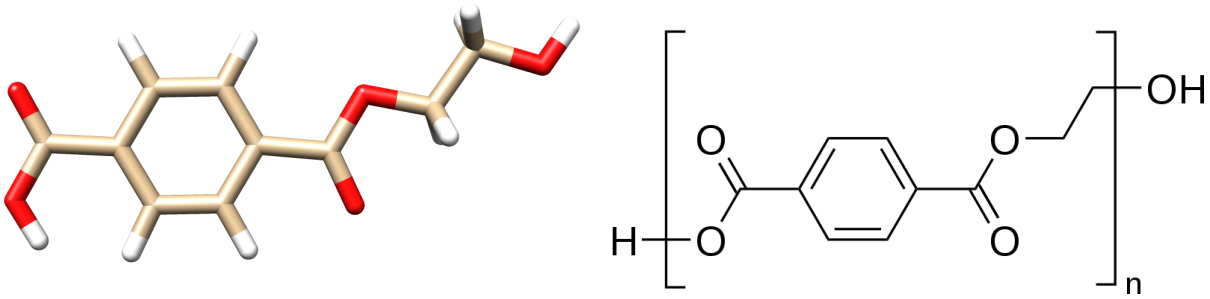

Figure 3. Computational 3D model of a fragment of a PET molecule built using Avogadro software and 2D structure of PET.

Polylactic acid (PLA) is a biodegradable and compostable thermoplastic derived from renewable resources such as corn starch or sugar cane. It is composed of repeating units of lactic acid and has a linear structure. PLA is used in various applications, including food packaging, disposable tableware and 3D printing filaments (Ranakoti et al., 2022).

Capa is a family of aliphatic polyester polymers that are biodegradable and compostable. Capa polymers are used in various applications, including food packaging, disposable cutlery and bags, as they are a sustainable alternative to traditional plastics (Ingevity, n.d.).

3.2 Enzymes

Enzymes are complex biomolecules that catalyze chemical reactions in living organisms. They are typically proteins with a unique three-dimensional structure that enables them to recognize and bind to specific substrates or reactant molecules and convert them into products. Enzymes play a crucial role in metabolic pathways, allowing living organisms to carry out the necessary chemical reactions for life. Enzymes work by lowering the activation energy required for a chemical reaction to occur. By doing so, they increase the rate of reaction and enable the reaction to take place at physiological temperatures and pressures, which would otherwise be too slow or energetically unfavorable. Enzymes are highly specific and typically catalyze only one type of reaction or a limited number of related reactions. Enzymes are essential for life and are involved in a wide range of biological processes including digestion, energy production, DNA replication and immune response. They are also widely used in various industrial applications such as food processing, pharmaceuticals and biofuels production. Enzymes can be produced through genetic engineering and modified to improve their performance or increase their stability under different conditions (Adlercreutz et al., 2021).

3.2.1 Plastic degrading enzymes

Plastic degrading enzymes are a group of enzymes that can break down and degrade plastic polymers. These enzymes are produced by microorganisms, such as bacteria and fungi, that are able to utilize plastic as a carbon source. The discovery of these enzymes has generated a lot of interest due to their potential use in waste management (Kaushal, Khatri and Arya, 2021). The most studied plastic degrading enzymes are the PETases which are able to break down PET plastic. PETases can break down PET into its constituent monomers, such as terephthalic acid and ethylene glycol, which can be recycled or degraded further by other microorganisms (Knott et al., 2020). Other plastic degrading enzymes include the cutinases which can degrade plastics made from natural polymers such as cellulose and chitin and the polyurethaneases, which can break down polyurethane plastics (Chen et al., 2013). These enzymes have been found in various environments such as soil, compost and marine sediments, suggesting that they play an important role in the natural cycling of organic materials. Research is currently ongoing to improve the efficiency and specificity of plastic degrading enzymes, as well as to identify new enzymes that can break down other types of plastic. One challenge is the low solubility and accessibility of plastics, which makes it difficult for enzymes to access the substrate. However, recent studies have shown that some enzymes can be engineered to increase their activity and stability under different conditions, such as high temperatures and alkaline pH, which could expand their potential applications in plastic waste management (Kaushal, Khatri and Arya, 2021).

3.2.2 Autotransporter domain containing esterase

Autotransporter domains containing esterases are a group of enzymes that belong to the esterase family and contain an autotransporter domain. The autotransporter domain is a type of protein

domain found in some bacterial proteins that enables the transport of the protein across the bacterial outer membrane (Cai et al., 2017). The esterase domain of these enzymes is responsible for their catalytic activity, which involves the hydrolysis of ester bonds (van den Berg, 2010). Autotransporter domains containing esterase are of interest due to their potential use in various industrial applications, such as in the production of biofuels and the degradation of plastic waste. These enzymes have been found in various bacteria, including *Pseudomonas*, *Escherichia coli* and *Bacillus sp* (Wells et al., 2007). One example of an autotransporter domain containing esterase is EstA from *Pseudomonas aeruginosa*. EstA is a lipase that is able to degrade a wide range of substrates, including triacylglycerols, phospholipids and esters of cholesterol. The autotransporter domain of EstA enables transport across the bacterial outer membrane, where it can interact with its substrates (Wilhelm et al., 2007).

The two autotransporter domains containing esterase evaluated in this report are referred to as "1800", due to the length of the gene, in base pairs, and "Thermo", due to its thermostable abilities. "1800" is expressed by the organism *Stenotrophomonas maltophilia* and has an optimal growth temperature of 37ºC (Mahdi, Eklund and Fisher, 2014). "Thermo" the thermostable autotransporter domain containing esterase is expressed in the organism *Chlorobium sp* that has an optimal growth temperature of 55ºC (Castenholz, Bauld and Jørgenson, 1990).

3.3 Methods

3.3.1 Protein sequence alignment

Protein sequence alignment plays a critical role in bioinformatics and molecular biology as it enables the identification of homologous proteins and the inference of their functional and structural characteristics. One widely used method for protein sequence alignment is the Basic Local Alignment Search Tool (BLAST). BLAST utilizes an algorithm to rapidly search large databases for sequences that show significant similarity to a query sequence. The underlying principle of BLAST is to identify local regions of high similarity, known as high-scoring segment pairs (HSPs), between the query sequence and sequences in the database. The alignment scoring in BLAST is based on a substitution matrix, such as the popular BLOSUM matrices, which assign scores to amino acid substitutions based on their likelihood in evolutionarily related proteins. The alignment is optimized by maximizing the alignment score, which corresponds to the similarity between the query sequence and a given database sequence. BLAST provides statistical measures, such as the E-value, to estimate the significance of a match. The E-value represents the number of alignments with similar or higher scores that would be expected by chance alone, lower E-values indicate more significant matches (Altschul, 1990).

3.3.2 Molecular modeling

Molecular modeling is a computational technique used to study the structure, properties and interactions of molecules at the atomic level. It involves the use of computer programs and algorithms to create three-dimensional models of molecules and to simulate their behavior under different conditions. Molecular modeling can be used to study a wide range of molecules, from small organic molecules to large biomolecules such as proteins and nucleic acids. There are several methods of molecular modeling including molecular mechanics, molecular dynamics and quantum mechanics. Molecular mechanics involves the use of classical mechanics to model the behavior of molecules, while molecular dynamics simulates the movement of molecules over time. Quantum mechanics, on the other hand, provides a more accurate description of the electronic structure of molecules. However, quantum mechanical calculations are more computationally demanding compared to molecular mechanics. The 3D modeling can be validated based on a scoring system called Z-score and it describes how many standard deviations the structure is from the average high-resolution X-ray structure (Laskowski et al., 1993). Molecular modeling can be used to study a wide range of properties of molecules, such as their conformation, stability and reactivity. It can also be used to design new molecules with specific properties, such as drugs or materials with desired mechanical, electronic or optical properties (Ishida and Agag, 2016, pp.103–110).

3.3.3 Molecular docking

Molecular docking is a computational technique used to predict the binding modes and affinities of molecules such as ligands, proteins or nucleic acid targets. It involves the use of computer algorithms and molecular modeling techniques to simulate the interactions between a ligand and a target molecule and to predict the most favorable binding orientation. Molecular docking typically involves three main steps: preparation of the target molecule and ligand, generation of potential binding modes or conformations and scoring of the resulting complexes. The preparation of the target molecule involves the removal of water molecules and other nonessential atoms from the structure (Coumar, 2021, pp.245–269). The ligand is similarly prepared and different conformations or orientations are generated. The generation of potential bindings is typically achieved using a search algorithm such as a genetic algorithm, simulated annealing or Monte Carlo methods. These algorithms generate different conformations of the ligand and evaluate their compatibility with the target molecule based on factors such as shape, electrostatics and hydrogen bonding. The scoring of the resulting complexes involves the calculation of the energy of the complex and the identification of the most favorable binding orientation. This is typically achieved using scoring functions which quantify the strength and nature of the interactions between the ligand and the target molecule. The scoring function can also be used to rank different ligands and to predict their binding affinities, typically a lower docking score is more favorable (Meng et al., 2011).

3.3.4 Production of recombinant enzymes

Recombinant enzymes are enzymes that are produced using recombinant DNA technology which involves the insertion of a gene encoding the enzyme into a host organism, typically a bacterial or yeast cell. The production of recombinant enzymes begins with the identification of the gene encoding the enzyme of interest. This gene is then cloned into a suitable vector, which is typically a plasmid that contains a promoter sequence for gene expression and a selection marker for identifying cells that have taken up the plasmid. The recombinant plasmid is then introduced into a host organism such as *Escherichia coli* or *Saccharomyces cerevisiae* using a variety of methods such as transformation or electroporation. Once inside the host cell the plasmid replicates and the gene is expressed, resulting in the production of the recombinant enzyme (Wennerberg et al., 2019).

To optimize the expression of the recombinant enzyme various parameters such as the type of promoter, the culture conditions and the choice of host organism are carefully selected. After expression the recombinant enzyme is typically purified using a combination of chromatography and other purification techniques. The purity and activity of the enzyme are then assessed using various assays such as activity assays or SDS-PAGE. Recombinant enzymes have a wide range of applications, including in the pharmaceutical, food and biotech industries. They can be used for the production of therapeutic proteins, the synthesis of fine chemicals and pharmaceutical intermediates and the development of diagnostic assays (Demain and Vaishnav, 2009).

3.3.5 IMAC

IMAC or Immobilized Metal ion Affinity Chromatography is a technique for the purification of proteins and other biomolecules that have affinity for specific metal ions. IMAC is based on the principle that metal ions can bind to certain protein domains such as polyhistidine tags or imidazole groups with high specificity and affinity. The IMAC process involves the immobilization of metal ions onto a solid support. The immobilized metal ions are then used as affinity ligands to selectively bind the target protein or biomolecule of interest which may be tagged with a polyhistidine tag or contain imidazole groups. During the purification process, the protein mixture is passed over the IMAC column, allowing the target protein to selectively bind to the immobilized metal ions. The column is then washed to remove non-specifically bound proteins and the target protein is eluted using a buffer containing a competitive ligand such as imidazole that disrupts the metal ion-protein interaction (Yip, Nakagawa and Porath, 1989). IMAC has several advantages over other protein purification methods such as ion exchange chromatography and gel filtration chromatography. IMAC is highly selective, allowing for the isolation of specific proteins from complex mixtures. Additionally IMAC can be used to purify proteins under native or denaturing conditions making it a versatile technique for protein purification. (Yip, Nakagawa and Porath, 1989).

3.3.6 SDS-PAGE

SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) is a widely used technique for separating and analyzing proteins based on their size. The process involves denaturing proteins with SDS, a detergent that unfolds and coats the proteins with a negative charge and then subjecting them to an electric field that moves them through a polyacrylamide gel matrix. The proteins migrate based on their size, with smaller proteins moving further through the gel. Once separated, the proteins can be visualized by staining or an imaging developing system and a UV/stain free tray. SDS-PAGE is a crucial tool for protein purification, identification and quantification in fields such as biochemistry, molecular biology and biotechnology (Hagiwara, 2022).

3.3.7 HPLC

High Performance Liquid Chromatography (HPLC) is a powerful analytical technique used for the separation, identification and quantification of complex mixtures of biomolecules and other compounds. HPLC is based on the principles of liquid chromatography where a mixture of compounds is passed through a stationary phase that separates the individual components based on their physical and chemical properties. The HPLC process involves the use of a liquid mobile phase that is passed through a column packed with a stationary phase. The stationary phase consists of a solid support, typically a gel matrix that is coated with a thin layer of chemically modified material that interacts with the sample molecules in a specific way. As the sample mixture is passed through the column the individual components interact differently with the stationary phase causing them to be separated and eluted from the column at different times. (Nielsen, 2017, pp.213–226).

3.3.8 Microwave reactor

Microwave reactors have been widely used in chemistry and biochemistry research as they provide a highly efficient method for heating and accelerating chemical reactions. The Biotage Initiator Microwave Reactor is a specialized type of microwave reactor that is designed specifically for synthetic chemistry applications. The Biotage Initiator Microwave Reactor uses microwave radiation to rapidly heat reaction mixtures allowing for highly efficient and controlled chemical reactions (Biotage, n.d.). The reactor is equipped with a microwave cavity which generates microwave radiation at a specific frequency and power level. The radiation is then directed into the reaction vessel where it interacts with the reaction mixture and causes rapid heating. The advantages of using a microwave reactor include reduced reaction times, improved reaction yields and increased selectivity. The rapid heating provided by the microwave radiation allows for faster reaction kinetics and more efficient energy transfer leading to higher yields of desired products. In addition, the controlled heating provided by the microwave reactor can improve selectivity by minimizing side reactions and reducing the formation of unwanted byproducts (Priecel and Lopez-Sanchez, 2018).

4. Material and method

4.1 Identification of protein expression organism

The sequence of an autotransporter domain containing esterase from the organism *Stenotrophomonas maltophilia* was used as a query in both a UniProt-BLAST and a NCBI-BLAST search to identify similar sequences (UniProt Consortium, 2018; Altschul, 1990). The meaning of this search was to identify similar sequences from thermophilic organisms as these organisms have made adaptations that allow them to grow and live in high-temperature environments and therefore may have unique enzymatic properties. Following the BLAST search a number of domains expressed by thermophilic organisms was selected for further analysis. To ensure that the active site of the autotransporter domain containing esterase from *Stenotrophomonas maltophilia* was conserved in the chosen domains, a multiple sequence alignment was performed in Clustal Omega (Sievers et al., 2014). This alignment was used to compare the sequences of the selected domains and identify any conserved residues that may be involved in the enzymatic activity of the esterase.

4.2 Modeling of target enzyme

In this study, the structural characterization of the autotransporter domain containing esterase enzymes was performed through the use of computational methods. The amino acid sequences of the targeted enzymes were acquired and used to generate accurate 3D models of their structures. The modeling process was conducted using the Yasara software which utilizes homology modeling algorithms to predict the 3D structure of proteins from their amino acid sequences (Land and Humble, 2018). In this case the amino acid sequences of the autotransporter domain containing esterase enzymes were inserted into the Yasara software to generate the predicted 3D structures of the enzymes. The 3D structures were based on the crystal structure of the autotransporter domain containing esterase presented by (van den Berg, 2010), with PDB accession number 3KVN.

4.3 Molecular docking

The molecular docking process began with the preparation of the different ligands which were modeled using the software Avogadro (Hanwell et al., 2012). This allowed the ligands to be constructed with high accuracy, taking into account their individual chemical properties and three-dimensional geometries. The ligands were then optimized using force field calculations to ensure they were in the most energetically favorable conformation. The molecular docking was performed in Chimera (Pettersen et al., 2004), a molecular modeling software, using the AutoDock Vina extension (Eberhardt et al., 2021). This process involved the prediction of the binding affinity between the ligands and the target molecules by calculating their binding energy.

The ligands were docked onto the active sites of the target molecules and the resulting conformations were analyzed to identify the most favorable binding modes.

4.4 Transforming cells

For this research project the plasmid pET-21b(+) was used, containing a His-tag used for purification. The solution with plasmids for expression of "1800" and the *E.coli* BL21(D3) cells were thawed, followed by mixing 5 µL of plasmids and 50 µL of cells. The mixture was put on ice for 30 min and the mixture was subjected to a heat shock treatment in a water bath at 42ºC for 45 s. The solution was immediately cooled on ice for 2 min. The cells were then incubated at 37ºC with shaking at 170 rpm for a period of 1 h. Finally 100 µL of the treated solution was plated on agar plates containing 100 µg/mL ampicillin and incubated overnight at 37ºC.

4.5 Production and purification of enzymes

In order to prepare for the expression and purification of the target protein all equipment and LB-media was autoclaved to eliminate any potential sources of contamination. The LB-media was prepared by mixing 15 g of LB-powder with 600 mL of distilled water, previously prepared petri dishes with the desired cell strains were used to collect healthy colonies. These colonies were transferred to 3 mL of LB-media in falcon tubes with an ampicillin concentration of 100 µg/mL which were stored in a shaking incubator overnight at 37ºC at 170 rpm. To induce protein expression an IPTG solution was prepared by mixing 2,386 g IPTG with 10 mL distilled water, the IPTG solution was then filtered to ensure no contaminants. Shaking flasks with 300 mL LB-media and an ampicillin concentration of 100 µg/mL were prepared and the 3 mL of cell culture in falcon tubes were added to the shaking flasks. The optical density (OD) of the culture was measured regularly and when the OD reached the interval 0.6-1.0 the IPTG solution was added to a concentration of 1 mM. The shaking flasks were then stored at 37ºC with shaking at 170 rpm overnight to allow for the expression of the target protein.

The purification was based on the work of (van den Berg, 2010). Once the protein expression was completed, the shaking flask was transferred to a centrifuge tube and centrifuged for 10 min at 8000 rpm. The supernatant was discarded and the pellets were dissolved in 15 mL binding buffer that contained 1% of the detergent Lauryldimethylamine oxide (LDAO). Depending on the usage of the cell extract different methods were used in the later steps. For purification and isolation of the enzymes the buffers were transferred to falcon tubes and the samples were sonicated for 15 min at 60% amplitude and 0.5 cycles. The samples were then centrifuged at 13000 rpm for 20 min to remove any cell debris, the supernatant was then further analyzed. If purification and isolation was not needed the enzyme was used in the reaction with the unsonicated cells or sonicated cells that were treated with the same sonication settings as for the purification.

4.6 IMAC

The supernatant of the cell suspensions were purified using an ÄKTA chromatography system. A tris-elution buffer and tris-binding buffer, both with pH 7.4, were used as mobile phases. As the enzymes contain a His-tag, an appropriate method was used to separate the soluble proteins in the supernatant. The column used was a HisTrap™ FF crude 5 mL, the flow rate was set to 5 mL/min, the sample volume was roughly 10 mL and the elution gradient started at 0% and increased until 100%.

4.7 Enzymatic reaction with plastic powder

The enzymatic reactions were carried out by adding 20 mg of the plastic powder, approximately 1 mm in diameter, in 2 mL reaction vials and adding a mixture of 100 µL of the sample with cells expressing the desired enzyme, 200 µL of dimethyl sulfoxide (DMSO), 1000 µL of a pH 8,5 sodium phosphate buffer with a concentration of 100 mM and 700 µL of milli-Q water. The resulting reaction mixture was placed into a shake incubator at the desired temperature, with a shaking speed of 200 rotations per minute for a period of 24 h. The final buffer concentration is presented in appendix 1.

4.8 Enzymatic reaction with plastic beads

The enzymatic reactions with plastic beads, approximately 10 mm in diameter, were executed as presented in section 4.7 Enzymatic Reaction With Plastic Powder with the exception of that one plastic bead was used in each reaction vial instead of 20 mg of plastic powder. After the reaction, a washing of the beads was necessary, this is performed by vortexing the plastic bead in a mixture of 15 mL Milli-Q water and 0,15 g of washing detergent for 1 min. After washing, the bead was vortexed for 1 min in pure Milli-Q water.

4.9 HPLC

The amount of TPA produced in the enzymatic reactions was measured using a Dionex Ultimate 3000 HPLC machine. In this report the HPLC utilized pure acetonitrile and 0,1% formic acid in H_2O as the mobile phases for the separated components. The HPLC analysis was performed by injecting 1 µL of the reaction mixture into the HPLC machine and then separating the components of the mixture based on their physical and chemical properties. The separated components were then quantified using a UV-vis detector, which measures the absorbance of the components at a wavelength of 260 nm. The samples used for the HPLC were prepared by mixing 500 µL of the reaction mixture from section 4.9 Enzymatic Reaction with 500 µL of DMSO, this mixture was then filtered through a filter with 0,2 µm pores and injected into HPLC-vials. During the analysis the flow rate was set to 0,4 mL/min for 3 min per sample and the column used was a Kinetex column with the dimensions 50 x 2,1 mm and a particle size of

1,7 micrometer. The stationary phase of the column was C18 with iso-butyl side chains and with TMS endcapping.

4.10 Microwave reactor

The microwave reactions were performed in a Biotage Initiator and the preparations were performed as presented in 4.9 Enzymatic Reaction. The reactor settings used for the reactions were a temperature of 50 ºC, a power of 40 W, the stirring was set to 300 rpm and the reaction lasted for 1 h. The reactions were carried out in glass vials designed for the microwave reactor and were of the size 2-5 mL.

4.11 SDS-PAGE

For preparation of the SDS-PAGE a SDS running buffer was prepared with the concentrations 25 mM TRIS, 192 mM glycine and 0,1% (w/v) with a pH of 8,3. 16 µL of the samples were mixed with 4 µL of loading buffer and then heated for 10 min at 100 ºC. The samples were then loaded onto the gel and the gel was treated with 180 Volts for 15 min and then 200 volts for 30 min. The picture of the gel was then developed with BIO-RAD GelDoc Go Imaging System and a UV/stain free tray.

5. Results

The result section will include a brief depiction of the protein sequence alignment, the 3D modelings and dockings performed with the two autotransporter domains containing esterase, "1800" and "Thermo", also the active site of the enzymes will be presented. The results from the HPLC and the IMAC analyses will be presented in respective sections and finally the effect of auto transporter domains containing estereas on plastic beads will be presented.

5.1 Protein sequence alignment

The protein sequence alignment performed in NCBI BLAST is shown in figure 4, the upper sequence representing "1800" and the lower one representing "Thermo". The protein alignment resulted in an identity percentage of 28,48%, an E-value of $5e^{-38}$ and a gap percentage of 16%.

Score	Expect	Method	Identities	Positives	Gaps
149 bits(375)	5e-38	Compositional matrix adjust.	176/618(28%)	262/618(42%)	103/618(16%)

```
Query  5    KRPIRTLMAAAIALAALPAMAGESPYSKAVFFGDSLTDAGYFRP--------LLDPGVQP  56
            KR I ++ A A+A +A      E+ Y+  + FGDSLTD G +          LL  G+
Sbjct  4    KRRIFSVFAFALAASANSFAHAETSYTSLITFGDSLTDGGSYSSNVIAGSGGLLPSGIHY  63

Query  57   VTGQFTTN----PGWVWSQQVANYYGL-------NGGANGNGQKGDNYAVGGARVSVDEA  105
               +FTTN        W++ +A   GL       +G  N +G  G NYA GGA+V+VD
Sbjct  64   ---RFTTNFLDGSARTWAEYLAASMGLQLEPNTIDGVFNLSGSAGTNYAQGGAQVTVDP-  119

Query  106  GGLGAIP-----SLKSQAARYLAANGGKADGNALYTVWGGANDLFAATRAAAGGASQAQV  160
             GL + P     S++ Q   +LA N  +   + L+ +W GANDLF A  +A G
Sbjct  120  -GLSSAPGAPARSVEKQIDAFLAVNP-QLHSSQLFVLWAGANDLFHANPSAHGVD-----  172

Query  161  QGIIGAAVTDQIALVGALKQAGAQYVLVPNLPDVGITPQFRGP---------NAAAATAL  211
               I  A  D    +  LK AGA Y++V  LP++G TP +            + A A A
Sbjct  173  ---IATAAVDLAGQIQRLKSAGATYIVVNGLPNLGQTPLYSNTFINNLLSSGDPALAAAA  229

Query  212  SAGYNKALYGGLKQAGIEFIPLDTFSILREVTANPAMYGFTNVTSTA----------CKI  261
            S  +N  L   +  +G   I +DT  +L  V A+P  +GF  V  T             I
Sbjct  230  STLFNTTLKQSI--SGKNVIYVDTAKLLGAVVADPVRFGFNPVAGTVPYGLYVANNGSLI  287

Query  262  DPNNATASIIGCNPTSYVSPDA---ANTYLFADGVHPTTAGHQLLGQYAVSVLEAP----  314
             P   T  +     TS V P     AN+++F D +HPT A H L GQ    VL A
Sbjct  288  YPPGDTTCLTHV-LTSLVDPREKAFANSFIFTDPIHPTDAAHALFGQAVFGVLRAVDQKA  346

Query  315  ---------------RLQQVLSHSAQTIGRSRADQVSMHLGGRPADGLSWWGGVRGDLQR  359
                        L+  LS +A   G     ++  H+G    D   W+GG  G  Q
Sbjct  347  TTLFETSYAIRQQGIDLEPRLSAAALLKGDGMNGELR-HVG----DNQFWFGGSLGGYQ-  400

Query  360  YDHADL---YDGLAPAGLFGID--WARDGMVVGGFAGFGRLNADFGNSRGDFTQKDTTAG  414
            ++   L   Y     AG  G+D     D ++   F+ +   ++ FGN    +  + + A
Sbjct  401  HNSTQLDPDYSANTEAGSVGVDRMITSDALLGAAFS-YSMGHSTFGNDVETYDSRLSLAT  459

Query  415  LF-AGWYGDRIWVNGQVSYTWLSY-DVNRKVQLGPATREHGGSPDGSNLTAALNAGYEFG  472
            L+   +    ++VN  + Y  LS  ++ R V LG  +    GS  G+   A +  GYE
Sbjct  460  LYGTAFLARHLYVNASLQYGDLSLRNIQRTVALGATSITSSGSTAGTYKAARIGLGYE-N  518

Query  473  TEGGFRHGPIASVIWQKVKIDGYTESAAAGTLATALGYDRQNVDSTVGRIGWQARFDGGT  532
            + G +   P  S+   +  I GYTES    +LA    Y     +S +       R +G
Sbjct  519  SYGSWSCNPSISLTAARTLIKGYTESDTPVSLA----YGDAEYNSNIVTFAISCRMNGSK  574

Query  533  LK--PYAQLTYDHEFEDT  548
             +  P  +L  DH+ + +
Sbjct  575  DRWLPAIRLGVDHDLDQS  592
```

Figure 4. The sequence alignment of the amino acid sequences of "1800", upper row, and "Thermo", lower row.

5.2 Modeling

The 3D modeling of the two autotransporter domains containing esterase called "1800" and "Thermo" were performed in Yasara software. The outcome is presented in figure 5 with the secondary structure visible in different colors. The lower half of the secondary structures which include the color red are the autotransporter domains that are bound to the outer membrane of the host organisms. The upper part of the enzymes are the esterases that contain the active site responsible for the cleavage of ester bonds in polyesters. Both the catalytic esterase and the membrane bound autotransporter domain consists of both α-helices and β-sheets, although the autotransporter domain consists mainly of β-sheets. The validation of the 3D structures were based on the Z-scoring and "1800" obtained a score of -1,76 and "Thermo" obtained a score of -1,912

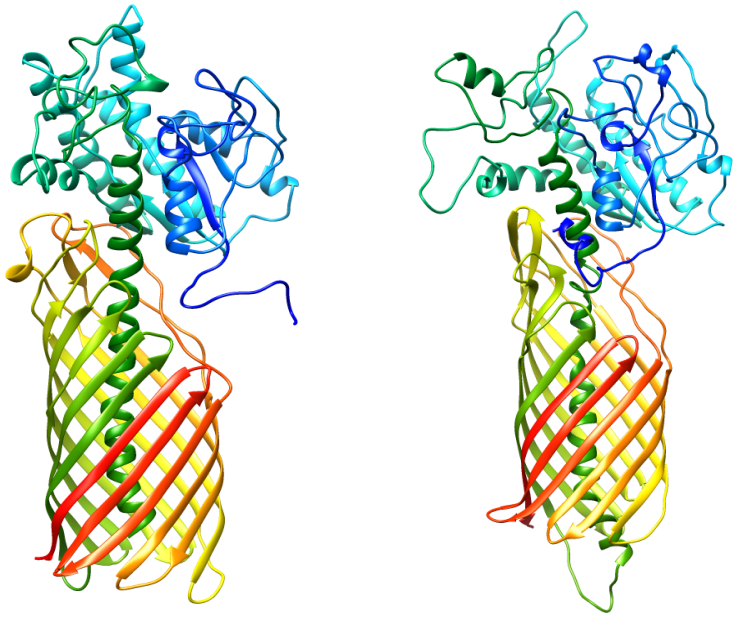

Figure 5. 3D models of the two autotransporter domains containing esterase, to the left "1800" and to the right "Thermo".

5.3 Active site

The active site in the autotransporter domain containing esterase is referred to as the catalytic triad and to find the catalytic triad in "1800" and "Thermo", a comparison of the 3D structures with another autotransporter domain containing esterase. That enzyme has the pdb accession number 3KVN and is presented by (van den Berg, 2010). The catalytic triad in 3KVN is the amino acids Ser14, Asp286 and His289. When comparing the catalytic triad of 3KVN with the 3D structure of "1800" and "Thermo" it suggests that the two active sites are Ser 39, Asp 291 and His 294 for "1800" and Ser 38, Asp 319 and His 322 for "Thermo", figure 6. As the multiple sequence alignment can confirm in figure 7, the catalytic triad is conserved in all three of the autotransporter domains containing esteras.

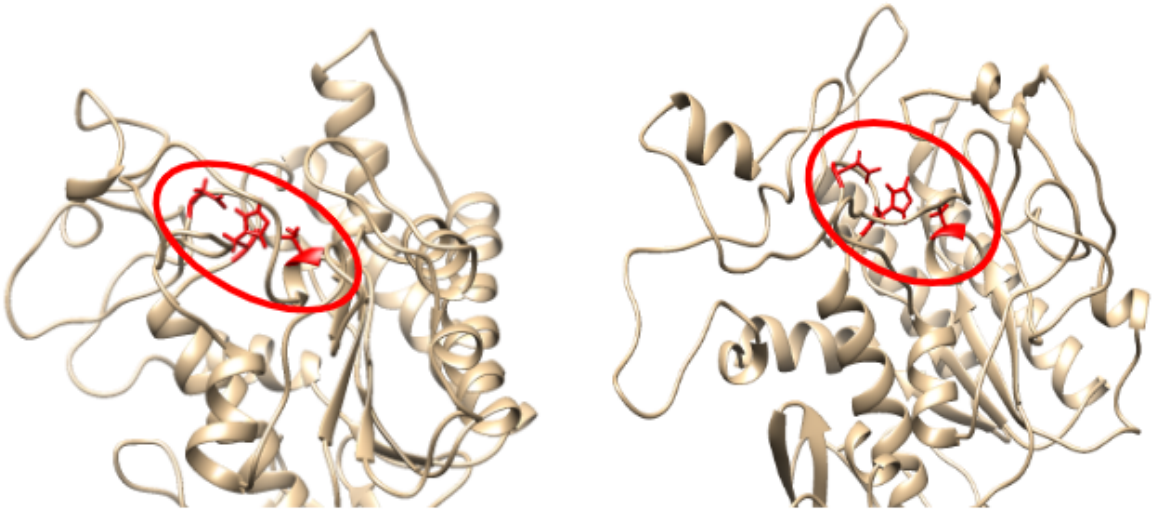

Figure 6. To the left is the catalytic triad of "1800" containing Ser 39, Asp 291 and His 294. To the right is the catalytic triad of "Thermo" containing Ser 38, Asp 319 and His 322.

```
tr|A0A533TZ12|A0A533TZ12_CHLSQ     -MKLKRRIFSVFAFA-LAASANSFAHAETSYTSLITFGDSLTDGGSYSSNVIAGSGGLLP      58
tr|A0A5K1NPK0|A0A5K1NPK0_STEMA     MLLSKRPIRTLMAAA-IALAALPAMAGESPYSKAVFFGDSLTDAGYFRPLLDPGVQ----      55
sp|O33407|ESTA_PSEAE               --MIRMALKPLVAACLLASLSTAPQAAPSPYSTLVVFGDSLSDAGQFDPPAGPAGS----      54
                                       :  :  :.* . :*  :     . : *:. : *** *:*.* :      .

tr|A0A533TZ12|A0A533TZ12_CHLSQ     SGIHYRFTTNFLDGSARTWAEYLAASMG--------LQ--LEPN-----TIDGVFNLSGS      103
tr|A0A5K1NPK0|A0A5K1NPK0_STEMA     -PVTGQFTT----NPGWVWSQQVANYYG------------LNGG----------ANGNGQ      88
sp|O33407|ESTA_PSEAE               ---TSRFTN----RVGPTYQNGSGEIFGPTAPMLLGNQLGIAPGDLAASTSPVNAQQGIA      107
                                      :**.      . .: :  .   *           :  .          : .

tr|A0A533TZ12|A0A533TZ12_CHLSQ     AGTNYAQGGAQVTVDPGLSSA--PGAP--ARSVEKQIDAFL----AVNPQLHSSQLFVLW      155
tr|A0A5K1NPK0|A0A5K1NPK0_STEMA     KGDNYAVGGARVSVDEAGGLGAIPS-------LKSQAARYL---AANGGKADGNALYTVW      138
sp|O33407|ESTA_PSEAE               DGNNWAVGGYRTDQIYDSITAANGSLIERDNTLLRSRDGYLVDRARQGLGADPNALYYIT      167
                                    * *:* ** :.        .   .       :  .   :*      .   . . *: :

tr|A0A533TZ12|A0A533TZ12_CHLSQ     AGANDLFHANPSAHGVD--------IATAAVDLAGQIQRLKSAGATYIVVNGLPNLGQTP      207
tr|A0A5K1NPK0|A0A5K1NPK0_STEMA     GGANDLFAATRAAAGGASQAQVQGIIGAAVTDQIALVGALKQAGAQYVLVPNLPDVGITP      198
sp|O33407|ESTA_PSEAE               GGGNDFLQGRILNDVQA-----Q----QAAGRLVDSVQALQQAGARYIVVWLLPDLGLTP      218
                                   .*.**:: .                  *.      :  *:.*** *::*  **::* **

tr|A0A533TZ12|A0A533TZ12_CHLSQ     LYSNTFINNLLSSGDPALAAAASTLFNTTLK--QSISGKNVIYVDTAKLLGAVVADPVRF      265
tr|A0A5K1NPK0|A0A5K1NPK0_STEMA     Q-FRGP--------NAAAATALSAGYNKALYGGLKQAGIEFIPLDTFSILREVTANPAMY      249
sp|O33407|ESTA_PSEAE               ATFGGP--------LQPFASQLSGTFNAELTAQLSQAGANVIPLNIPLLLKEGMANPASF      270
                                                 *:  *  :*  *    . :* :.* ::   :*    *:*. :

tr|A0A533TZ12|A0A533TZ12_CHLSQ     GFNPVAGTVPYGLYVANNGSLIYPPGDTTCLTHV-LTSLVDPREKAFANSFIFDQHHPT      324
tr|A0A5K1NPK0|A0A5K1NPK0_STEMA     GFTNVTS----------TACKIDPNNATASIIGCNPTSYV---SPDAANTYLFADQHHPT      296
sp|O33407|ESTA_PSEAE               GLAADQNLI--GTCFSGNGCTMNPTYG---ING----------STPDPSKLLFADQHHPT      315
                                   *:    .          ... : *      :           .    .. :*  :  .*

tr|A0A533TZ12|A0A533TZ12_CHLSQ     DAAHALFGQAVFGVLRAVDQKATTLFETSYAIRQQGIDLEPRLSAAALLKGDGMNGELRH      384
tr|A0A5K1NPK0|A0A5K1NPK0_STEMA     TAGHQLLGQYAVSVLEAPRLQQVLSHSAQTIGRSRADQVS----------MHLGG--RP      343
sp|O33407|ESTA_PSEAE               ITGQRLIADYTYSLLSAPWELTLLPEMAHGTLRAYQDELR----------SQWQADWEN      364
                                    :.: *:.: . .:* *          :    *    ::              .  .

tr|A0A533TZ12|A0A533TZ12_CHLSQ     VGDNQFWFGGSLGG----YQHNSTQLDPDYSA----NTEAGSVGVDRMITSDALLGAAFS      436
tr|A0A5K1NPK0|A0A5K1NPK0_STEMA     -ADGLSWWGG-VRGDLQRYDHA-----DLYDG----LAPAGLFGIDWARDG-MVVGGFAG      391
sp|O33407|ESTA_PSEAE               -WQNVGQWRGFVGGGGQRLDFDSQDSAASGDGNGYNLTLGGSYRIDEA----WRAGVAAG      419
                                     :.   : * : *     :.          ..     : .*  :*         *   .

tr|A0A533TZ12|A0A533TZ12_CHLSQ     YSMGHSTFGNDVETYDSRLSLATLYGTAFLARHLYVNASLQYGDLSLRNIQRTVALGATS      496
tr|A0A5K1NPK0|A0A5K1NPK0_STEMA     FGRLNADFGNSRGDFTQKDTTAGLF-AGWYGDRIWVNGQVSYTWLSYD-VNRKVQLGPAT      449
sp|O33407|ESTA_PSEAE               FYRQKLEAGAKDSDYRMNSYMASAF-VQYQENRWWADAALTGGYLDYDDLKRKFALGGGE      478
                                   :   :   * .   : .   *  : . :   : :.:. :    *.   ::*.. **

tr|A0A533TZ12|A0A533TZ12_CHLSQ     ITSSGSTAGTYKAARIGLGYENS---YGSWSCNPSISLTAARTLIKGYTESDTP----VS      549
tr|A0A5K1NPK0|A0A5K1NPK0_STEMA     REHGGSPDGSNLTAALNAGYEFGT--EGGFRHGPIASVIWQKVKIDGYTESAAAGTLATA      507
```

Figure 7. Multiple sequence alignment of three autotransporter domains containing esterase. Conserved catalytic trial circled in yellow.

The cleft of the active site on the two enzymes was measured using tools in chimera, the distance of the cleft leading to the active site of "1800" is 14,968 Å and for "Thermo" 13.922 Å, this is presented in figure 8.

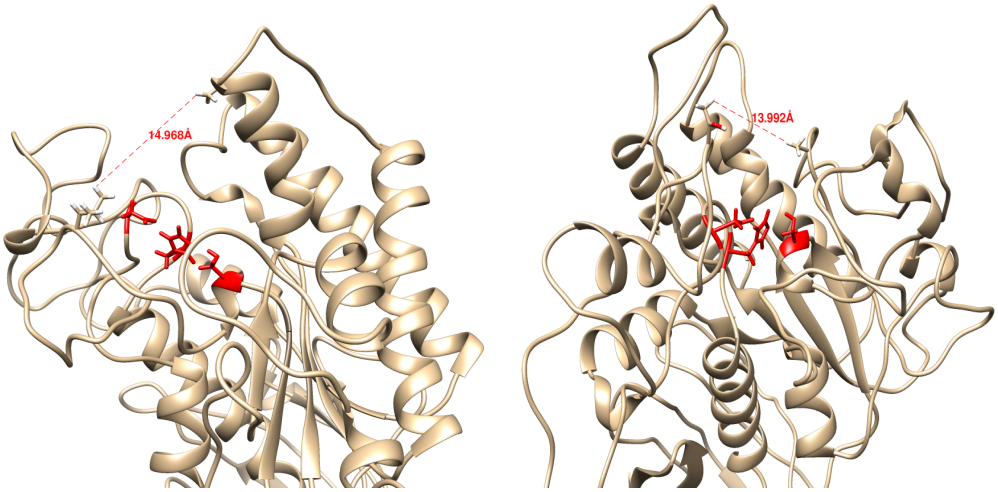

Figure 8. Measurement of the smallest opening in the cleft to the catalytic triad, to the left, the enzyme "1800" and to the right, the enzyme "Thermo".

5.4 Docking

The docking of ligands to the active site was performed in chimera using the extension tool Autodock vina. For the enzyme "1800" a dimer of Akestra^{TM} was used, figure 9, the presented docking got a docking score of -9,3. For "Thermo" a trimer of PET was used as ligand, figure 10, the presented docking got a docking score of -6,4. Both dockings generated several different interactions, the active site consists of charged and polarized amino acids generating electrostatic interactions between the ligand and the enzyme. Hydrogen bonds were generated in both cases and a big part of the amino acids surrounding the active site has a hydrophobic side chain leading to hydrophobic interaction between the ligand and the cleft of the active site. It can also be assumed that Van der Waals interactions are present in both dockings. When comparing the docking of the same ligand to the two autotransporter domains containing esterase it is obvious that the dockings look very unsimilar.

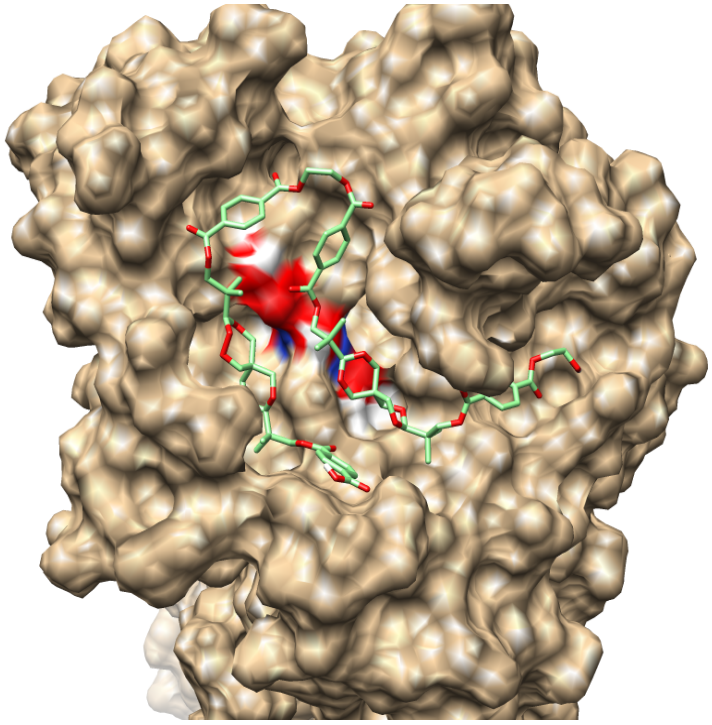

Figure 9. The docking of an Akestra™ dimer to the active site of "1800".

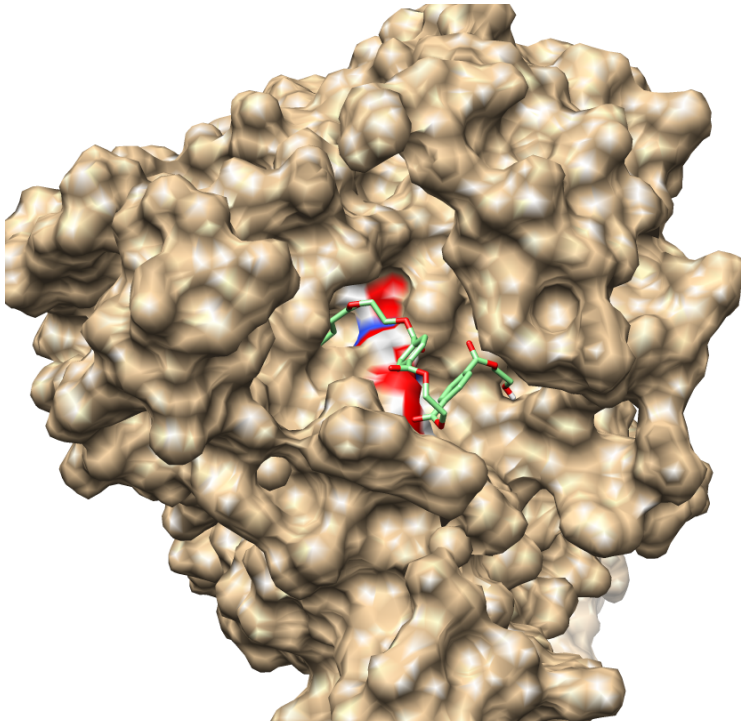

Figure 10. The docking of an PET trimer to the active site of "Thermo".

5.5 HPLC

5.5.1 First Run

In the first run of HPLC, "1800" and "Thermo" were tested on the three plastics Akestra™ 90, Akestra™ 110 and PET. The reaction mixtures consisted of unsonicated and sonicated cells that expressed the respective enzymes, suspended in a binding buffer with 1% LDAO. The reactions occurred at 50ºC for 24 h. The concentration was calculated with equation (1), derived from a linear regression of the calibration points, y is the peak area and x is the concentration of TPA. The concentration, in mM, of produced TPA in the enzymatic reactions are presented in table 1 showing that the highest concentration of TPA is when using Akestra™ 90 mixed with "1800" expressed in unsonicated cells and when mixing PET with unsonicated cells expressing "Thermo". In figure 11 and 12 the chromatograms of the enzymatic reactions containing "1800" and Akestra™ 90 with both unsonicated and sonicated cells are presented. In both figures, a peak representing TPA is clearly visible and indicates that degradation of Akestra™ 90 was successful using "1800". However, the peak of TPA is significantly higher when using unsonicated cells. In figure 13, the chromatogram of PET mixed with unsonicated cells expressing "Thermo" is presented and a very small peak representing TPA is presented, although this is the highest peak when comparing the chromatograms of "Thermo". The retention time of both DMSO and TPA is longer compared to available data (Wagner-Egea et al., 2022).

(1) $$y = 6,0223x - 1,4162$$

Table 1. The average concentration of TPA in mM produced in the enzymatic reactions.

"1800"	Unsonicated cells	Sonicated cells
Akestra™ 90	2,44	1,81
Akestra™ 110	0,85	0,49
PET	0,25	0,28
"Thermo"		
Akestra™ 90	0	0
Akestra™ 110	0	0
PET	0,25	0,24

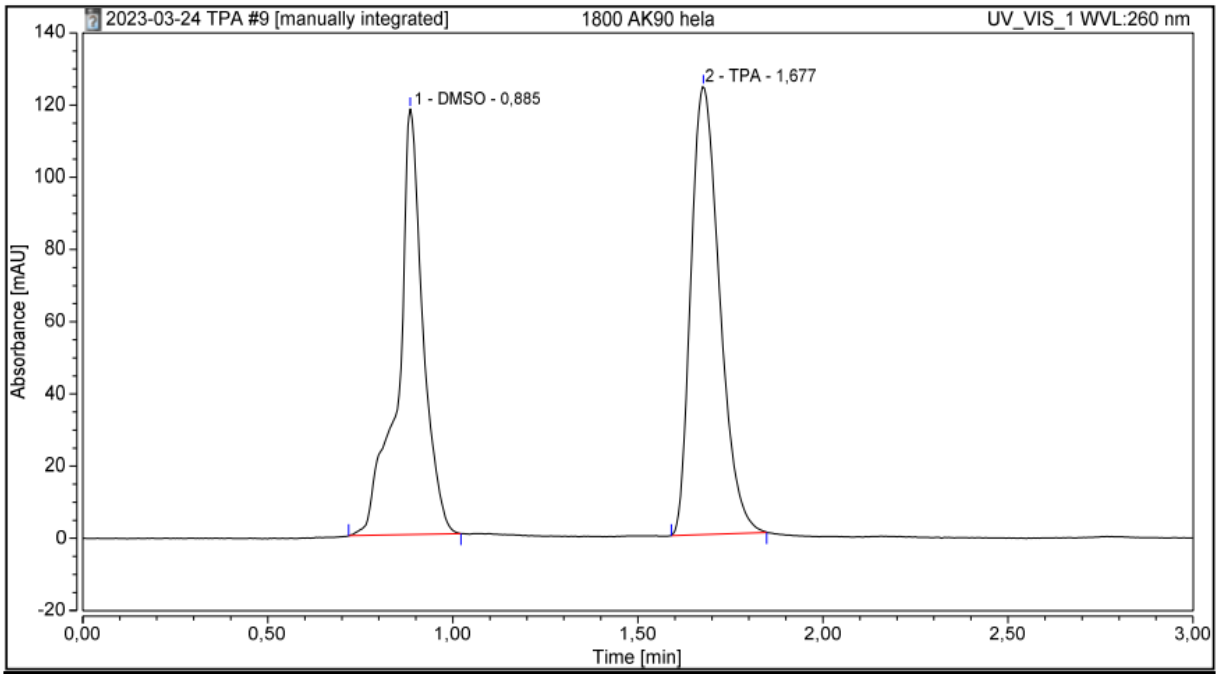

Figure 11. The chromatogram of the enzymatic reaction sample with Akestra^{TM} 90 mixed with unsonicated cells expressing "1800", at 50^{o}C.

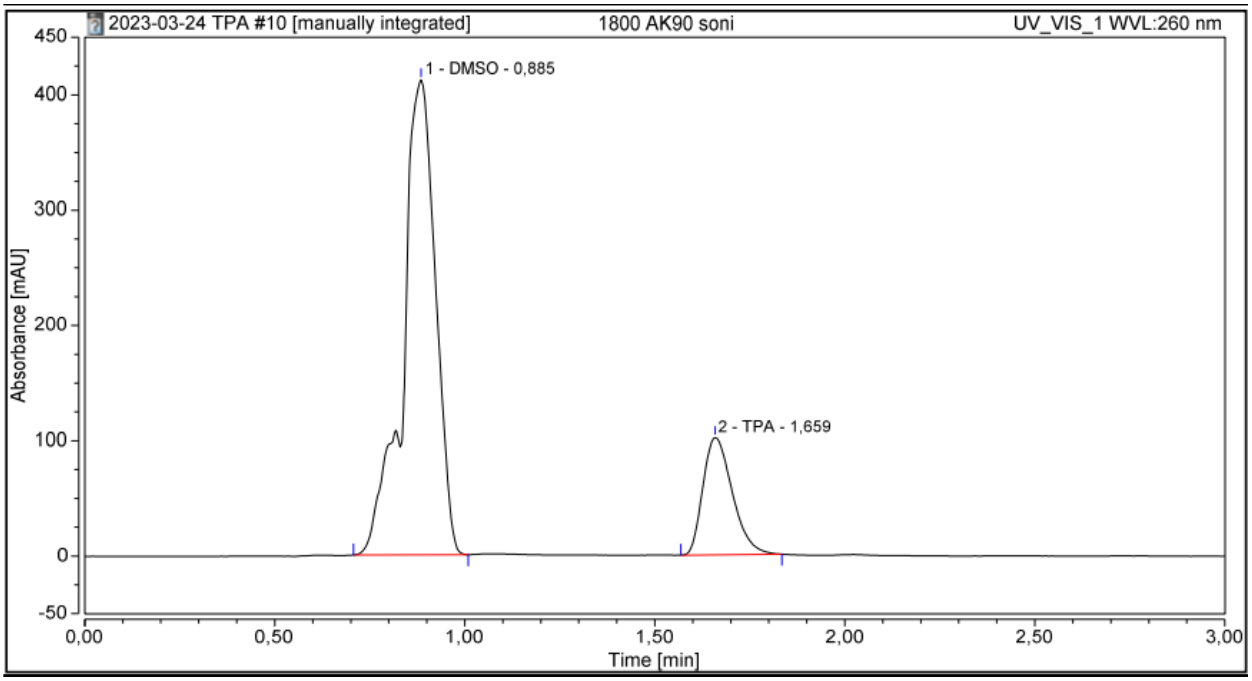

Figure 12. The chromatogram of the enzymatic reaction sample with Akestra^{TM} 90 mixed with sonicated cells expressing "1800", at 50^{o}C.

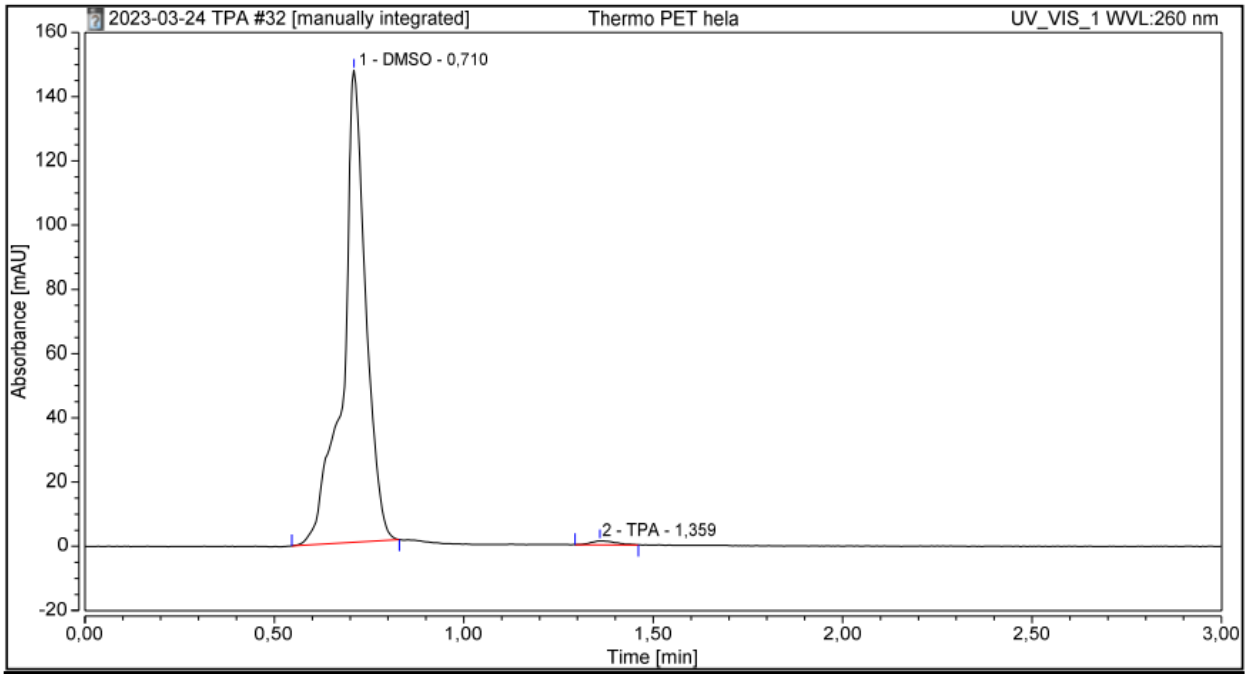

Figure 13. The chromatogram of the enzymatic reaction sample with PET mixed with unsonicated cells expressing "Thermo", at 50ºC.

5.5.2 Second Run

In the second run of HPLC another column of the same kind was used due to problems, such as pressure drops and irregular chromatograms. In the chromatogram, figure 14, it is clear that the retention time for DMSO has decreased due to the change of column and the new retention time is closer to documented values. All of the samples that were analyzed in both the first and second run had lower peaks representing TPA in the second run compared to the first run. In figure 14, the same conditions are used as in figure 11 but the resulting peak representing TPA is smaller. In all peaks representing produced TPA the concentration was lower than all the calibration points, leading to negative concentrations of TPA in all samples.

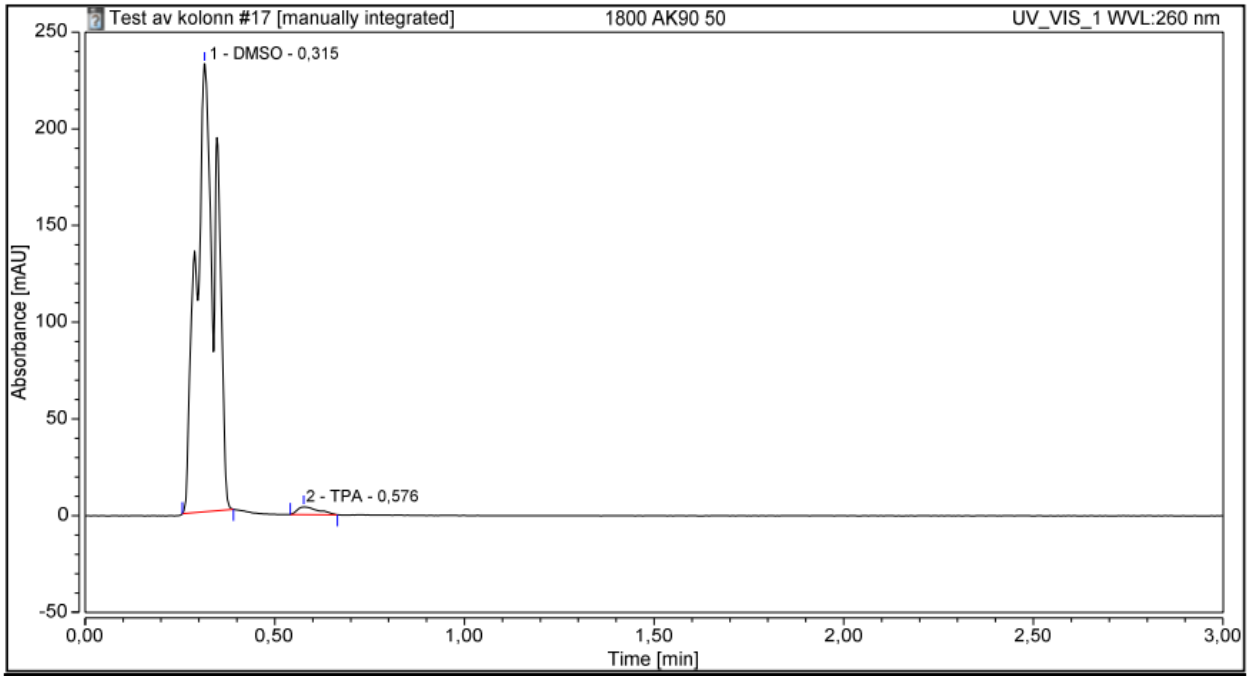

Figure 14. The chromatogram from the second run of the enzymatic reaction sample with Akestra^{TM} 90 mixed with unsonicated cells expressing "1800", at 50^{o}C.

5.5.3 Third Run

In the third run of HPLC the temperature was varying to decide the optimal temperature, also the age of the enzyme samples were tested to see if the storage time affects the enzymatic activity, the comparison was carried out with the same conditions at 50^{o}C and for 24h and 200 rpm. The effect of heating using a microwave reactor compared to conventional heating was also analyzed during the third run. In figure 15, the reaction that occurred in a microwave reactor is presented, it has a small peak representing TPA, however, as presented in figure 16 to 19 no peak representing TPA is present in any of the other chromatograms.

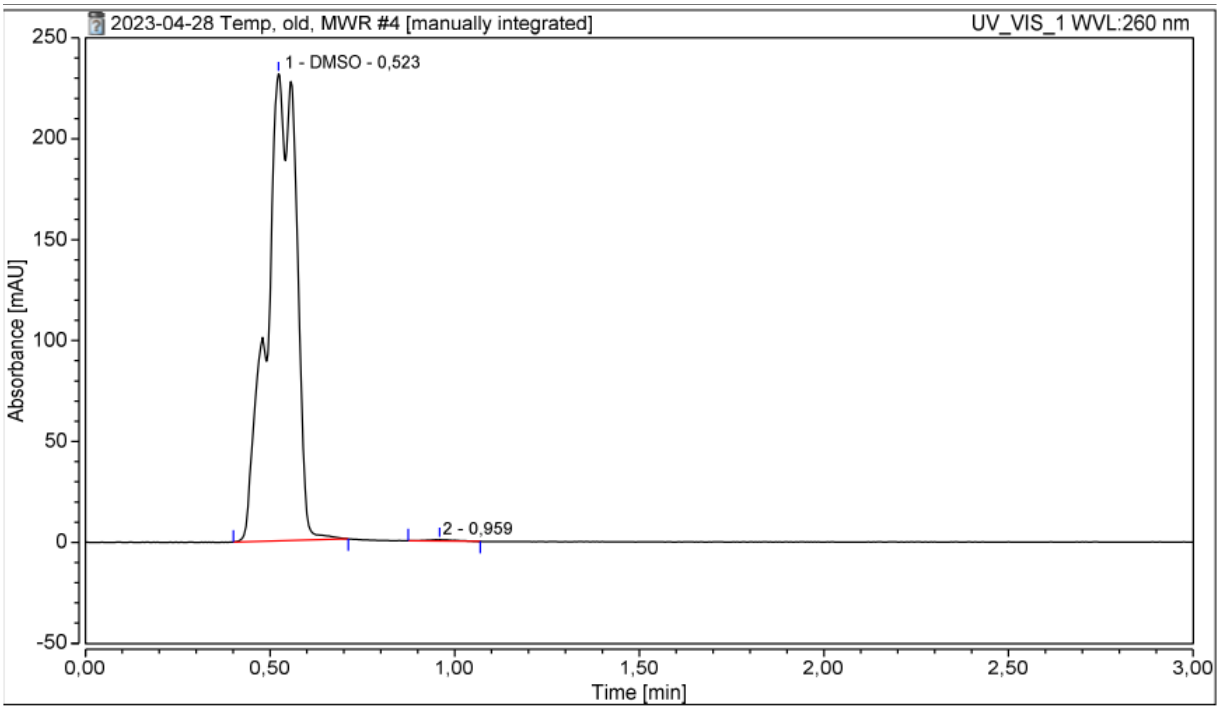

Figure 15. The chromatogram of the enzymatic reaction sample, heated in a microwave reactor, with Akestra^{TM} 90 mixed with unsonicated cells expressing "1800", at 50^{o}C.

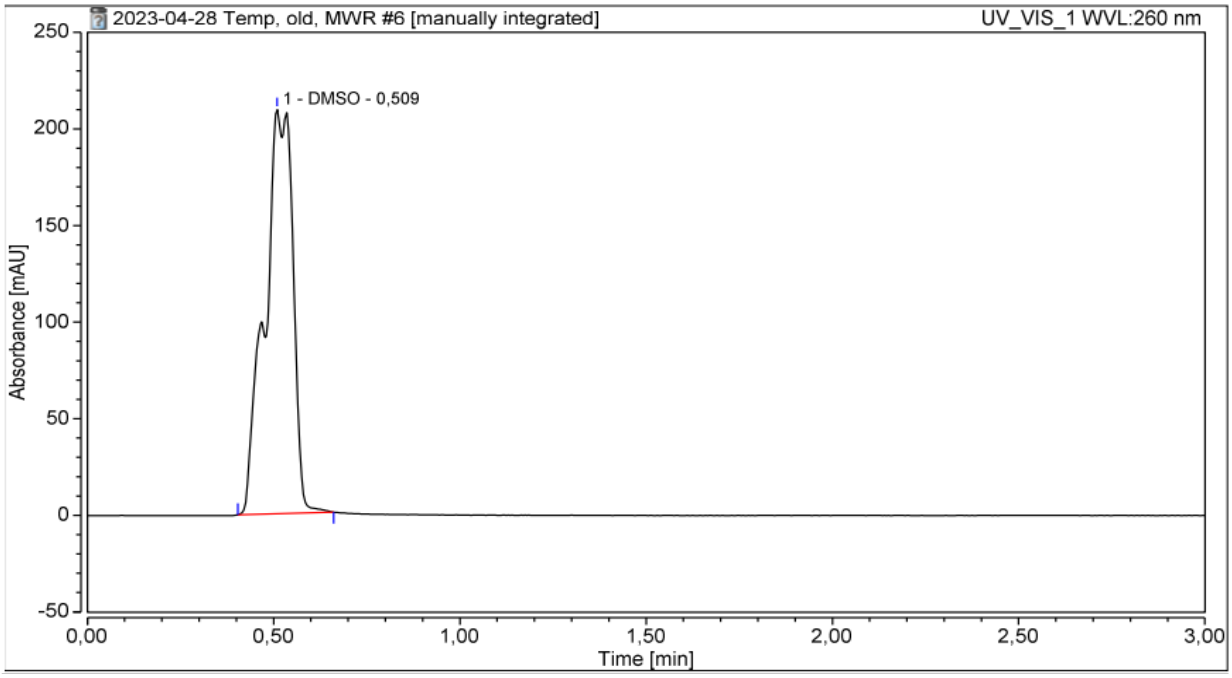

Figure 16. The chromatogram of the enzymatic reaction sample with PET mixed with an old batch of unsonicated cells expressing "Thermo", at 50^{o}C.

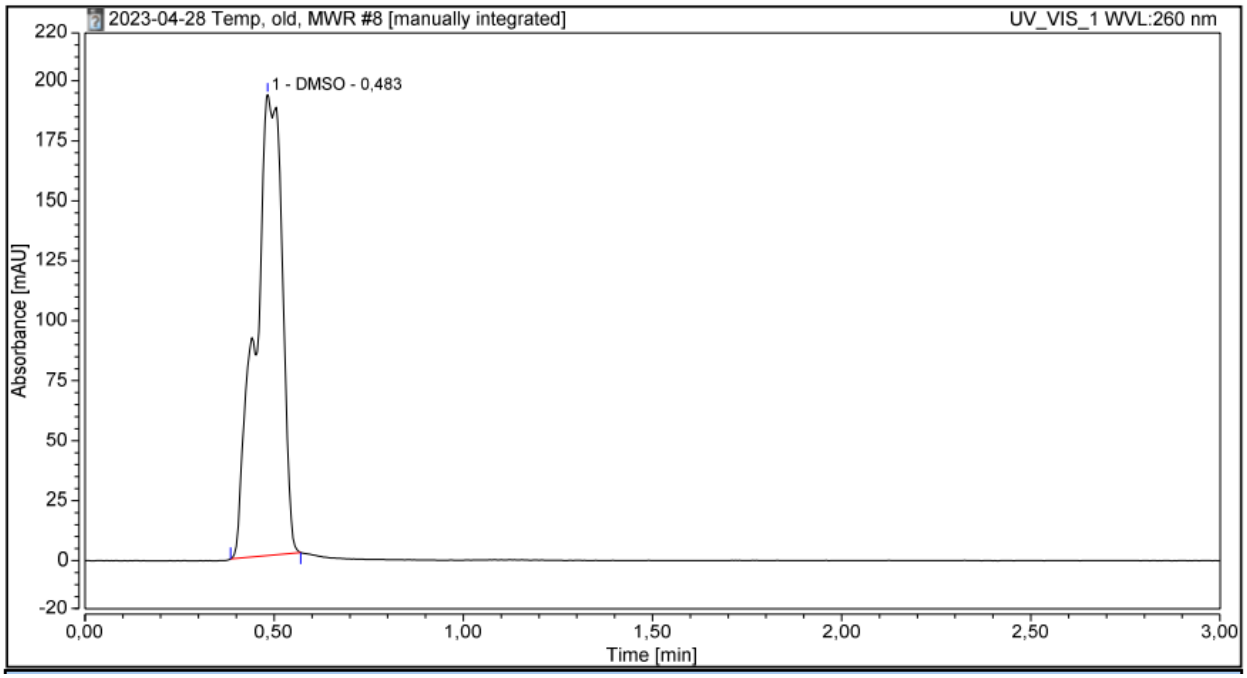

Figure 17. The chromatogram of the enzymatic reaction sample with Akestra^{TM} 90 mixed with an old batch of unsonicated cells expressing "1800", at 50ºC.

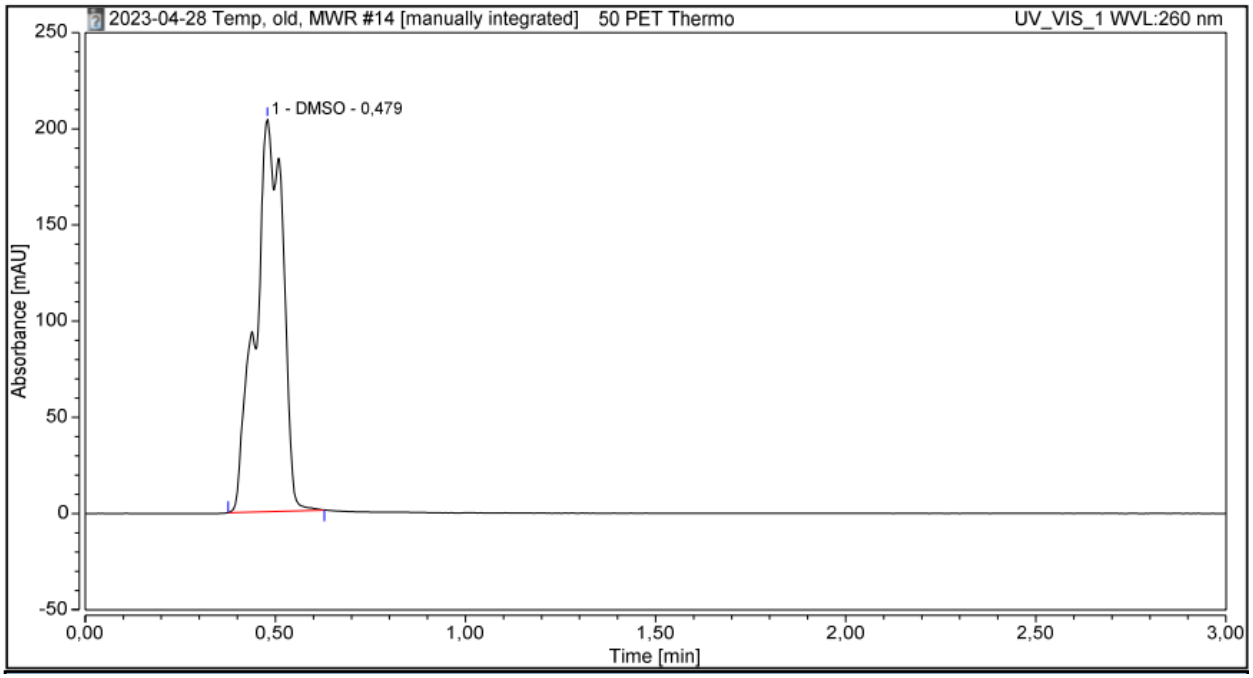

Figure 18. The chromatogram of the enzymatic reaction sample with PET mixed with unsonicated cells expressing "Thermo", at 50ºC.

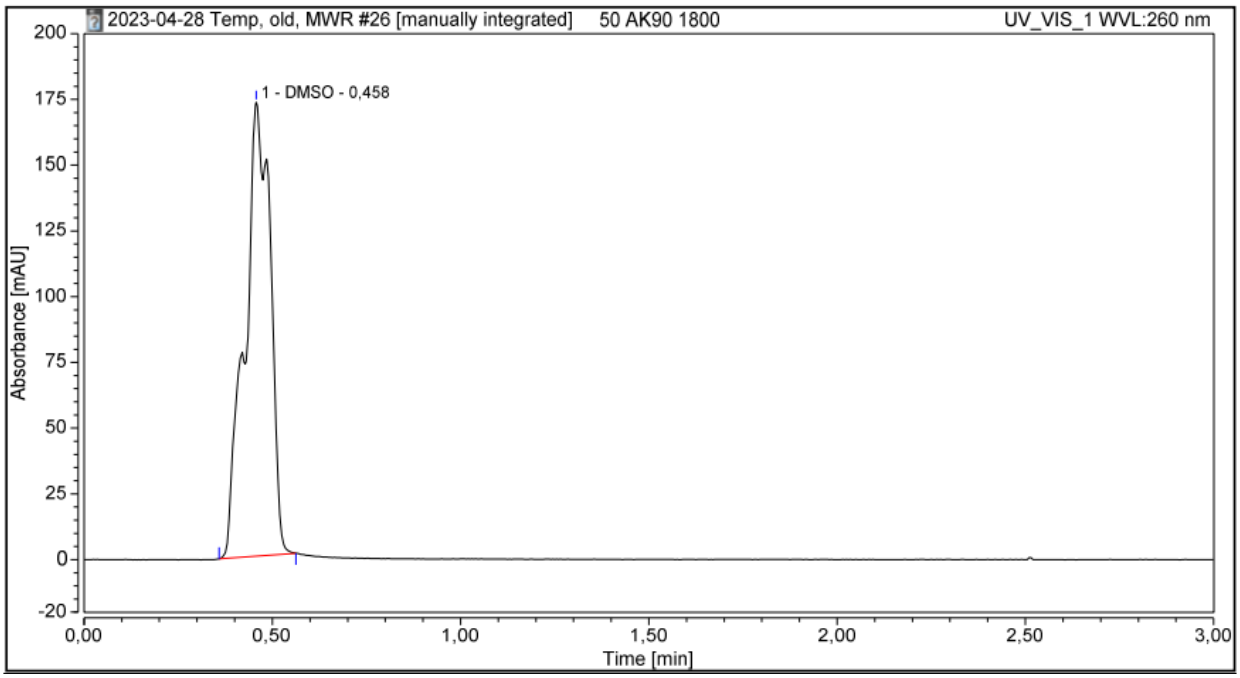

Figure 19. The chromatogram of the enzymatic reaction sample with Akestra^{TM} 90 mixed with unsonicated cells expressing "1800", at 50^{o}C.

5.6 IMAC

In an attempt to isolate and collect a pure sample of enzyme a purification using IMAC was performed. In figure 20 the measured absorbance of the purified samples of "Thermo" are presented, using both 1 and 2% of added detergent, LDAO, the absorbance is overlapped for comparison. The pink line represents the gradient of the elution buffer, the peaks after the increase of gradients is assumed to be nonspecific proteins that are flushed out by the elution buffer. No peak representing the target enzyme is noted, neither when using 1 or 2% detergent.

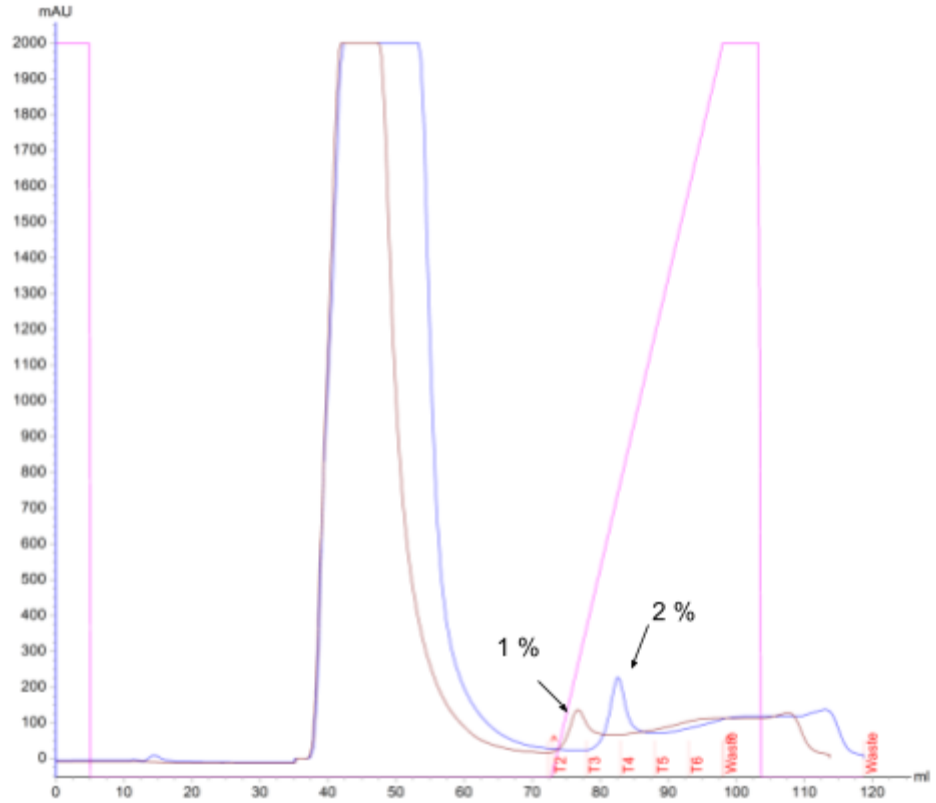

Figure 20. The Absorbance and gradient curves of the IMAC for purification of "Thermo" with 1 and 2% of LDAO.

5.7 SDS-PAGE

The SDS-PAGE gel after it has been treated with electricity is shown in figure 21, in lane 1 the staining ladder is shown. Lane 2 shows the cell suspension containing unsonicated cells expressing "1800", in lane 3 the cell suspension containing unsonicated cells expressing "Thermo" is presented and finally, in lane 4 is the purified sample fraction corresponding to the peak in figure 20 representing "Thermo" 1%. In lane 2 there is a band with high concentration representing a protein with a molecular weight between 50 and 75 kDa. In lane 4, the sample is the purified sample of lane 3, but no clear band with the same molecular weight can be seen when comparing lane 3 and 4. Lane 3 lacks a band with high concentration.

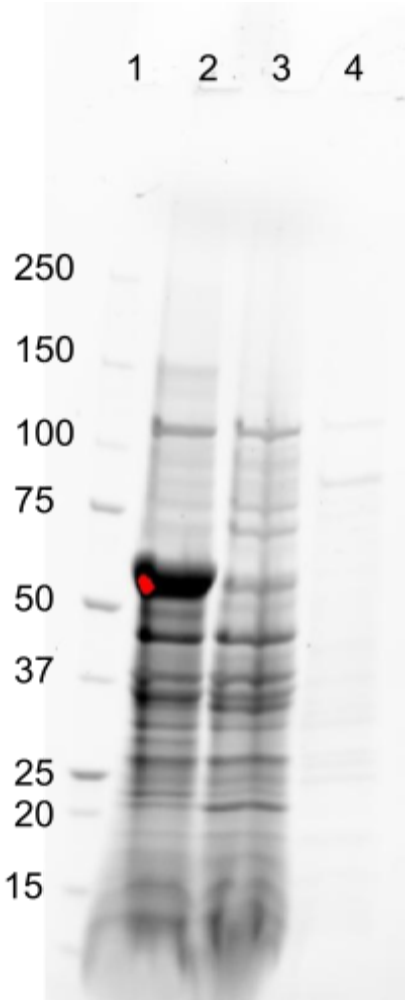

Figure 21. The SDS-PAGE gel after it has been treated. Lane 1 is the ladder, lande 2 is the cell suspension containing unsonicated cells expressing "1800", lane 3 is the cell suspension containing unsonicated cells expressing "Thermo" and lane 4 is the purified sample fraction corresponding to the peak in figure 20 representing "Thermo" 1%

5.8 Plastic Beads

The result of the enzymatic reactions with plastic beads are presented in figure 22, there was no documented method to determine the concentration of product during the degradation, this is why the results are visibly determined by comparing the treated plastic bead to the control bead. No visible difference is noticed under microscope when comparing the control capa bead with the capa bead reacting with unsonicated cells expressing "1800".

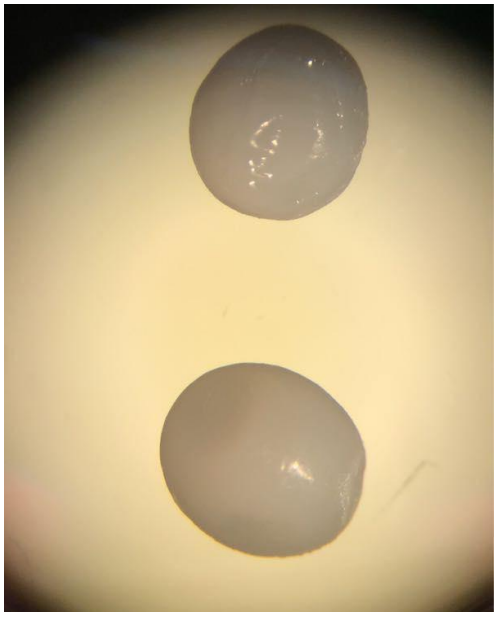

Figure 22. Plastic beads of Capa under a visual test in a microscope. The upper bead has been treated with unsonicated cells expressing "1800" and the lower bead is a control.

6. Discussion

6.1 Protein sequence alignment

The sequence alignment performed using a BLAST search indicates that the two amino acid sequences have a relatively low identity percentage of 28,48%. Which means that only 28,48 percent of the amino acids is conserved in both sequences. The obtained E-value of $5e^{-38}$ indicates that there is a high probability of this alignment and that it is unlikely that the alignment happened by chance. The sequence alignment also presents a gap percentage of 16%, indicating that there has been an occurrence of insertions and deletions during the evolutionary divergence of the sequences. The obtained values from the protein sequence alignment indicates that there is a distant relationship between the two enzymes. However, the low identity percentage can indicate a lack of similar functions and this has to be further analyzed with 3D modeling.

6.2 Modeling

The 3D models that are shown in figure 5, generated by Yasara have a very similar structure to other comparable autotransporter domains containing esterases, this is an indication that the modeling is rather successful and that the real enzyme has the same appearance. It is visible that the autotransporter domain has a tube looking appearance that is bound to the outer membrane of the cell and that the esterase is bound to the outside of the cell where it can react with substrate.

The autotransporter domain looks more or less the same in the two models but the esterases have a difference in appearance. It looks like the esterase on "Thermo" has more steric hindrance than the one in "1800", this can be an indication that the enzymatic activity in "Thermo" is lower than the activity of "1800". The steric hindrance of "1800" covering the active site consists of loops built up by amino acids 43-60, 143-159 and 258-288. While the steric hindrance covering the active site of "Thermo" consists of loops built of amino acids 43-64, 111-116, 123-137, 161-169, 207-222 and 288-297. Hence does the steric hindrance of the active site on the esterase belonging to "Thermo" consist of the double amount of loops covering the active site. Which explains the low degradation of plastic when using "Thermo" in the enzymatic reactions. The Z-score of -1,76 and -1,912 is relatively bad and indicates a big deviation from the average high-resolution X-ray structure of the enzymes and this is likely due to the esterases. The esterases contain a lot of loops that have a high likelihood of being flexible making the 3D model harder to predict and causing a lower Z-score. However the validation classifieds the 3D structures as satisfactory even though the Z-score was low.

6.3 Active site

The active site of the auto transporter domain containing esterase is located on the esterase where the breaking of the ester bond occurs. The active site of esterase is referred to as the catalytic triad as it consists of the active amino acids Serine, Aspartic acid and Histidine. The catalytic triad is located in the same place in both "1800" and "Thermo" but the difference is that there visibly is more steric hindrance that covers the catalytic triad in "Thermo" and as is shown in figure 6 the active site is surrounded by more steric hindrance and the esterase is bulkier than compared to "1800". The smallest opening to the active site is smaller for "Thermo", 13,992 Å, compared to "1800", 14,968 Å and this can cause a problem because the plastic polymers are generally large and this requires a relative open access to the active site to ensure a good enzymatic activity. Hence, this does also imply that the enzymatic activity is lower for "Thermo" compared to "1800". The multiple sequence alignment indicates that the active site is preserved in both "1800" and "Thermo", indicating that there is a catalytic activity, however, as mentioned, the steric hindrance does affect how well the polyesters can interact with the active site.

6.4 Docking

The docking of ligands to the two autotransporter domains containing esterase indicates that the binding can be inhibited due to the small cleft openings leading to the active site. The dockings presented in figure 9 and 10 are the docking that got the best docking score in chimera when using Autodock vina but even though these have the best score value it is clear that there are some problems with the docking. Plastic polymers are usually in a straight formation and the ligands in figure 9 and 10 are curved to fit the active site and this can be a cause of worry because it indicates that the polyesters can have a hard time binding to the active site and decreasing the enzymatic activity. The generated dockings between the enzyme and the ligands

indicates that the binding can occur in real life, also the hydrogen bond can be an indication that the ligand interacts with the enzyme in a beneficial manner. When docking the same ligand to the two enzymes and the docking has very different outcomes might be due to the greater presence of steric hindrance in the thermostable enzyme, also indicating that the thermostable enzyme might be less suitable for degrading plastic.

6.5 HPLC

In the first run of HPLC the results looked promising, especially when combining "1800" with Akestra™ 90 and combining "Thermo" with PET. These two combinations generated the highest peaks which is why further testing was performed solely on these two combinations with different conditions. When using unsonicated cells compared to sonicated cells there were in most cases a bigger peak with unsonicated cells which is why unsonicated cells were used in further reactions. The reason for this can be that the sonication disrupts the enzyme which leads to a lower degradation. In the first run the retention time of both DMSO and TPA was almost twice as high as previous data from other articles. This was thought to be because of the column which is why another column of the same kind was tested in the second run to compare the retention times. The retention time decreased and gave a more expected retention time for the two monomers with the other column. In the second run the effect of temperature on the enzymatic activity was researched. In the second run the peaks was much smaller even with the same reaction conditions as in the first run, the cell suspension used for the second time was 3 weeks old and due to the fact that no purification was made it is though that a degradation of the enzyme was undergoing, decreasing the enzymatic activity and that is thought to be the reason for the lower peaks in run two. This led to the comparison that was made in the third run, comparison of a new vs old cell suspension, with the exact same conditions. In the third run also the microwave reactor was tested to see if there was an effect of that compared to conventional heating. In the third run even though the good column was used the peaks were non existing in almost all chromatograms, except in one of the microwave reactor tests. The absence of peaks is best explained by the fact that the cells did not express any enzymes at all or at least in such small amounts it did not affect the plastic, for different reasons. However the peak in the microwave reactor can just be an error but it can also be an indication that the microwave reactor increases the enzymatic activity compared to conventional heating, this is very interesting and something that has to be elaborated even more to draw a conclusion of it. The whole third run was a disappointment except for the Microwave reactor test.

6.6 IMAC

In this report the detergent LDAO is used in an attempt to extract the enzyme and make the enzyme soluble before the purification of the enzymes. Both 1 and 2% of the detergent was tested but in both cases as seen in figure 20, there was no peak present of the desired enzyme. This is probably caused by the fact that the enzyme never detaches from the membrane hench

when the cell debris is removed and discarded the enzymes follow with it. That is why no enzyme is present in figure 20, presenting the absorbance of proteins. There is one peak after the elution has begun but this is thought of as nonspecific proteins that elute when an elution buffer is added. It is shown that the peak of the nonspecific proteins is higher when using a higher percentage of detergent, this is believed to be caused by the detergent dissolving more proteins from the cells and increasing the final concentration of nonspecific proteins in the final solution that is analyzed using IMAC.

5.7 SDS-PAGE

The gel, figure 21, indicates a high concentration of a protein band in lane 2, which contains the cell suspension with unsonicated cells expressing "1800", the protein in that band has a molecular weight in the range of 50 to 75 kDa. In lane 3 where the "Thermo" is expressed no band is of significant high concentration indicating that there might not be an expression of the enzyme at all. In lane 4 where the fragment corresponding to the peak in figure 20, after purification in ÄKTA, there is no band that is of the same molecular weight as the bands in lane 3 with the highest concentration, indicating that the purification was unsuccessful and that the peak just consists of unspecific proteins that are suspended in the detergent.

5.8 Plastic beads

The enzymatic reactions with plastic beads could only be analyzed by comparing a control bead with the treated bead under a microscope and as is presented in figure 22 there is no visible difference between the two beads and due to this no further testing was carried out.

7. Conclusion

The use of plastic degrading enzymes can have a great potential in solving the plastic pollution crisis around the world and help us make a smaller emission footprint. In this report an assessment of the plastic degrading abilities of two enzymes belonging to the group of autotransporter domains containing esterase. Even though the results in this report are ambiguous there are still indications that the two enzymes "1800" and "Thermo" have potential as plastic degrading enzymes in the industry. From the results of the first run HPLC it can be concluded that the enzyme "1800" has the biggest plastic degrading effect on the plastic Akestra 90 and the enzyme "Thermo" has the biggest plastic degrading ability on the plastic PET, however, this result could not be replicated and more research is needed.

Another implication from the results is that heating the reactions in a microwave reactor might have potential benefits compared to conventional heating, although more research is needed for this to be an obvious conclusion. All in all the enzymatic family, autotransporter domains

containing esterase has potential in the field of plastic recycling but there is still more research needed to declare its benefits compared to conventional heating and before it can be implemented industrially.

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Appendix

1 Buffer concentration

In section 4.7 Enzymatic reaction with plastic powder a buffer with concentration 100 mM is used. The volume of buffer in the total reaction volume is half which means the final concentration of buffer is 50 mM

2 UniProt ID

The two autotransporter domains containing estease can be further analyzed in UniProt with the IDs A0A5K1NPK0 for "1800" and A0A533TZ12 for "Thermo".