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Declaration

I hereby declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or processional qualification except as specified.

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1. Introduction

1.1 Plastic and the world

Synthetic polymers of various compositions are used in literally all service sectors of today's world. The unique combination of features such as stability, inertia, and low weight causes a steadily increasing demand. Therefore, a future without plastic is hardly imaginable. However, it is these very features that make plastic become an increasing threat to the environment, as discarded plastic waste is hardly degraded naturally, due to its resilience to biodegradation. In 2015, about 322 million tons of plastics have been produced with a rising global tendency. Plastics used in sectors, such as construction, medicine or science, stay in use for up to 40 years (Geyer et al., 2017), while plastics used for single-use packaging of mail or beverages often leave the "use-phase" after a short time. These materials then become problematic, as they cannot be reused for same-value purposes and often take several centuries to degrade naturally. Often, governments deal with excessive waste by shipping it to countries in Southeast Asia, primarily China (Velis C.A., 2014). China was the biggest importer of plastic waste until January 1st of 2018, when the Chinese government finally banned imports of different waste materials which were considered as health hazards (Brooks et al., 2018).

Plastic waste essentially faces three different destinations: storage, recycling, and incineration. The use of landfills falls into the first category. A detailed comparison of the countries using landfills has been made public (OECD Environment Statistics (database), 2015). Landfills pose many disadvantages: sunlight weakens the material and causes particle emissions that contaminate soil. However, little data on the effects of this contamination is available (Rillig, 2012). Moreover, plastic waste often gets dumped into the ocean, causing an ever increasing threat to the marine environment. Seawater animals often mistake plastic for food and eventually starve to death because their stomachs are filled with indigestible materials. If not properly handled, waste circles in the environment and eventually ends up in the food chain.

There are various forms of recycling. The term 'recycling' commonly refers to the use of used plastics for the same or less-value. This process delays the material from being incinerated or discarded, and can therefore not be considered a solution for the problem of increasing waste. In Europe, about 30% of all plastics are reused annually. According to a study from the European Environmental Bureau in 2017, Germany is at the top of the list with a recycling rate of 53%, while Austria reaches just above 48% (Eunomia Research & Consulting Ltd, 2017). Globally, among the countries with the highest plastic waste output, many of them have significantly lower recycling rates, bringing the global average of all plastics ever recycled down to 9%, of which only 10% have been reused more than twice (Geyer et al., 2017).

Incineration means heating up plastic waste and causing combustion with high efficiency, emitting ash, flue gas and heat. This heat can be used to provide energy, which is also considered a form of recycling (Eriksson and Finnveden, 2009). Energy-generating incineration of plastic waste also has a more efficient ratio of emitted greenhouse gases to produced energy than the burning of fossil fuels, and can be considered a more eco-friendly alternative. The volume of the incinerated waste decreases by more than 90%, which ultimately makes storage in landfills easier, but doesn't prevent it. Another form of incineration is pyrolysis, which describes heating of plastic waste and the subsequent separation of the heated gaseous phase and condensation in order to create useable fuels (Sogancioglu et al., 2017). The effects of incineration on the environment strongly depend on operation and design of incinerators. Incomplete combustion is difficult to prevent and leads to

emission of Dioxins, which represent a health hazard for humans (UNILABS Environmental, 2001; Verma et al., 2016). About 12% of all plastics ever produced have been incinerated, with or without energy recovery. About 4.9 billion metric tons of plastics are no longer in use and accumulate in landfills and in the natural environment ever since 1950 (OECD Environment Statistics (database), 2015).

In conclusion, plastic waste is and has been produced with increasing quantities each year, while little attention has been paid to responsible handling of the unwanted byproducts. Investigating new technologies that provide a way of closing the life cycle of plastic could lead to a sustainable and reproductive handling of synthetic polymers in the future. Ideally, all collected plastic waste is completely degraded into its basic components via an industrial process, which can then be reused to produce new synthetic polymers of the same or different composition.

1.2 Different kinds of plastics

322 million tons of plastic have been produced in 2015. Of those, about 150 million tons was polyalkenes, like polyethylene and polypropylene (PlasticsEurope, 2016). Of the remainder, 43 million tons was polyvinyl chloride (PVC), and 56 million tons were polyethyleneterephthalate (PET) (Bornscheuer, 2016), of which 18.8 million tons was non-fiber PET (PlasticsEurope, 2016), which was used mainly for packaging of beverages, while the remainder consists of PET fibers, commonly used in clothing, and known as Polyester. Since this work deals primarily with PET degradation, we will focus on the production and properties of PET in this chapter.

PET is formed by polycondensation, which is a form of step-growth-polymerization, at which a leaving group is split from the dimers and polymers as they form. The basic material for PET production is terephthalic acid (TPA) and ethylene glycol (EG). TPA is mixed with EG and a basic catalyst, like antimony(III)-oxide. The mixture is then melted to induce esterification. First reaction products are mono- and bis(hydroxyethyl)-terephthalate (MHET and BHET) and small oligomers. Excess glycol, as well as the leaving group are distilled and recovered. Then, in the polycondensation step, the reaction is continued under a vacuum, forming long chains of PET. The reaction happens at high pressure and temperatures of about 200°C. One molecule of water splits from each end of one molecule of terephthalic acid. The reaction mechanism is shown in Figure 1:

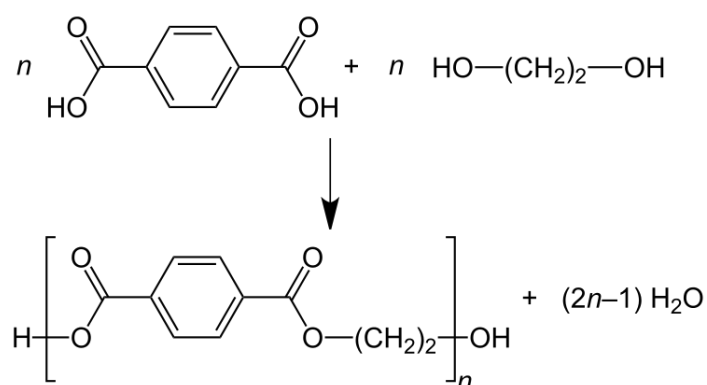


Figure 1: Esterification reaction of terephthalic acid and ethylene glycol, forming polyethylene terephthalate and water.

An outdated method to produce PET involves the use of dimethyl terephthalic acid and ethylene glycol. In this case, the leaving group is methanol (Michel, 1990). After polycondensation, PET is slowly cooled down to its crystallization temperature at which it can be formed and crystallized.

During the cooling process, byproducts like acetaldehyde, water, and EG are continuously distilled. Once PET passes the glass transition temperature, it becomes stable. An accurate depiction of transition temperatures is shown in Figure 2 in the form of a Differential Scanning Calorimetry (DSC) spectrum. DSC is a thermo-analytical method, which analyses the amount of heat required to keep a substance constantly at the same temperature. With increasing heat, the substance undergoes phase transitions which either require more heat (endothermic transition process), or set free heat (exothermic transition process). The amount of required heat either results in a positive or a negative peak of heat flow. Figure 2 shows the DSC spectrum of PET. The melting temperature (T_m) is indicated by an endothermic peak at 230-260°C. The crystallization temperature (T_c) is indicated as an exothermic peak and is found in between 140-160°C. The glass transition temperature (T_g) of crystalline PET is at about 80°C

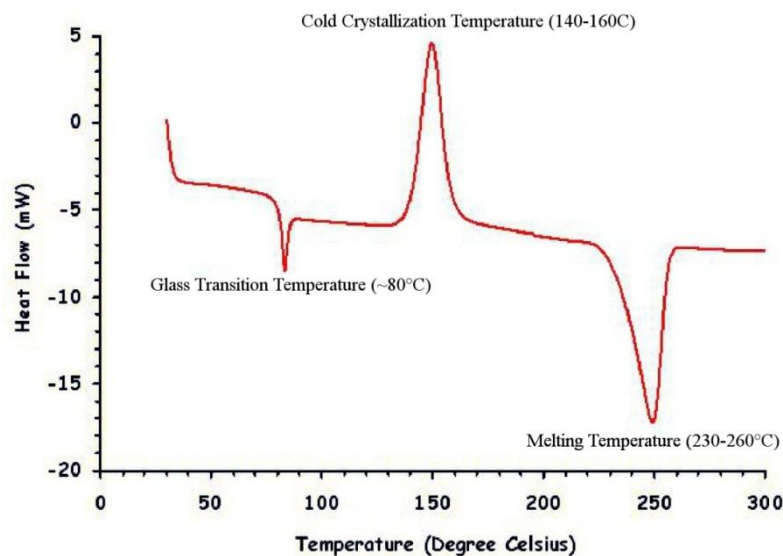


Figure 2: Differential Scanning Calorimetry spectrum of PET. (Demirel et al. 2011)

T_g is at 81°C for crystalline PET and at 67°C for amorphous PET (Demirel et al., 2011). Depending on later use, additives can be added during the production process to give PET more desirable features or to facilitate crystallization (Scheirs and Long, 2003).

Some methods exist for PET degradation, which usually require high temperatures and the use of catalysts (MMA Nikje and F. Nazari, 2009; Yue et al., 2011) and often include an irradiation step using microwaves. Methanolysis degrades PET into dimethyl terephthalic acid, which can be used for PET production (Michel, 1990).

Polybutylene succinate (PBS) is a crystalline, thermoplastic polymer with similar properties as polypropylene. It is produced from succinic acid and 1,4-butanediol, which can be derived from renewable sources. Furthermore, PBS is biodegradable by several microorganisms (Jarerat and Tokiwa, 2001; Kleeberg et al., 1998; Mergaert and Swings, 1996; Pranamuda et al., 1995; Tomita et al., 2000). It also naturally degrades over time, releasing H_2O and CO_2 (Xu and Guo, 2010). The structure of PBS and of poly-(butylene succinate)-co-adipate (PBSA) are shown in Figure 3.

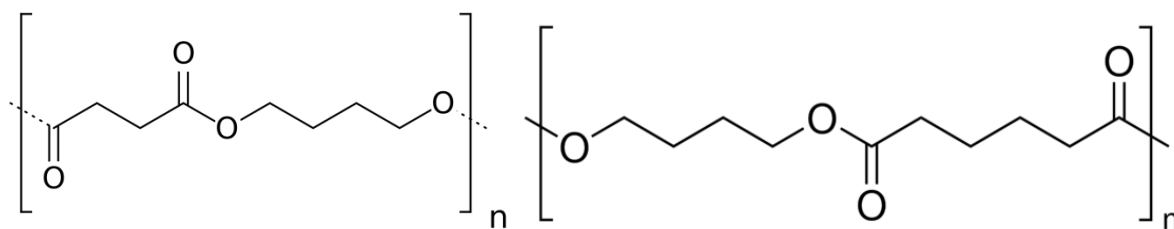


Figure 3: Molecular structure of PBS (left) and PBSA (right).

1.3 PET-hydrolysing enzymes

A small number of enzymes are capable of degrading PET. Several species of fungi and bacteria produce PET-hydrolyzing enzymes, for example *Glomerella cingulata* (Seman et al., 2014), *Fusarium oxysporum* (Dimarogona et al., 2015), , *Thermobifida alba* (Ribitsch et al., 2012), *Thermomonospora curvata* (Wei et al., 2014b), *Saccharomonospora viridis* (Kawai et al., 2014). Three of the most notable PET-hydrolyzing enzymes are *Thermobifida fusca* cutinase (Müller et al., 2005), Leaf and branch compost cutinase, which is a cutinase that has been discovered through a metagenomic screen of DNA from a leaf and branch compost (Sulaiman et al., 2012), and PET-hydrolase (“PETase”) from the recently discovered *Ideonella sakaiensis* (Yoshida et al., 2016). Yoshida tested PET-hydrolyzing activity of several enzymes and compared quantitative data as shown in Figure 4 and Figure 5:

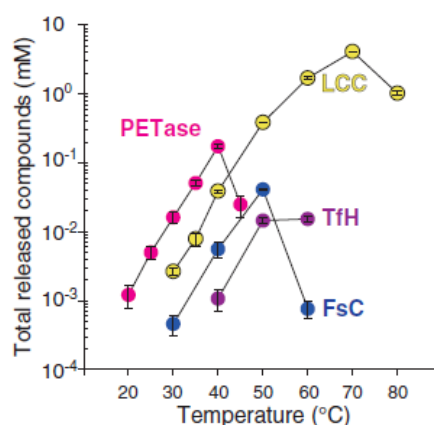


Figure 4: Comparison of PET-hydrolyzing activity of four different enzymes at different temperatures. A PET-film with 6 mm diameter was incubated for an hour with solutions of 50 nM *Ideonella Sakaiensis* PETase (pink) and 200 nM of each Leaf-branch compost cutinase (LCC, yellow), *Thermobifida fusca* hydrolase (TfH, purple) and *F. solani* cutinase (FsC, blue). Experiments were conducted in a pH 9.0 bicine NaOH buffer. (Yoshida et al., 2016)

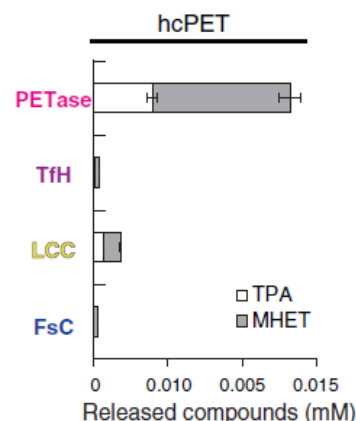


Figure 5: Comparison of PET-hydrolyzing activity of four different enzymes using a 6 mm diameter PET-film with high crystallinity. The PET film was incubated for 18 hours at 30°C with solutions of 50 nM *Ideonella sakaiensis* PETase and 200 nM of each Leaf-branch compost cutinase (LCC), *Thermobifida fusca* hydrolase (TfH) and *F. solani* cutinase (FsC), from a fungus in a pH 9.0 bicine NaOH buffer. (Yoshida et al., 2016)

From this data alone, it appears as if *Ideonella sakaiensis* PETase has the strongest potential activity, however lacking the thermostability required for efficient hydrolyzation of amorphous PET at its T_g (67°C). *Ideonella sakaiensis* uses PET as its main carbon source (Yoshida et al., 2016). It does this in two steps: first, it attaches itself to the substrate, using filamentous extensions, and then secretes the PET hydrolase, which degrades the PET substrate into shorter PET chains, bis-(hydroxyethyl-

terephthalate, mono-(hydroxyethyl)-terephthalate, and small amounts of terephthalic acid and ethylene glycol. These components are then transported into the cytoplasm of the cell, where another enzyme, MHET-hydrolase can further degrade BHET and MHET into TPA and EG (Yoshida et al., 2016). A schematic overview of PET degradation is shown in Figure 6.

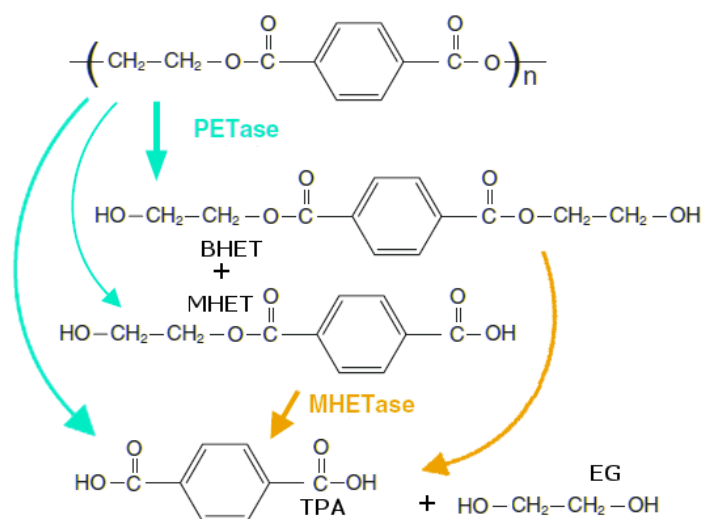


Figure 6: Schematic overview of PET-hydrolysis by the concerted efforts of PETase and MHETase of *Ideonella sakaiensis*. BHET: bis(hydroxyethyl)-terephthalate, MHET: mono(hydroxyethyl)-terephthalate, TPA: terephthalic acid, EG: ethylene glycol. PETase degrades PET by cutting one EG moiety from a TPA moiety, creating one EG-terminal end and one TPA-terminal end. Upon further catalysis, only BHET and MHET remain, which PETase cannot degrade any further. The second enzyme, MHETase then hydrolyses all remaining EG residues from BHET and MHET, releasing EG and TPA as end products.

In general, PET-hydrolyzing enzymes share an α,β -hydrolase structure, which comprises six α -helices that surround a central β -sheet, consisting of nine β -strands (Figure 7). PET-degrading enzymes usually have a catalytic triad consisting of serine, histidine and aspartic acid. The serine can be found within a characteristic motive, that comprises 5 residues: G_1, X_2, S_3, X_4, G_5 . The first and last of these residues are always glycine, while the middle residue is the serine of the catalytic triade. Residue X_2 is usually a histidine, while X_4 is always a methionine. In case of PETase and PBS-depolymerase, X_2 is a tryptophane (Danso et al., 2018).

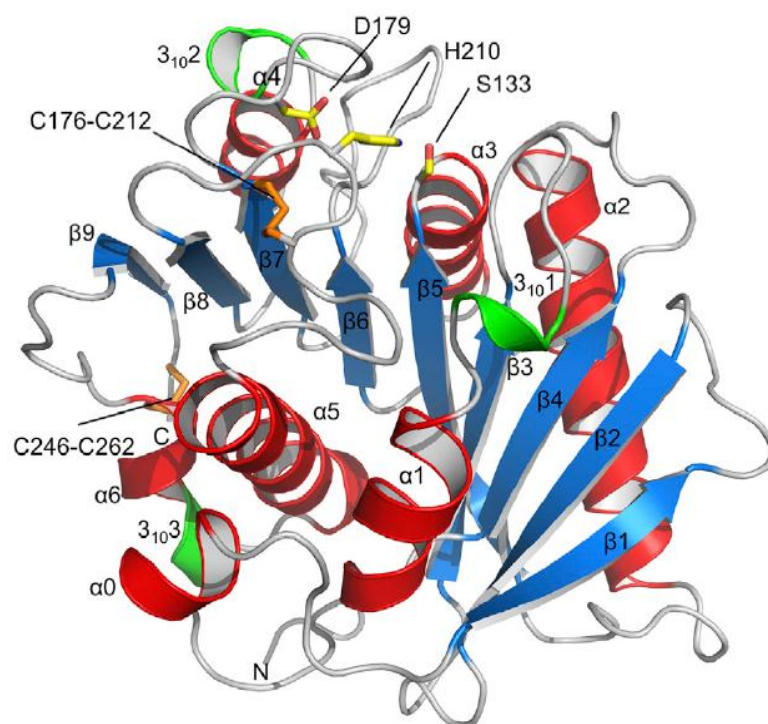


Figure 7: The α - β -hydrolase structure of PETase with secondary structure elements in blue (α -helices), red (β -strands) and green (3_{10} -helices). D179, H210 and S133 are part of the catalytic triad (shown as yellow sticks). The two disulfide bonds are shown as orange sticks. (Fecker, 2018)

Recent studies have investigated the structural differences between PETase and other enzymes that are capable of PET degradation, and found two distinct differences (Han et al., 2017; Joo et al., 2018; Liu et al., 2018; Chen et al., 2018; Fecker et al., 2018). The disulfide bond that connects $\alpha 7$ to $\beta 9$ is present in many PET-hydrolyzing enzymes. The second disulfide bond, which brings the catalytic histidine and aspartic acid closer together, is a feature unique to a small group of PET-hydrolyzing enzymes (Figure 8). This specific disulfide bond is required for catalytic functionality of the enzyme, since an extended loop near $\alpha 6$ would cause the elements of the catalytic triad to be too far apart from each other. Substitution of a cysteine, in order to disrupt this disulfide bond, results in a loss of 95% – 100% of activity (Chen et al., 2018; Han et al., 2017; Joo et al., 2018; Liu et al., 2018). The second distinct feature is the broader substrate binding cleft. In PETase, the residue W158 is able to exhibit various conformations (Han et al., 2017). Current opinion is that the flexible tryptophane facilitates substrate binding (Chen et al., 2018; Han et al., 2017). The reason for the increased flexibility of W158 compared to other PET-hydrolyzing enzymes is the substitution of H187 for a serine residue. The smaller serine leaves more space for W158 to change its conformation.

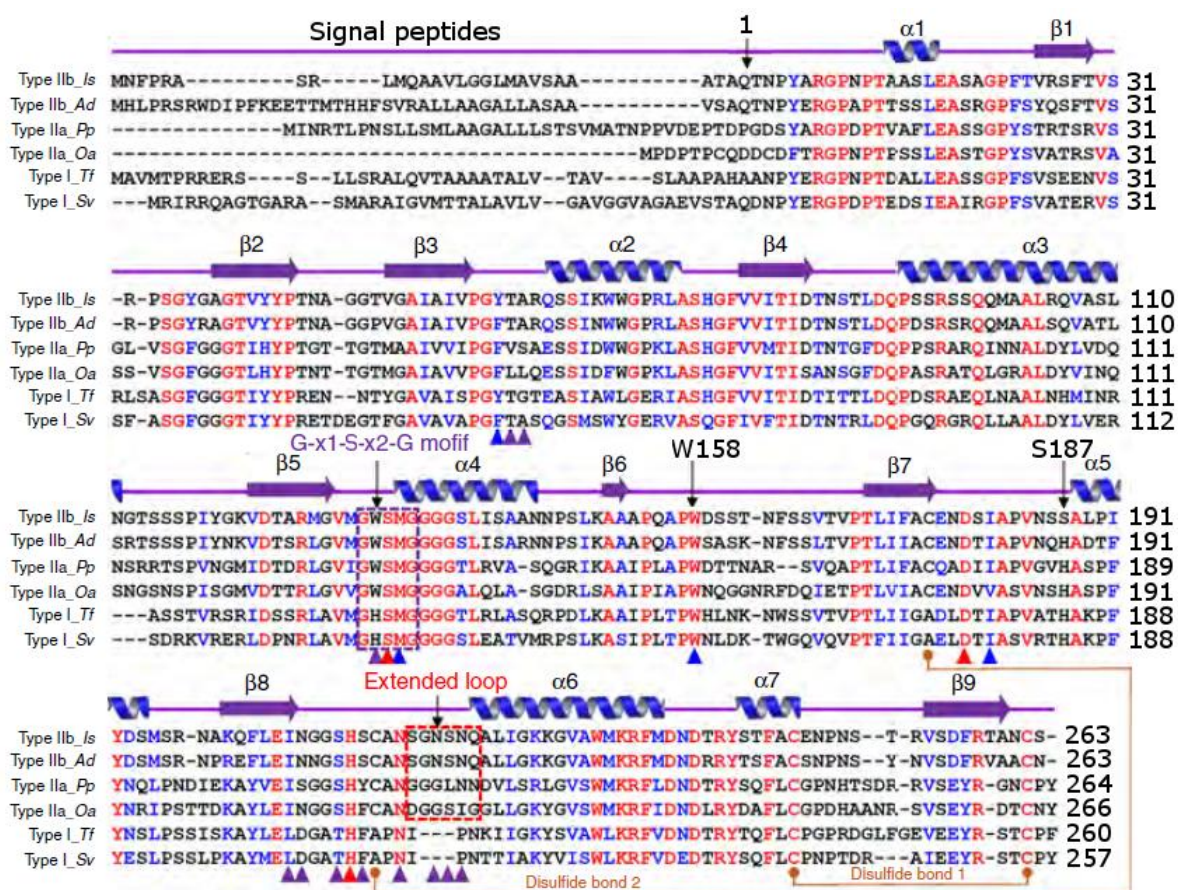


Figure 8: Amino acid sequence alignment of various PET-degrading enzymes of. The abbreviations represent the following species: Is: *Ideonella sakaiensis*, Ad: *Acidovorax delafieldii*, Pp: *Pseudomonas pseudoalcaligenes*, Oa: *Oleispira antarctica*, Tf: *Thermobifida fusca*, and Sv: *Saccharomonospora viridis*. The Gly-x1-Ser-x2-Gly motif is represented as a purple color box. The extended loop is represented as a red color box. Residues involved in enzyme catalysis and constitution of subsite I and subsite II are indicated by red-, blue- and purple-colored triangles, respectively. Disulfide bonds are marked as orange-colored lines. Source: Original Graphic taken from Joo et al., 2018. Amino acids were numbered starting from the cleavage point of the signal peptide of PETase by *I. sakaiensis* and PBSase by *A. delafieldii*, marked with "1".

In terms of industrial application, the major advantage of PETase over other PET-hydrolyzing enzymes is the strong activity at 30°C (Figure 4). PETase's low thermostability however impedes the enzyme's capability of hydrolyzing PET at higher temperatures. The PET-hydrolyzing activity of enzymes that operate at 60 - 70°C increases exponentially with rising temperature. In a recent publication, the activity of PETase against low- and high crystallinity PET film of 6 mm was determined and compared to the PET hydrolyzing enzymes TfCut2 and LCC (Furukawa et al., 2018). The amount of released compounds after incubation of IcPET film with PETase at 30°C for 3 hours was at 0.07 nmol min⁻¹ cm⁻². In contrast to this, TfCut2 produces 2.2 nmol min⁻¹ cm⁻² at its temperature optimum of 60°C, and LCC produces 51 nmol min⁻¹ cm⁻² at 70°C. At 30°C PETase achieved the highest activity (Yoshida et al., 2016). PETase's high activity at 30°C is accounted to W158 being able to change its conformation freely.

A recent paper stirred some controversy; two residues in the substrate-binding cleft have been "back-mutated", making it narrower and more like in the *Thermobifida fusca* cutinase (Austin et al., 2018). Surprisingly, the loss of crystallinity on a PET film has increased after incubation with PETase at 30°C, compared to normal PETase. Although this is indeed an interesting discovery, it should be noted that the output of BHET, MHET, TPA and EG did not change after incubation with this mutant protein. In 2002, the gene *pbsA* from *Acidovorax delafieldii* was described (entrynumber Q8RR62,

UniProtKB). It encodes a PBS-depolymerase ("PBSase"), which incidentally is the closest relative to PETase with a nucleotide sequence similarity of 81%. In sequence analysis, it shows the same characteristic extended loop with the additional disulfide bond, the same GWSMG-motive surrounding S133 and the exact same amount of amino acids for the mature protein. The signal peptide is 41 amino acids long in PBSase and 27 amino acids in PETase. However, despite the fact that PBSase was discovered in 2000, it has only been described in two publications up until delivery of this work (Uchida, 2000; Uchida et al., 2002). Uchida et al. have synthesized PBSase in its native host and isolated the enzyme successfully. Several kinetic experiments were performed using live *Acidovorax delafieldii*, using both emulsified and solid PBS and PBSA as substrate (Uchida, 2000). The bacterium was capable of degrading PBS and PBSA. A year later, Uchida managed to prove expression of PBSase in an *E. coli* strain (Uchida et al., 2002). However, in both publications, no quantifiable data has been obtained regarding the catalytic activity or thermostability of PBSase. If PBSase were more thermostable than PETase, the enzyme could help with future protein engineering experiments that aim at increasing thermostability of PETase. The goal of this work was the characterization of *Acidovorax delafieldii* PBS-depolymerase, specifically in regard to PET-hydrolysis and thermostability.

1.4 Heterologous gene expression in *E. coli*

In order to study a new protein, it has to be synthesized and isolated. Using the native host for this often causes problems, as it often is an unstudied organism with various unknown features that contribute to protein synthesis and/or proper folding. Therefore, researchers often tend to use well studied model organisms to synthesize a new protein. When preparing heterologous protein expression, various factors have to be considered: The host has to be capable of post-translational modifications on the polypeptide chain in the same manner as the native host (Glick, 1995); the transformed gene has to be modified with regard to codon bias (Gustafsson et al., 2004); the model organism should be easy to handle and produce large amounts of protein. Different bacterial model organisms are available for heterologous protein synthesis.

Escherichia coli, a Gram-negative coli bacterium, is to date the best-characterized bacterium. It has a short generation time and grows to high numbers in a short time, making it a valid choice for attempts to synthesize foreign proteins. However, despite its short generation time and easy handling, *E. coli* has a few distinct disadvantages, that have to be considered when attempting heterologous gene expression: *E. coli* is incapable of adding post-translational modifications to polypeptide chains, which are commonly required for most eukaryotic proteins. It has no means for extracellular secretion, and it does not support the formation of disulfide bonds in its cytoplasm.

The growth of *E. coli* in medium can be divided in four essential phases, as shown in Figure 9. Usually, the medium is inoculated with cells from an overnight culture. Such overnight cultures have little nutrients left in the medium, and a high cell density. Therefore, the cells have down-regulated their metabolism to achieve less growth and therefore higher survivability in this stressful environment. Right after inoculation of the fresh medium, the cells need some time to adjust their metabolism to the nutrient-rich medium ("lag-phase"), after which they accelerate their generation time to its maximum, which defines the exponential growth phase ("log-phase"). During this phase, the growth rate always exceeds the death rate. This exponential growth is limited by the amount of nutrients in the medium and slowed down by the increasing amount of toxic byproducts from the bacterial metabolism that accumulate over time, until the growth rate equals the death rate, which can be

described as the “stationary phase”. During this time nutrients in the medium start to deplete, causing the growth rate to shrink, while waste products keep accumulating until the medium is toxic enough for the death rate to rise above the growth phase. The cell count decreases (“death phase”) until the remaining viable cells go into the long-term stationary phase, when it feeds on released nutrients from dead cells. In this phase, bacterial titer increases and decreases several times and the population remains viable for weeks (Pletnev et al., 2015).

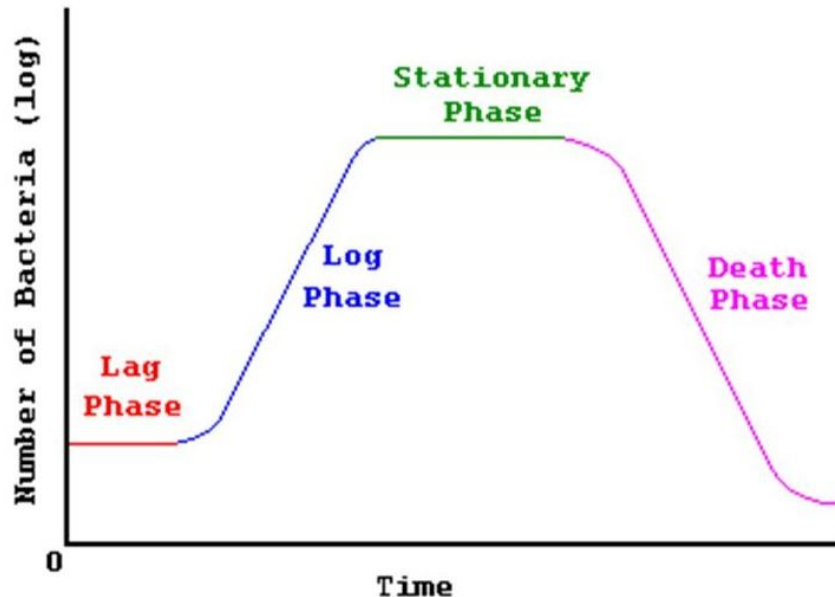


Figure 9: Bacterial growth curve.

Prior to heterologous gene expression, the gene of interest has to be cloned into an expression plasmid, which is a small, circular strand of DNA. Expression conditions have to be tailored to support expression of a particular gene. Multiple different expression plasmids are available as well as mutated strains that provide different expression conditions for this purpose. A plasmid has to comprise certain elements. First, a plasmid cannot be proliferated without an origin of replication (ORI). Furthermore, plasmids do not convey any specific advantages to the bacteria; on the contrary, expression of the gene of interest puts metabolic stress on the cells. Therefore, plasmids are usually degraded after a short time. In order to force bacteria to keep a plasmid undegraded, a resistance gene for an antibiotic also has to be present on the plasmid. The antibiotic is later added to the expression medium. Finally, genes are easily cloned into a plasmid by cutting the plasmid at specific restriction sites. Many different restriction enzymes are used for this. Therefore, a plasmid has to comprise a multiple restriction site, which is located downstream of the T7-promoter. The T7-promoter is about five times stronger than the equivalent *E. coli* RNA-polymerase, and it is very specific for T7-polymerase, a DNA-dependent RNA polymerase, that is a derivative of the phage T7. Usually, commercial *E. coli* strains already carry a gene that encodes the T7-polymerase (these strains carry the abbreviation “DE3” in their names). The gene that encodes T7-polymerase is under control of a L8-UV5 lac-promoter, which is derived from a normal lac-promoter, and is usually induced by addition of a galactose-analog called Isopropyl- β -D-1-thiogalactopyranoside (IPTG). The L8-UV5 lac promoter has three mutations compared to a wild-type lac promoter: two in the -10 region, which strengthen binding of the promoter to transcriptional elements, while the third mutation is in the -35 -region and cancels the promoter’s sensitivity to glucose. This way, expression efficiency will not

decrease when the cell suffers stress. Synthesis of T7-polymerase is induced by adding IPTG to the expression medium, which in turn encodes for the gene of interest on the transformed plasmid. Over-expression puts heavy metabolic pressure on the bacteria, as it can amount to up to 50% of all proteins in the cell. Expression of the resistance gene also takes away resources from other metabolic pathways. Also, pET-plasmids often amount to more than 15 copies, of which several get lost during cell division. Maintenance of these plasmids, expression of the resistance gene, and basal transcription of T7-polymerase are factors that slow the bacteria's growth. Therefore, usually induction is performed shortly after the beginning of the exponential growth phase, or even a bit later (Glick, 1995).

Heterologous expression in general means expressing a protein in an environment that differs from its host environment (Rosano and Ceccarelli, 2014). A change in pH, osmolarity, or redox-potential could influence protein folding; specific chaperones or co-factors might be missing that are crucial for proper folding. When unfavorable conditions are met, proteins often fail to fold and the free polypeptide chains entangle themselves with one another and aggregate, forming so-called inclusion bodies. These are depots of insoluble protein that are often not folded, or misfolded (Sørensen and Mortensen, 2005). Therefore, when expressing an unknown protein in *E. coli*, the protocol that works best has to be established through trial and error. Inclusion body formation can be reduced by lowering the expression speed; this can be achieved by using a weaker promoter, lower temperatures, or less inducer. Co-expression of chaperones like GroES-GroEL can be considered (Hayer-Hartl et al., 2016).

Folding of proteins with disulfide bonds is barely possible in the reducing environment of the cytoplasm of *E. coli*. However, the periplasm has an oxidizing environment, as well as two oxidases, disulfide oxidoreductase (DsbA) and disulfide isomerase (DsbC), that catalyze the formation and isomerization of disulfide bonds (Berkmen et al., 2005). Secretion of a polypeptide chain into the periplasm usually is achieved by fusing it to a leader sequence *pelB* or *OmpT*. This however reduces yield of maximum properly folded protein significantly.

Formation of inclusion bodies can also be prevented by usage of a different expression strain. In the reducing environment of *E. coli* cytoplasm, the formation of disulfide bonds is repressed, and proteins that form disulfide bonds do not fold properly. The strain SHuffle T7 Express is capable of producing properly folded proteins with multiple disulfide bonds (Lobstein et al., 2012).

Mutating thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) results in nonviable cells because essential proteins rely on the reducing capabilities of the mutated enzymes. Therefore, a mutation in the gene encoding for the peroxidase *AhpC* is required to grant reducing power to the cell. Additionally, the oxidizing power of thioredoxins often causes misfolded proteins if more than one disulfide bond is to be formed. The strain Origami does not take this effect into account. The isomerase DsbC, also predominantly present in *E. coli* periplasm, is capable of refolding such misfolded proteins (Berkmen et al., 2005). By co-producing the isomerase DsbC without its signal peptide, DsbC remains in the cytoplasm and rescues misfolded proteins, which increases the yield. Also, like most BL21 strains, SHuffle T7 Express lacks two proteases, *Lon* and *OmpT*, which would degrade the newly produced foreign protein.

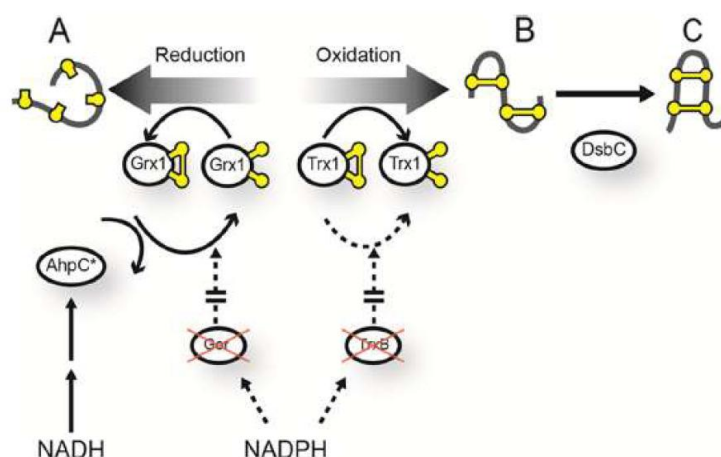


Figure 10: Display of redox-pathways in SHuffle T7 Express. Cystein residues are shown as yellow balls; disulfide bonds are shown as yellow sticks. Reducing and oxidizing pathways are marked as black arrows. Inhibited reducing pathways are marked as dashed arrows. The mutated *gor* and *TrxB* allow the formation of disulfide bonds in the cytoplasm. (A) The mutated AhpC is able to reduce Grx1, which restores the reducing power required for essential proteins. (B) Trx1 is not reduced anymore by TrxB and therefore is able to help the formation of disulfide bonds by oxidizing, which also leads to misfolded proteins. (C) The isomerase DsbC refolds proteins. (Lobstein et al., 2012)

1.5 Enzyme characterization

In order to understand the potential of an enzyme's catalytic mechanism, its specific activity against its main substrate (preferably also against various other substrates) has to be determined. An enzyme might not show its full potential against the substrate it was initially linked to, and could exhibit higher activity against a different substrate. Enzyme activity can be measured by following either the decrease of substrate, or the increase of product. This can be done by measuring absorbance values of samples at an adequate wavelength; measuring the amount of acid or base that is titrated in order to maintain a certain pH-level; or by measuring the weight of a precipitate, obtained at distinct time points. In this work, all measurements of enzyme activity have been absorbance measurements. Measured absorbance is converted into enzyme activity (U/ml) using the Lambert-Beer law:

$$A = \epsilon * c * d$$

Whereas A is the measured absorbance, ϵ is the molar extinction coefficient in $\text{L} * \text{mmol}^{-1} * \text{cm}^{-1}$, C is the concentration of the absorbing substance in mol/L and d is the thickness of cuvette in cm.

By dividing enzyme activity by the protein content, specific activity against a substrate of a certain concentration is obtained. The enzyme's specific activity can be further characterized by repeating these measurements with various substrate concentrations, and fitting the resulting data for specific activity into a Michaelis Menten model.

Interest in PETase has mainly been motivated by its potential for industrial application for sustainable waste disposal, or "total recycling" of waste PET, which is mainly present in a low- or high crystallinity conformation. Therefore, PETase has been previously characterized mainly with the use of 6 mm thick PET films as substrate (Austin et al., 2018; Fecker et al., 2018; Furukawa et al., 2018; Han et al., 2017; Joo et al., 2018). Proper characterization however sometimes requires the substrate to be in a different conformation, which provides a larger surface area for enzymes to adhere. Therefore in this

work, PET nanoparticles have been used to determine PET-degrading activity of enzymes instead of PET films.

1.6 Current knowledge about PETase and PBSase

PETase can be generally described as an aromatic polyesterase rather than solely a PET-hydrolase, since it is also capable of degrading PEF (Polyethylenefuranoate), a non-fossil fuel based polymer (Austin et al., 2018). Austin states that PETase will become easily modifiable to not only degrade PET, but also a broad spectrum of aromatic polyesters. Sadly, PETase is unable to degrade aliphatic polyesters such as PBS and Polylactic acid (PLA) (Austin et al., 2018).

Recently, a group incubated PET-film with anionic surfactants (Furukawa et al., 2018), which increased the enzyme's activity against a PET-film about 122-fold. In a preceding work, specific activity of PETase on the substrate *para*-nitrophenylacetate (*p*NPA) was determined to be 4.77 U/mg, when using a substrate concentration of 1 mM *p*NPA (Walczak, 2017). A recent publication presented different numbers: PETase's specific activity towards 1 mM *p*NPA was determined to be 14 U/mg. After fitting the data into a Michaelis Menten model, Fecker et al. calculated the theoretical maximum specific activity of PETase against *p*NPA to be at about 48 U/mg, when using a substrate concentration of above 11 mM (Fecker et al., 2018), Galaz-Davison, P., personal communication) the parameters obtained for the Michaelis Menten model were such: $V_{\max} = 31.3$ U and $K_m = 2.6$ μ g substrate. 25 nM of enzyme were used for each measurement (Fecker et al., 2018), supplementary material).

Uchida performed degradation experiments on both PBS and PBSA, using the strain BS3 (Uchida, 2000). The strain was able to use emulsified PBS and PBSA, as well as solid PBSA as its main carbon source, but was unable to grow on solid PBS. Activity of up to 3.5 U was observed when using solid PBSA. The isolated PBS-depolymerase was able to degrade PBS. Moreover, it was stated that PBSase was unable to degrade two other polyesters: poly(3-hydroxybutyric acid)-co-(4-hydroxybutyric acid) and poly(L-lactic acid). In a second publication, Uchida produced PBSase in *E. coli* strain JM109 and analyzed the sequence of the gene *pbsA*. The enzyme does not have the H187S substitution that is thought to account for PETase's highly flexible substrate binding cleft and comparably high activity at 30°C.

Carmen Walczak previously attempted to synthesize PBSase, using the strains Codon Plus RIPL, which was used by Yoshida and Liu (Liu et al., 2018; Yoshida et al., 2016), Arctic Express RIL, Rosetta gami B, which was used by Joo (Joo et al., 2018)), and SHuffle T7 Express. Synthesis of PETase proved to be detrimental to cell growth. This effect was remedied by removing the sequence encoding for the signal peptide by PCR. While synthesis of PETase was successful in many strains, always resulting in reasonably large amounts of soluble protein, synthesis of PBSase resulted in functional enzyme only when expressing the PBSase gene in cells of the strain SHuffle T7 Express (Walczak, 2017).

2. Aim

With more and more knowledge about plastic-degrading enzymes accumulating, the concept of plastic biodegradation becomes a reasonable long-term-solution to deal with the global problem of plastic pollution. However, a method for PET-degradation involving PET-degrading enzymes has not been developed so far, in part because an enzyme with sufficient catalytic activity and thermostability was not available. Protein engineering has already been attempted in order to widen the substrate binding cleft of various PET-hydrolyzing enzymes (Araújo et al., 2007; Silva et al., 2011; Wei et al., 2016). However, the respective enzymes were still dependent on high temperatures for PET degradation. PETase surpasses other PET-degrading enzymes in their PET-hydrolyzing activity at 30°C, but loses its activity at higher temperatures, since it has a melting temperature of 46-48°C (Austin et al., 2018; Joo et al., 2018). A PETase mutant with increased thermostability that enables the enzyme to function at temperatures of 70°C could have a high potential activity. Thermostability of a protein can be increased via various protein engineering techniques (Cohen, 2001). The goal of this work was to provide a foundation to future protein engineering approaches, using PETase and its closest relative PBSase. As there is next to no quantitative data available for PBSase, this work focused on characterizing its catalytic activity and thermostability. Additionally, the enzyme would be compared to PETase in regard to activity and thermostability against *para*-nitrophenylacetate and against a PET nanoparticle solution.

3. Results

3.1 Production of PBSase and PETase in various expression strains

Previously, PBSase has been successfully synthesized in SHuffle T7 Express (Walczak, 2017), but yield was very low. Therefore, in this study, in addition to SHuffle T7 Express, additional expression hosts were drawn into consideration for the attempted synthesis of PBSase. The genes encoding for PETase (GenBank accession number, GAP38373.1) and PBSase (GenBank accession number, BAB86909.1) were commercially synthesized with codon optimization for expression in *E. coli* cells (GenScript). Both genes were present in pET21b plasmids. The PETase-gene was present without the sequence that codes for the protein's native signal peptide. Negative effects on cell growth were observed in the culture that synthesized PBSase (Walczak, 2017). The removal of the signal peptide solved this issue. PBSase, encoded by the shortened gene will be referred to as "mature PBSase", while PBSase that contains the native signal peptide will be referred to as "full length PBSase". After each expression attempt, the cells were lysed, the lysates were centrifuged in an ultracentrifuge, and the cleared supernatants were analyzed via SDS-PAGE and via esterase-assays, to confirm the presence of soluble and insoluble protein. Obtained PETase- and PBSase samples were subjected to esterase-assays, PET-degradation assays, and Differential Scanning Fluorimetry (NanoDSF) experiments. In Table 1, the different combinations of strains and plasmids that were employed to synthesize PBSase and PETase are shown. In some cases, different induction times and concentrations of IPTG were tested.

Table 1: Strains of *E. coli* that served as expression host for mature PBSase and full length PBSase. pET21b expression vectors include a T7 promoter and a C-terminal histidine tag. pET22b expression vector includes a N-terminal pelB-signal sequence and a C-terminal histidine tag. Performed experiments are marked with a ✓

Strain	Conditions	Mature PBSase		PBSase	PETase
		pET21b	pET22b(pelB)	pET21b	pET21b
<i>E. coli</i> BL21(DE3) C41 Overexpress	LB, induction at OD 1	✓	-	-	-
<i>E. coli</i> BL21(DE3) C43 Overexpress	LB, induction at OD 1	✓	-	-	-
<i>E. coli</i> T7 Express LysY/lq	LB, induction at OD 1	✓	-	-	-
<i>E. coli</i> BL21(DE3)	LB and TB, induction at OD 1	-	✓	-	-
<i>E. coli</i> SHuffle T7 Express	LB and TB, early and late inductions, using 1 mM, 2 mM, 6 mM, 12 mM IPTG	✓	-	✓	✓

3.1.1 Synthesis of PBSase without its native signal peptide, using *E. coli* BL21 C41(DE3) and C43(DE3)

When expression of heterologous genes is induced in *E. coli*, cells are often affected by an unforeseen toxic effect. While this effect is more commonly true for membrane proteins, it does not exclude globular proteins (Miroux and Walker, 1996). Miroux and Walker originally described the “Walker strains” C41(DE3) and C43(DE3) which have been used to synthesize proteins which were toxic to wild type cells. Also, a variety of proteins that cannot be synthesized in *E. coli* BL21(DE3), were synthesized in Walker strains. Finally, overall yield of proteins that were known to be toxic increased when synthesized in these strains. Later studies have revealed a mutation in the *lacUV5* promoter to be the cause for this phenotype, as synthesis of T7 RNA polymerase was dampened, resulting in a much slower production of the protein of interest (Wagner et al., 2008).

PBSase might be toxic to *E. coli* cells and its synthesis could be repressed in *E. coli*, as described by Miroux and Walker (Miroux and Walker, 1996). The Ability of C41 to produce PETase has been demonstrated previously (Austin et al., 2018). Therefore, synthesis of mature PBSase was attempted in C41(DE3) and C43(DE3) cells.

For each cultivation, 50 ml of LB-medium in a 250 ml baffled flask containing 100 µg/ml ampicillin was inoculated with C41(DE3) and C43(DE3) cells, each carrying pET21b-plasmids with the coding sequence for mature PBSase. The cells were incubated at 33°C until they reached an optical density at 600 nm (OD_{600}) of 1, at which point gene expression was induced by adding IPTG to a final concentration of 1 mM. Induction had no effect on the growth of both strains (supplementary Figures S1, S2). Samples were withdrawn from the culture at specific time points (“time samples”). After six hours of continuous growth, the expression temperature was decreased to 16 °C. After 18 hours of expression at 16°C, the cells of both cultures were harvested and disrupted by sonication in 50 mM sodium phosphate buffer. Soluble and insoluble fractions were separated from each other by centrifuging at 17 000 g. SDS-PAGE analysis of supernatants and insoluble fractions of these time samples revealed no significant bands at a molecular size of 29 kDa in either strain. pNPA-hydrolysis assays were performed using 50 µl of undiluted cleared lysate. No significant activity was measured, therefore it appears that strains C41(DE3) and C43(DE3) failed to synthesize mature PBSase, and no further experiments were conducted using these strains.

3.1.2 Synthesis of PBSase without its native signal peptide, using *E. coli* T7 Express LysY/Iq

The introduction of the natural inhibitor of T7 RNA-polymerase, T7 lysozyme, into the genome of *E. coli* represses basally transcribed T7 RNA-polymerase and mimics the phenotype of C41(DE3) and C43(DE3) (Wagner et al., 2008). By adding the lacIq-repressor gene as an additional control feature, basal expression is even further repressed, providing the highest possible control over the synthesis of T7 RNA polymerase. The ability of a strain called T7 Express lysY/Iq to express PETase has been previously demonstrated in 2016's iGEM competition by the Harvard team (iGEM, 2016).

As synthesis of mature PBSase failed in the strains C41(DE3) and C43(DE3), T7 Express LysY/Iq was used for the next expression attempt. 50 ml of LB-medium in a 250 ml baffled flask containing 100 µg/ml ampicillin was inoculated with T7 Express LysY/Iq cells, each carrying the pET21b-plasmid with the coding sequence for mature PBSase. The cells were incubated at 33°C until they reached an OD₆₀₀ of 1, at which point gene expression was induced by adding IPTG to a final concentration of 1 mM. Induction did not affect growth of the culture (supplementary Figure S3). Samples were withdrawn from the culture at specific time points ("time samples"). After six hours of continuous growth, the expression temperature was decreased to 16 °C. After 18 hours of expression at 16°C, the cells were harvested and disrupted by sonication in 50 mM sodium phosphate buffer and soluble and insoluble fractions were separated from each other by centrifuging at 17 000 g. SDS-PAGE analysis of supernatants and insoluble fractions of these time samples revealed no significant bands at a molecular size of 29 kDa. pNPA-hydrolysis assays were performed using 50 µl of undiluted cleared lysate. No significant activity was measured, therefore it appears that T7 Express LysY/Iq failed to synthesize mature PBSase, and no further experiments were conducted using this strain.

3.1.3 Synthesis of PBSase containing its native signal peptide, using SHuffle T7 Express

As SHuffle T7 Express has previously been successfully employed as expression host to synthesize PETase, and PBSase, the strain was used again for a new line of experiments. It is a common habit among researchers working with PETase, to express the enzyme without its native signal peptide (Austin et al., 2018; Fecker et al., 2018; Han et al., 2017; Joo et al., 2018; Liu et al., 2018). So far, no negative consequences have been derived from this. However, it was hypothesized that in the case of PBSase, the signal peptide could play a role in folding. Therefore, synthesis of PBSase containing its native signal peptide (full length PBSase) was attempted in SHuffle T7 Express. 50 ml of LB-medium in a 250 ml baffled flask containing 100 µg/ml ampicillin was inoculated with SHuffle T7 Express cells, carrying the pET21b-plasmid with the coding sequence for full length PBSase. The cells were incubated at 33°C until they reached an OD₆₀₀ of 1, at which point gene expression was induced by adding IPTG to a final concentration of 1mM. From this point onward, growth was carefully monitored via spectrophotometric measurements, in order to detect even small disturbances in exponential growth, and samples were withdrawn from the culture. Judging from absorbance data at an OD of 600 nm, growth was not affected in any way. However, after retrieving stored samples from overnight storage, the cells of “time samples” that were taken at least 30 minutes after induction of gene expression refused to suspend in cold 50 mM sodium phosphate buffer, and aggregated to form a precipitate. These cells were considered dead, presumably as a result of the successfully synthesized full length PBSase. After 18 hours of expression at 16°C, the cells of both cultures were harvested and disrupted alongside time samples by sonication in 50 mM sodium phosphate buffer and soluble and insoluble fractions were separated from each other by centrifuging at 17 000 g. SDS-PAGE analysis of supernatants and insoluble fractions of time samples revealed no significant bands at a molecular size of 29 kDa. pNPA-hydrolysis assays were performed using 50 µl of undiluted cleared lysate. No significant activity was measured.

Two important revelations came from this experiment: The attempt to synthesize full length PBSase had a clear negative effect on the viability of cells; and because of this, synthesis of full length PBSase was considered successful in SHuffle T7 Express, albeit no protein bands were observed on an SDS-gel, and no esterase activity was measured. Therefore, synthesis of full length PBSase was again attempted using this strain as expression host. In order to increase yield of obtained PBSase, the time of induction was set to a later point, when the culture would have reached a higher cell count. For this purpose, gene expression in a culture of SHuffle T7 Express cells was induced after cell count reached an OD₆₀₀ of 6, by adding IPTG to a final concentration of 1 mM. The amount of inducer was not sufficient to induce gene expression. Therefore, for the next experiment, the final concentration of IPTG was matched to the OD in a ratio

$$\Delta OD_{600} : 1 \text{ mM IPTG}$$

whereas ΔOD_{600} is equal to the current OD₆₀₀ of the culture.

Three times 200 ml TB in 1L baffled flasks containing 100 µg/ml ampicillin were inoculated with SHuffle T7 Express cells, of which two carried plasmids that encoded for full length PBSase; and the last medium was inoculated with transformants that carried the “empty” pET15b plasmid. This culture served as negative control.

Cells were incubated at 33°C. Gene expression of the two expression cultures was induced at an OD₆₀₀ of 1 for one culture, and at an OD₆₀₀ of 6 for the other. Gene expression of the cells that carried the empty pET15b plasmid was also induced at an OD₆₀₀ of 6. Accidentally, double the amount of

IPTG was added (2 mM and 12 mM final concentration). The media of the “later” induced cultures were exchanged prior to induction, by centrifuging at 4°C with 4000 g for 30 minutes. Then the media were discarded, and the cultures were suspended in fresh media of the same composition. A high concentration of inducer can influence folding. Therefore only a small yield of functional PBSase was expected to be obtained from this cultivation. Nonetheless, the experiment proceeded as planned. The addition of 12 mM IPTG visibly affected growth of the expression culture, as it grew to just above an OD₆₀₀ of 12, compared to the culture that carried the pET15b plasmid, which grew to an OD₆₀₀ of above 20 (Figure 11). Samples were withdrawn from both expression cultures at specific time points (“time samples”). After 16 hours of expression at 16°C, the cells of both cultures were harvested. Harvested cells and cells in time samples exhibited the same behavior as described above: an inability to suspend in cold 50 mM sodium phosphate buffer and the formation of a precipitate. Cells were disrupted by sonication in 50 mM sodium phosphate buffer and soluble and insoluble fractions were separated from each other by centrifuging at 17 000 g. SDS-PAGE analysis of supernatants and insoluble fractions of these time samples revealed bands with high intensities at a molecular size of 29 kDa for the insoluble fractions of every time sample. Band intensity increased heavily after induction, showing a strong basal transcription of the PBSase gene. The soluble fractions appeared to not contain any soluble PBSase. These results suggest successful synthesis of misfolded PBSase, despite a concentration of 12 mM IPTG in the expression medium. *p*NPA-hydrolysis assays were performed using 50 µl of undiluted cleared lysate. Of the two expression cultures, the culture whose expression was induced at an OD₆₀₀ of 6 showed three times the activity of the culture whose expression was induced at an OD₆₀₀ of 1 (data not shown). The soluble fraction containing His-tagged PBSase was loaded onto a column of Roti®garose-His-beads, in order to purify PBSase. Fractions obtained from purification were analyzed via SDS-PAGE, and showed successful synthesis and purification of a soluble protein of a molecular size equal to the size of PBSase (Figure 12B). Protein content of the last two fractions depicted in Figure 12B, was determined by Nanoquant-assay to be 38 µg/ml in total, and specific activity of the purified enzyme against *p*NPA was determined to be 0.8 U/mg. The role of the native signal peptide of PBSase in proper folding of the enzyme could not be discerned in this experiment. However, as the synthesis of mature PBSase in a simultaneously conducted experiment showed more promising results in terms of yield of functional protein, further expression experiments aiming at the synthesis of full length PBSase were not conducted. The obtained soluble protein was further subjected to experiments aiming at characterizing its thermostability and ability to degrade PET-nanoparticles (chapter 3.2.1, 3.2.2).

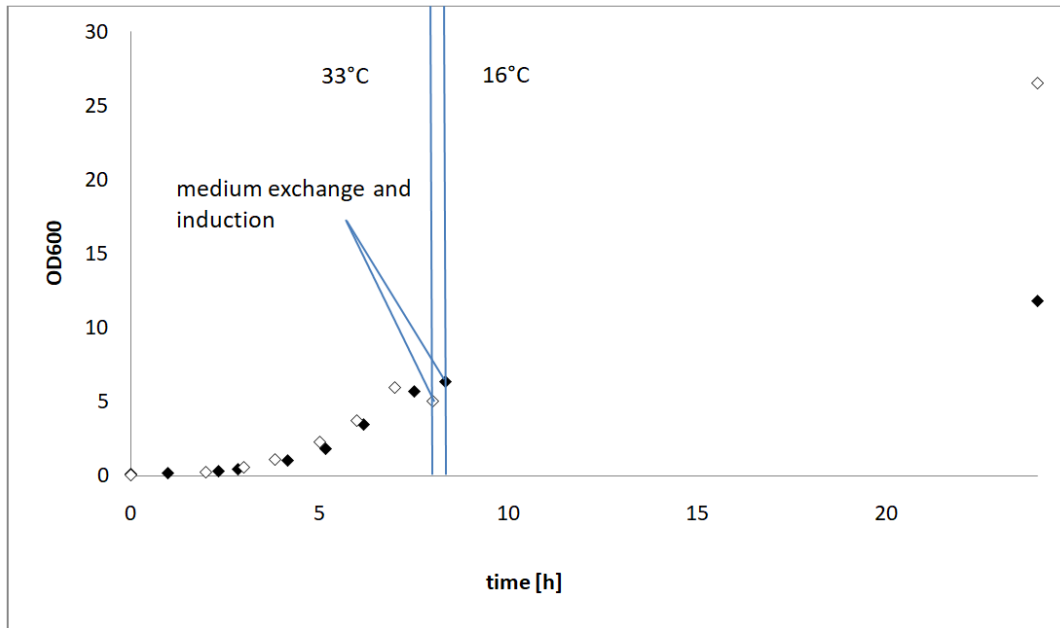


Figure 11: Growth curve of SHuffle T7 Express cells, synthesizing full length PBSase (black symbols) after induction at an OD₆₀₀ of 6. Growth is plotted against a culture carrying an “empty” pET15b plasmid (white symbols), grown under the same circumstances. Both cultures were set up in 200 ml TB-medium in 1 L baffled flasks, grown continuously at 33°C until they reached an OD of 6 (negative control), and 6.35 (expression culture). The cultures were then spun down for 30 minutes at 4000 g, and suspended in fresh medium of the same composition. The cultures were then induced by adding IPTG to a final concentration of 12 mM. Growth was continued at 16°C over night. After 24 hours, both cultivations were terminated.

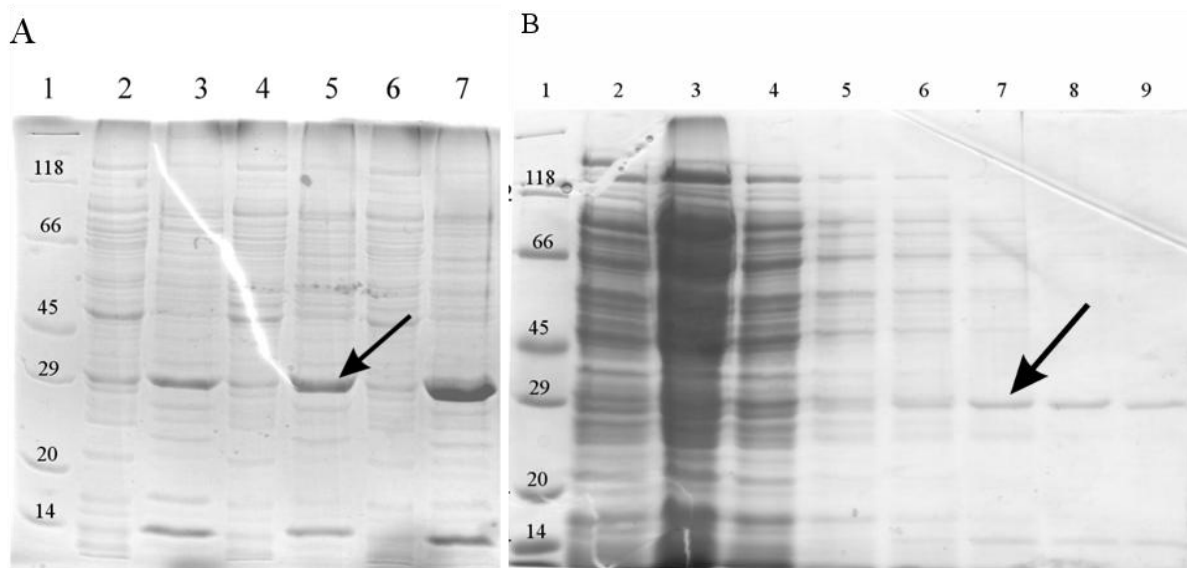


Figure 12: TCE-stained SDS-gels, following the synthesis and purification of PBSase, synthesized from a plasmid construct that contains the PBSase gene containing its native signal peptide in SHuffle T7 Express cells, after induction with a final concentration of 12 mM IPTG at an OD₆₀₀ of 6. Figure 12A shows the protein contents of the soluble and insoluble fractions of crude lysates, obtained from disrupted cells of time samples. The wells show the following fractions; 2-3: soluble and insoluble fraction of t₀, taken before induction, after the culture reached an OD of 1; 4-5: soluble and insoluble fraction of t₁, taken right after induction; 6-7: soluble and insoluble fraction of t₂, taken after 16 hours of gene expression. The arrow marks a band that represents a synthesized insoluble protein with a molecular size of 29 kDa. For size comparison, a protein marker Roti-Mark STANDARD was loaded onto the first well. Figure 12B shows a TCE-stained SDS-gel displaying various fractions obtained from IMAC of the soluble fraction of the culture. 2: Crude Lysate (diluted 1:10); 3: Flow-through; 4-5: Wash fractions 1 & 2. 6-10: Elution fractions 1-5. Elution fractions 1-4 were taken by eluting protein with 2 ml of Elution buffer. Elution fraction 5 was taken by eluting with 7 ml of Elution buffer. For size comparison, a protein marker Roti-Mark STANDARD was loaded onto the first well.

3.1.4 Synthesis of PBSase without its native signal peptide in SHuffle T7 Express

In order to compare expression efficiency of SHuffle T7 Express cells synthesizing full length PBSase to cells synthesizing mature PBSase, SHuffle T7 Express cells were transformed with the plasmid pET21b-PBSase-SP, which encodes for mature PBSase. Two times 200 ml TB in 1 L baffled flasks, containing 100 µg/ml ampicillin, were inoculated with these cells and the cultures were grown together with the cultures introduced in chapter 3.1.3. The cells were incubated at 33°C and gene expression of one culture was induced at an OD₆₀₀ of 1 by adding IPTG up to a final concentration of 2 mM. After the other culture reached an OD₆₀₀ of 6, its medium was exchanged prior to induction, by centrifuging at 4°C with 4000 g for 30 minutes. Then the medium was discarded, and the culture was suspended in fresh medium of the same composition. Gene expression was induced by adding IPTG up to a final concentration of 12 mM IPTG. Samples were withdrawn from both expression cultures at specific time points (“time samples”). After ~16 hours of expression at 16°C, the cells of both cultures were harvested and disrupted by sonication in 50 mM sodium phosphate buffer and soluble and insoluble fractions were separated from each other by centrifuging at 17 000 g. The soluble fractions of both cultures were analyzed with a *p*NPA-assay before purification. The culture that started synthesizing mature PBSase after induction at an OD₆₀₀ of 1 showed almost the same activity against *p*NPA as its sister culture, which started gene expression at an OD₆₀₀ of 6. This was rather unexpected, as the “early induced culture” grew to a lower final OD₆₀₀ of only 3.5 (Figure 13), and thusly should not have been able to synthesize as much protein as the “later induced culture”, which was harvested at a final OD₆₀₀ of 10 (data not shown). Together with results obtained in chapter 3.1.3, these results suggest that synthesis of PBSase generally results in higher yields of functional protein, when synthesized without its native signal peptide, and when induced at a late stage of exponential growth phase.

The soluble fraction containing His-tagged PBSase was loaded onto a column of Roti®garose-His-beads, in order to purify PBSase. Fractions obtained from purification were analyzed via SDS-PAGE, and showed successful synthesis and purification of a soluble protein of a molecular size equal to the size of PBSase. Yield of soluble protein obtained from both expression cultures was similar to the yield obtained from inducing expression of the PBSase gene containing its signal peptide at an OD₆₀₀ of 6 (Figure 12B, 14B, Supplementary Figure S4B). This suggests an equal efficient synthesis of PBSase in those three cultures. When analyzing the soluble and insoluble fractions of samples obtained from the expression culture which was induced at an OD₆₀₀ of 1, the band intensities increase in the second and third time sample (taken after 3 hours of expression at 33°C, and after 16 hours of expression at 16°C)(Figure 14A). The first sample (“t0”) shows basal transcription. However, *p*NPA-assays of the soluble fractions of these samples show increased activity only for the latest time sample (data not shown). This suggests that synthesis of PBSase at 33°C doesn’t produce functional PBSase, compared to synthesis at 16°C, which produces a small yield of functional PBSase.

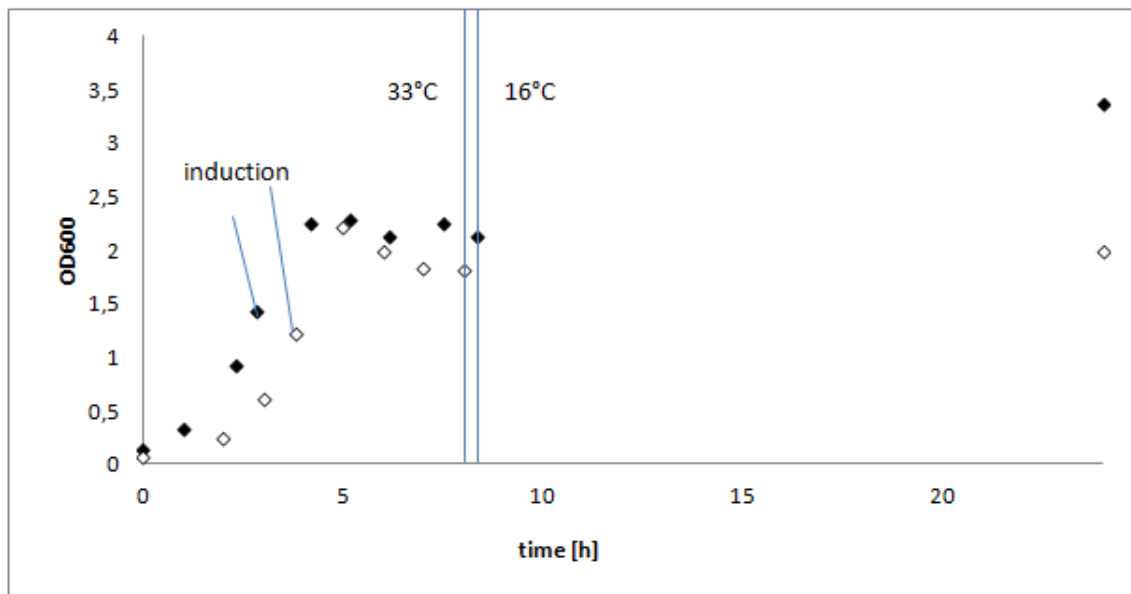


Figure 13: Growth curve of SHuffle T7 Express cells, synthesizing mature PBSase (black symbols) after induction at an OD₆₀₀ of 1. Growth is plotted against a culture carrying an “empty” pET15b plasmid (white symbols), grown under the same circumstances. Both cultures were set up in 200 ml TB-medium in 1 L baffled flasks, grown continuously at 33°C until they reached an OD of 1.2 (negative control), and 1.45 (expression culture). The cultures were then induced by adding IPTG to a final concentration of 2 mM. Growth was continued for about 3 hours, before expression temperature was lowered to 16°C for overnight expression. After 16 hours of overnight expression, both cultivations were terminated.

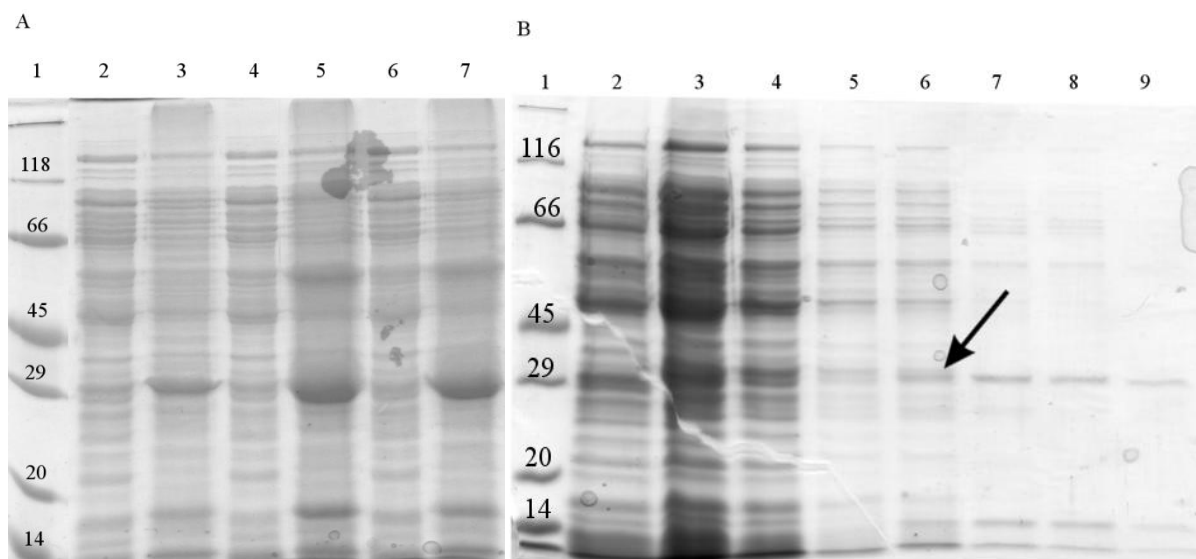


Figure 14: InstantBlue™-stained SDS-gels, following the synthesis and purification of mature PBSase, synthesized from a plasmid construct that contains the shortened PBSase gene in SHuffle T7 Express cells, after induction with a final concentration of 2 mM IPTG at an OD₆₀₀ of 1. Figure 14A shows the protein contents of the soluble and insoluble fractions of crude lysates, obtained from disrupted cells of time samples. The wells show the following fractions; 2-3: soluble and insoluble fraction of t₀, right after induction, after the culture reached an OD of 1; 4-5: soluble and insoluble fraction of t₁, taken after 3 hours of gene expression at 33°C; 6-7: soluble and insoluble fraction of t₂, taken after an additional 16 hours of gene expression at 16°C. The arrow marks a band that represents a synthesized insoluble protein with a molecular size of 29 kDa. For size comparison, a protein marker Roti-Mark STANDARD was loaded onto the first well. Figure 14B shows a TCE-stained SDS-gel displaying various fractions obtained from IMAC of the soluble fraction of the culture. 2: Crude Lysate (diluted 1:10); 3: Flow-through; 4-5: Wash fractions 1 & 2. 6-10: Elution fractions 1-5. Elution fractions 1-4 were taken by eluting protein with 2 ml of Elution buffer. Elution fraction 5 was taken by eluting with 7 ml of Elution buffer. The arrow marks a band that represents a synthesized insoluble protein with a molecular size of 29 kDa. For size comparison, a protein marker Roti-Mark STANDARD was loaded onto the first well

Synthesis of mature PBSase was attempted again with the same expression conditions, but using a higher volume. 400 ml of TB-medium in a 2 L baffled flask, containing 100 µg/ml ampicillin, was inoculated with SHuffle T7 Express cells, carrying pET21b-plasmids with the coding sequence for mature PBSase. The cells were incubated at 33°C until they reached an OD₆₀₀ of 6, at which point the culture was centrifuged at 4°C with 4000 g for 30 minutes. The medium was discarded, and the culture was suspended in fresh medium of the same composition. Then, gene expression was induced by adding IPTG to a final concentration of 6 mM, and the culture was incubated at 16°C. Samples were withdrawn from the culture at specific time points (“time samples”). After ~16 hours of expression at 16°C the cells were harvested and disrupted by sonication in 50 mM sodium phosphate buffer and soluble and insoluble fractions were separated from each other by centrifuging at 17 000 g. pNPA-hydrolysis assays of the soluble fraction were performed using 50 µl of undiluted cleared lysate and esterase activity was calculated to be at about 1 U/ml.

The soluble fraction containing His-tagged PBSase was loaded onto a column of Roti®garose-His-beads, in order to purify PBSase. Fractions obtained from purification were analyzed via SDS-PAGE, and showed successful synthesis and purification of a soluble protein of a molecular size equal to the size of PBSase (Figure 16B). All fractions containing purified protein were poured together, producing 7ml of protein solution, and activity against pNPA was calculated to be 0.081 U/ml. Protein content was not determined until two weeks later, at which point the sample exhibited no more esterase activity. Protein content was determined to be 24 µg.

SDS-PAGE analysis of the insoluble fractions of time samples revealed bands with high intensities at a molecular size of 29 kDa for each time sample (Figure 16A). Band intensity increased heavily after induction, showing a strong basal transcription of the PBSase gene. This suggests successful synthesis of mature PBSase, albeit again mostly resulting in inclusion bodies.

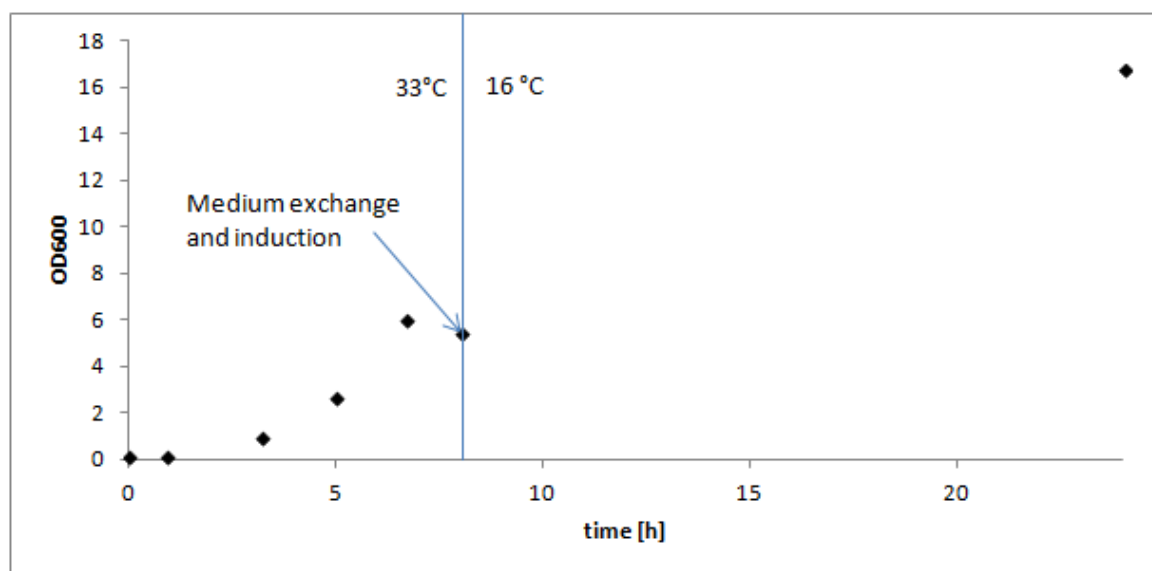


Figure 15: Growth curve of SHuffle T7 Express cells, synthesizing a shortened version of PBSase after induction at an OD₆₀₀ of 6. The culture was set up in 400 ml TB-medium in a 2 L baffled flask, and grew continuously at 33°C until it reached an OD of 6, at which point the medium was exchanged with fresh medium and the culture was induced by adding IPTG to a final concentration of 6 mM. Growth was continued at 16°C for overnight expression. After 16 hours of overnight expression, the cultivation was terminated.

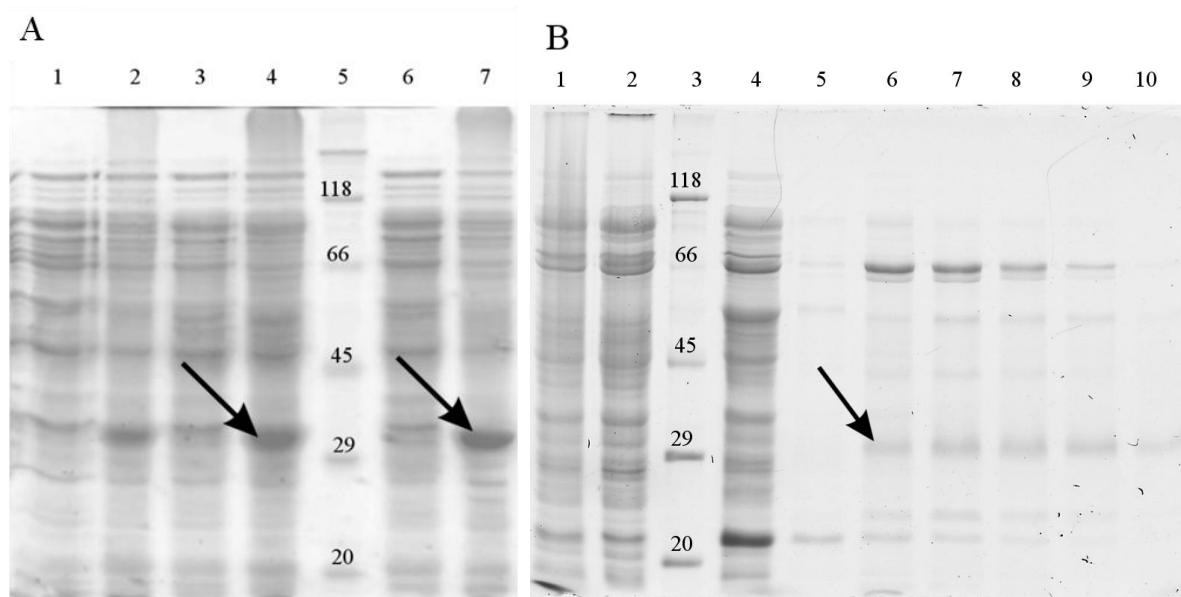


Figure 16: InstantBlue™-stained SDS-gels, following the synthesis and purification of PBSase, synthesized from a plasmid construct that contains the shortened PBSase in SHuffle T7 Express cells, after induction with a final concentration of 6 mM IPTG at an OD₆₀₀ of 6. Figure 16A shows the protein contents of the soluble and insoluble fractions of crude lysates, obtained from disrupted cells of time samples. The wells show the following fractions; 1-2: soluble and insoluble fraction of t₀, right after induction, after the culture reached an OD of 1; 3-4: soluble and insoluble fraction of t₁, taken after 3 hours of gene expression at 33°C; 6-7: soluble and insoluble fraction of t₂, taken after an additional 16 hours of gene expression at 16°C. The arrow marks a band that represents a synthesized insoluble protein with a molecular size of 29 kDa. For size comparison, a protein marker Roti-Mark STANDARD was loaded onto the fifth well. Figure 16B shows an InstantBlue™-stained SDS-gel displaying various fractions obtained from IMAC of the soluble fraction of the culture. 1: Crude Lysate; 2: Flow-through; 4-5: Wash fractions 1 & 2. 6-10: Elution fractions 1-5. Elution fractions 1-4 were taken by eluting protein with 2 ml of Elution buffer. Elution fraction 5 was taken by eluting with 7 ml of Elution buffer. The arrow marks bands that represent a synthesized insoluble protein with a molecular size of 29 kDa. For size comparison, a protein marker Roti-Mark STANDARD was loaded onto the third well.

Matrix assisted laser desorption/ionization using time-of-flight analysis mass spectrometry (MALDI-TOF MS)

In order to verify the identity of the purified protein, MALDI-TOF-MS analysis was performed. Using this technique, the amino acid sequence of a protein can be determined. MALDI-TOF combines the ionization technique MALDI with a time-of-flight mass spectrometry analysis of ions that are set free. A UV-laser hits the dried sample usually at a wavelength of 337 nm, and ionizes the molecules of interest with protons from the matrix. Choice of matrix depends on the features of the sample molecule. The molecule is ionized as a whole, and dissolves from the matrix, entering a gaseous phase. Molecules are accelerated and travel a certain distance which is dependent on the size of the molecules to a detector.

12 µg PBSase were loaded onto a SDS-PAGE, cut out, and digested with Trypsin. Then, MALDI-TOF was performed by the Institute of Microbiology, Department for Microbial Physiology and Molecular Biology, Greifswald University. Table 2 shows the protein sequence of PBSase with all residues that were covered by the peptides identified with MALDI-TOF, marked by underlining. Identified peptides exhibited 100% complementarity to the equivalent parts of the PBSase sequence. According to these results, it is highly likely for the purified soluble esterase to indeed be functional PBSase.

Table 2: Protein sequence of PBSase. 14 different peptide species were detected, ranging from 9 – 32 amino acids. All sequence parts that are complementary to a peptide discovered by MALDI-TOF-MS, are marked by underlining. These peptides match their complementary sequences on the PBSase sequence with 100% complementarity. Trypsin digest was unable to cut the protein at certain Arginin residues (marked with stars).

1	QTNPYER <u>GPA</u>	<u>PTSSLEASR</u>	<u>GPFSYQSFTV</u>	<u>SRPSGYRAGT</u>	<u>VYYPTNAGGP</u>
51	<u>VGAIAIVPGF</u>	<u>TAR</u> QSSINWW	GPRL <u>LASHGFV</u>	<u>VITIDTNSTL</u>	<u>DQPSRSRQQ</u>
101	<u>MAALSQVATL</u>	<u>SRTSSSPIYN</u>	<u>KVDTSR</u> LGVM	GWSMGGGGSL	ISARNNPSIK
151	AAAPQAPWSA	SKNFSSLTVP	TLIIACENDT	IAPVNQHADT	FYDSMSR*NPR*
201	EFLEINNGSH	SCANSGNSNQ	ALLGKKGVAV	MKR <u>FMDNDRR</u>	<u>YTSFACSNPN</u>
251	<u>SYNVSDFR</u> VA	ACN			

3.1.5 Synthesis of PBSase using the PelB-signal sequence

So far, attempts to synthesize PBSase have resulted in high amounts of insoluble protein and little amounts of soluble enzyme, which indicates severe difficulties during the folding process. By exporting the nascent protein into the periplasmic space of *E. coli*, the folding process can be assisted by the residing isomerases DsbA and DsbC (Berkmen et al., 2005). This can be achieved by fusing a protein to the pelB-sequence, a sequence that encodes for a signal peptide which is responsible for the translocation of *E. coli* proteins into periplasmic space. So far, this has been attempted only once before (Walczak, 2017) using a plasmid that encodes for pelB-PETase, and using SHuffle T7 Express or *E. coli* BL21(DE3) as expression host. In case of SHuffle T7 Express, a small amount of an enzyme with the corresponding size has been obtained. In order to test the expression efficiency of PBSase, using the pelB-sequence, a plasmid construct containing the PBSase gene fused N-terminally to the pelB-sequence, was created by FastCloning (Li et al., 2011). FastCloning is a technique that uses the host's crossing-over machinery to fuse two fragments together, if the fragments have overlapping sticky ends. The fragments are generated by PCR using a high-fidelity DNA polymerase, and the methylated DNA templates are digested with the use of the restriction enzyme *DpnI*. Primers for PCRs are designed in such a way that the resulting fragments have overlapping ends of a length of about 16 bp. Both DNA-fragments are then transformed into *E. coli* cells, and constructed into a plasmid.

For the construction of the plasmid pET22b-PBSase, two fragments have been synthesized via PCR: One fragment consisted of the PBSase sequence without its native signal peptide, but including the C-terminal histidine-tag (Figure 17A, Insert). The second fragment was made from the plasmid pET22b-PETase which encodes for PETase fused to the pelB-sequence, under excision of the PETase gene (Figure 17A, Vector). As a template for the insert, the plasmid pET21b_PBSase-SP, which encodes for mature PBSase was used. The PCR conditions that were used are listed in the methods section. The concentrations of obtained insert and fragment have been calculated photometrically. 8 µl of insert DNA and 8 µl of vector fragment DNA have been transformed into chemocompetent *E. coli* Top10 cells and grown on LB-ampicillin-agar plates. Two colonies were counted. Their plasmids were isolated and analyzed on a 1% analytical agarose gel (Figure 17B). The gel confirms a successful assembly of insert and vector into a new plasmid. Sequencing of the plasmids revealed a silent mutation at the position of T5610 for one of the clones.

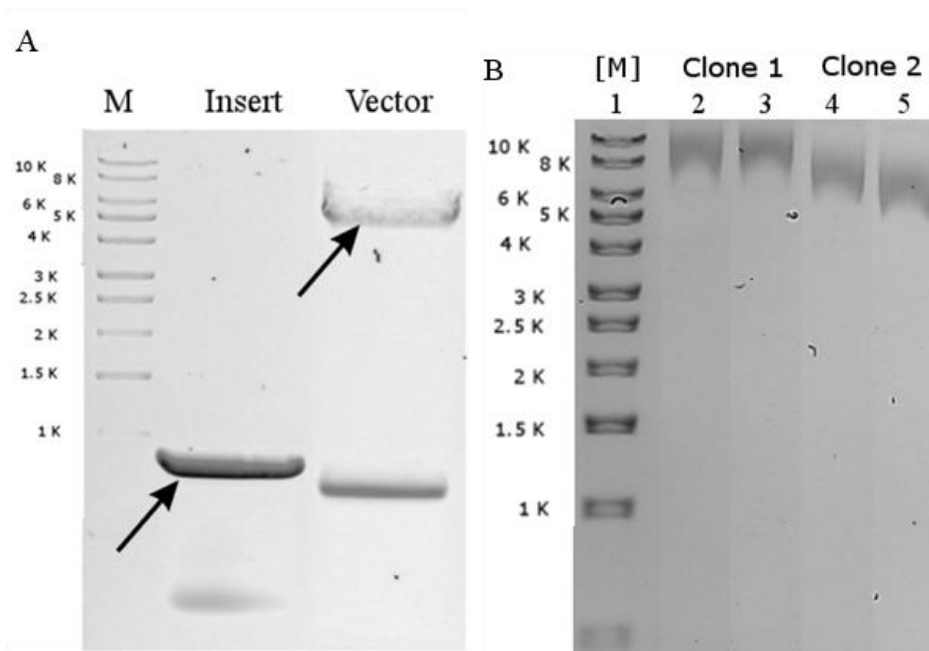


Figure 17A: 1% preparative agarose gel of insert and vector and a 1kb-DNA marker (M). Arrows point at the positions of PBSase-sequence (Insert) and pET22b-fragment (Vector). The vector fragment is about 5500 bp long and forms a band in between the lengths of 5000 and 6000 bps, together with a band below the 1 K marker, which represents an unidentified PCR-amplificate. The insert is about 900 bp long and forms a band underneath the length of 1000 bp. **17B:** 1% analytical agarose gel, showing the isolated plasmids of two colonies obtained after transformation with the two fragments created by PCR. The gel picture was digitally altered using GIMP. Albeit the different positions on the gel suggest different plasmid sizes, sequencing revealed no differences in between both clones, save a silent mutation in one clone.

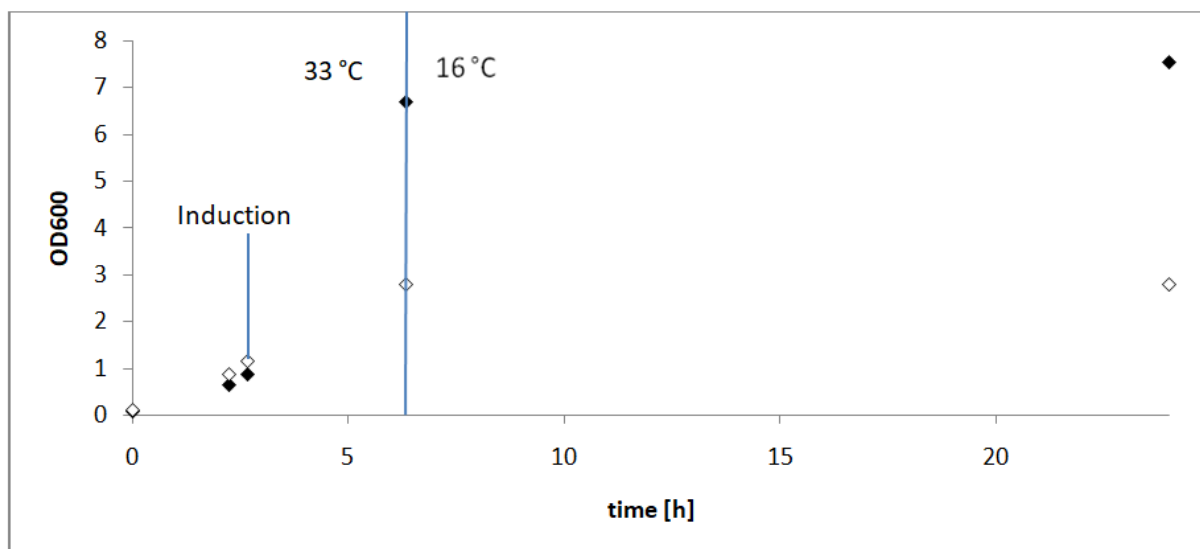
The plasmids were then transformed into *E. coli* BL21(DE3) chemo competent cells and glycerol stocks were prepared from the overnight cultures. 50 ml of LB-medium and 200 ml of TB-medium, each containing 100 µg/ml ampicillin were inoculated cells from these glycerol stocks. Additionally, for each expression culture, a culture of *E. coli* BL21(DE3), carrying an empty pET15b-plasmid, was grown under the same growth conditions. The cells were incubated at 33°C until they reached an OD₆₀₀ of 1, at which point gene expression was induced by adding IPTG to a final concentration of 1 mM. Cell count of both expression cultures increased up to an OD of 7-9 after 3 hours of expression at 33°C, while the cultures that served as negative control showed a repressed growth (Figure 18 A and B). The final ODs were quite similar to the ODs measured after 3 hours of expression, when expression temperature was decreased to 16°C.

Samples were withdrawn from the culture at specific time points ("time samples"). After six hours of continuous growth, the expression temperature was decreased to 16 °C. After 16 hours of expression at 16°C, the cells of both cultures were harvested and disrupted by sonication in 50 mM sodium phosphate buffer and soluble and insoluble fractions were separated from each other by centrifuging at 17 000 g. pNPA-hydrolysis assays were performed using 50 µl of undiluted cleared lysate, and showed no activity.

The soluble fraction containing His-tagged PBSase was loaded onto a column of Roti®garose-His-beads, in order to purify PBSase. Fractions obtained from purification were analyzed via SDS-PAGE, and showed successful synthesis and purification of a soluble protein of a molecular size equal to the size of PBSase (Figures 19B and 20). SDS-PAGE analysis of supernatants and insoluble fractions of these time samples shows no presence of PBSase in the soluble or insoluble fraction (Figures 19A and 20A). This leads to the conclusion that either gene expression did not work, and the faint bands observed on the SDS-gels in Figures 19B and 20B are unspecific protein, or PBSase was correctly

synthesized, but is found somewhere else than in the soluble or the insoluble fraction of cell lysates. There have been reports of protein synthesis and subsequent translocation mediated by the pelB-sequence, that resulted in the majority of protein to be found in the culture medium (Nikolaivits et al., 2016; Su et al., 2015)).

A



B

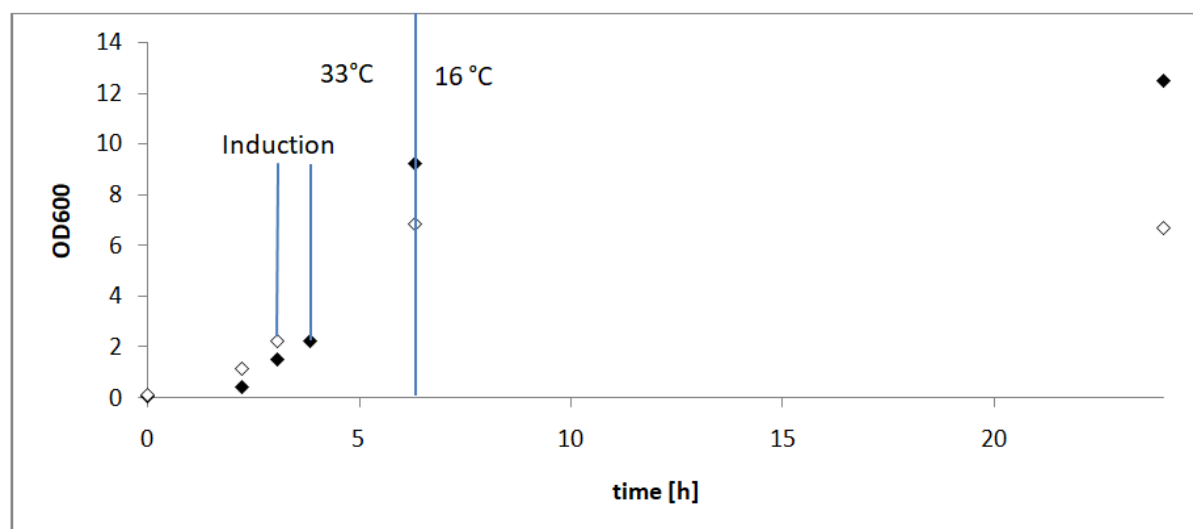


Figure 18: Growth curves of *E. coli* BL21 (DE3) cells, carrying the plasmid pET22b-PBSase, which encodes for “pelB-PBSase” (black symbols). Both experiments were set up with a sister culture of BL21(DE3) cells that carried the “empty” plasmid pET15b and served as negative controls (white symbols). 18A: The two cultures were set up in 50 ml LB-medium in two 250 mL baffled flasks. Both cultures were grown continuously at 33°C until they reached an OD of 1.2 (negative control), and 0.9 (expression culture) at which point the cultures were induced by adding IPTG to a final concentration of 1 mM. After 6 hours of growth, the cultivation temperature for both cultures was decreased to 16°C. After 16 hours of overnight expression, both cultivations were terminated. 18B: The two cultures were set up in 200 ml TB-medium in two 1 L baffled flasks. Both cultures were grown continuously at 33°C until they reached an OD of 2.25 at which point the cultures were induced by adding IPTG to a final concentration of 2 mM. After 6 hours of growth, the cultivation temperature for both cultures was decreased to 16°C. After 16 hours of overnight expression, both cultivations were terminated.

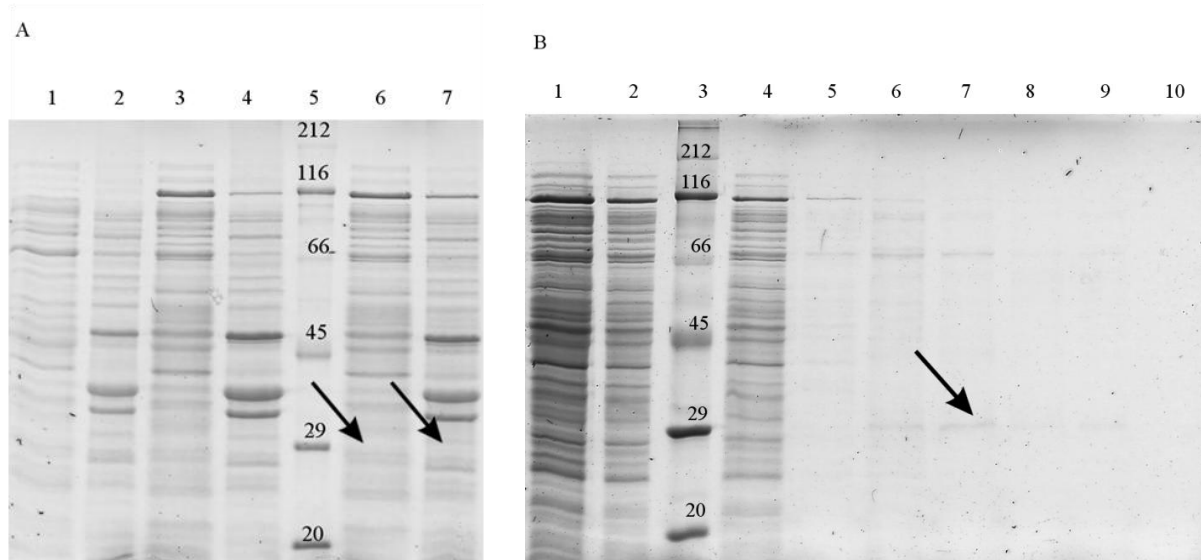


Figure 19: TCE-stained SDS-gels, following the synthesis and purification of PBSase, synthesized from a plasmid construct that encodes for PBSase, fused to the pelB-sequence, in *E. coli* BL21(DE3) cells, after induction with a final concentration of 1 mM IPTG at an OD₆₀₀ of 1. Figure 19A shows the protein contents of the soluble and insoluble fractions of crude lysates, obtained from disrupted cells of time samples. 1-2: soluble and insoluble fraction of t0, right after induction, after the culture reached an OD of 1; 3-4: soluble and insoluble fraction of t1, taken after 3 hours of gene expression at 33°C; 6-7: soluble and insoluble fraction of t2, taken after an additional 16 hours of gene expression at 16°C. The arrow marks the position at which a band representing PBSase should be found. For size comparison, a protein marker Roti-Mark STANDARD was loaded onto the fifth well. Figure 19B shows a TCE-stained SDS-gel displaying various fractions obtained from IMAC of the soluble fraction of the culture. 1: Crude Lysate; 2: Flow-through; 4-5: Wash fractions 1 & 2. 6-10: Elution fractions 1-5. Elution fractions 1-4 were taken by eluting protein with 2 ml of Elution buffer. Elution fraction 5 was taken by eluting with 7 ml of Elution buffer. The arrow marks a band that represents a synthesized soluble protein with a molecular size of 29 kDa. For size comparison, a protein marker Roti-Mark STANDARD was loaded onto the third well.

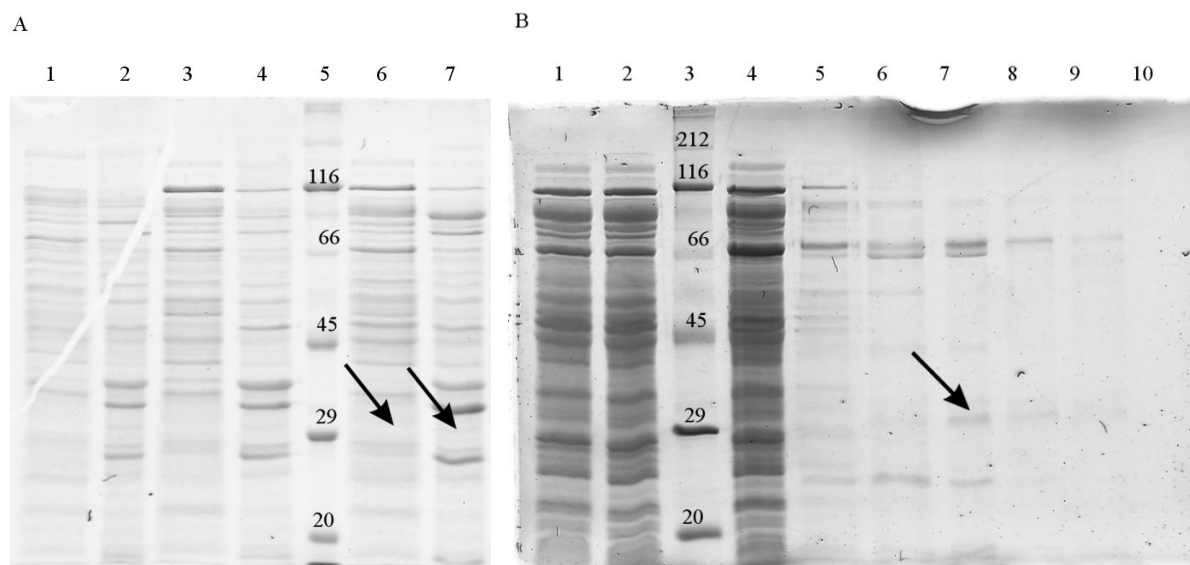


Figure 20: TCE-stained SDS-gels, following the synthesis and purification of PBSase, synthesized from a plasmid construct that encodes for PBSase, fused to the pelB-sequence, in *E. coli* BL21(DE3) cells, grown in 200 ml of TB-Amp. Gene expression was induced with a final concentration of 2 mM IPTG at an OD₆₀₀ of 2. Figure 20A shows the protein contents of the soluble and insoluble fractions of crude lysates, obtained from disrupted cells of time samples. The wells show the following fractions; 1-2: soluble and insoluble fraction of t0, right after induction, after the culture reached an OD of 1; 3-4: soluble and insoluble fraction of t1, taken after 3 hours of gene expression at 33°C; 6-7: soluble and insoluble fraction of t2, taken after an additional 16 hours of gene expression at 16°C. The arrow marks the position at which a band representing PBSase should be found. For size comparison, a protein marker Roti-Mark STANDARD was loaded onto the fifth well. Figure 20B shows a TCE-stained SDS-gel displaying various fractions obtained from IMAC of the soluble fraction of the culture. 1: Crude Lysate; 2: Flow-through; 4-5: Wash fractions 1 & 2. 6-10: Elution fractions 1-5. Elution fractions 1-4 were taken by eluting protein with 2 ml of Elution buffer. Elution fraction 5 was taken by eluting with 7 ml of Elution buffer. The arrow marks a band that represents a synthesized soluble protein with a molecular size of 29 kDa. For size comparison, a protein marker Roti-Mark STANDARD was loaded onto the third well.

3.1.6 Synthesis of PETase in SHuffle T7 Express

Synthesis of PETase in common *E. coli* BL21(DE3) usually produces misfolded or unfolded PETase in the form of inclusion bodies, which then has to be refolded by dialysis against an adequate buffer (Fecker et al., 2018; Furukawa et al., 2018). Several research groups have tried to avoid this by expressing the PETase gene in one of many different genetically engineered strains. However, strains expressing mutated *trxB* and *gor* have not been used with one exception (Joo et al., 2018). Therefore, synthesis of PETase in SHuffle T7 Express could potentially be more efficient in providing high yields of correctly folded PETase, than other methods. Also, the properties of a PETase, produced by SHuffle T7 Express could change relative to PETase produced in other strains. Previously thermostability of *Fusarium Oxysporum* Cutinase was increased by 60%, when producing Cutinase in Origami-B cells, a strain similar to SHuffle T7 Express (Nikolaivits et al., 2016).

200 ml of TB-medium in a 1 L baffled flask, containing 100 µg/ml ampicillin, was inoculated with SHuffle T7 Express cells, carrying pET21b-plasmids with the coding sequence for PETase. The cells were incubated at 33°C until they reached an OD₆₀₀ of 2, at which point gene expression was induced by adding IPTG to a final concentration of 2 mM. Samples were withdrawn from the culture at specific time points ("time samples"). After two hours of expression at 33°C, the expression temperature was decreased to 16 °C. After 16 hours of expression at 16°C, the cells were harvested and disrupted by sonication in 50 mM sodium phosphate buffer and soluble and insoluble fractions were separated from each other by centrifuging at 17 000 g. pNPA-hydrolysis assays were performed using 50 µl of undiluted cleared lysate and esterase activity of the soluble fraction was determined to be 106.5 U/ml.

Cleared lysates containing His-tagged PETase were loaded onto columns of Roti®garose-His-beads and purified via IMAC. Fractions obtained from purification were analyzed via SDS-PAGE, and showed successful synthesis and purification of a soluble protein with a molecular size of 29 kDa (Figure 22B). SDS-PAGE analysis of time samples revealed bands with low intensities at a molecular size of 29 kDa in the insoluble fraction of the second time sample, indicating no or little proper folding of PETase at 33°C. In the time sample taken after overnight expression, bands of equal intensity can be seen in both fractions.

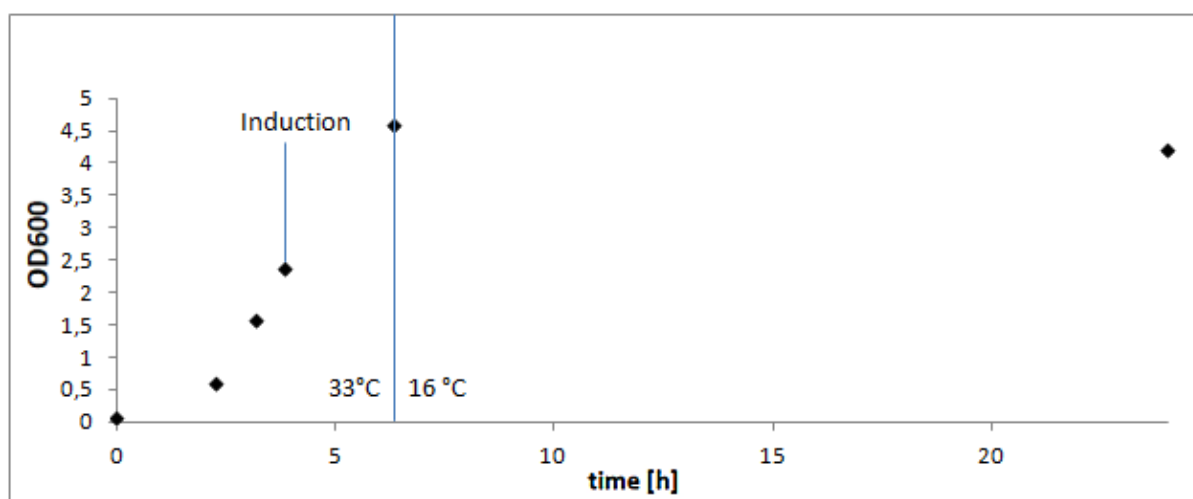


Figure 21: Growth curve of SHuffle T7 Express cells, synthesizing a shortened version of PBSase. The culture was set up in 200 ml TB-medium in a 1 L baffled flask, and grew continuously at 33°C until it reached an OD of 2.37, at which point the culture was induced by adding IPTG to a final concentration of 2 mM. Growth was continued for an additional 2 hours, until expression temperature was decreased to 16°C for overnight expression. After 16 hours of overnight expression, the cultivation was terminated.

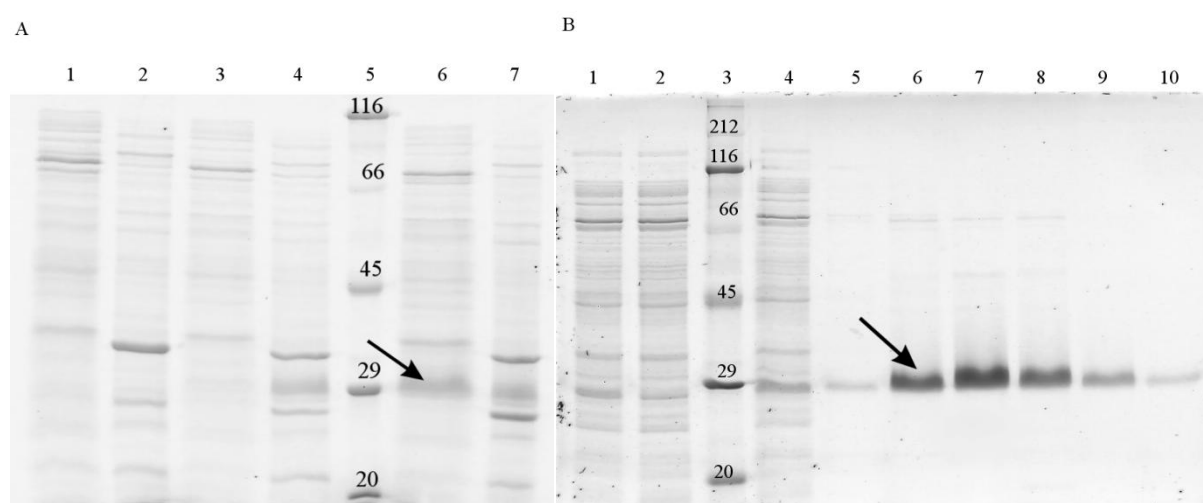


Figure 22 (A): TCE-stained SDS-gels, following the synthesis and purification of PBSase, synthesized from a plasmid construct that encodes for PBSase, fused to the pelB-sequence, in *E. coli* BL21(DE3) cells, grown in 200 ml of TB-Amp. Gene expression was induced with a final concentration of 2 mM IPTG at an OD₆₀₀ of 2.37. Figure 22A shows the protein contents of the soluble and insoluble fractions of crude lysates, obtained from disrupted cells of time samples. The wells show the following fractions; 1-2: soluble and insoluble fraction of t0, right after induction, after the culture reached an OD of 1; 3-4: soluble and insoluble fraction of t1, taken after 3 hours of gene expression at 33°C; 6-7: soluble and insoluble fraction of t2, taken after an additional 16 hours of gene expression at 16°C. The arrow marks the position at which a band representing PBSase would expectedly be found. For size comparison, a protein marker Roti-Mark STANDARD was loaded onto the fifth well. Figure 22B shows a TCE-stained SDS-gel displaying various fractions obtained from IMAC of the soluble fraction of the culture. 1: Crude Lysate; 2: Flow-through; 4-5: Wash fractions 1 & 2. 6-10: Elution fractions 1-5. Elution fractions 1-4 were taken by eluting protein with 2 ml of Elution buffer. Elution fraction 5 was taken by eluting with 7 ml of Elution buffer. The arrow marks a band that represents a synthesized soluble protein with a molecular size of 29 kDa. For size comparison, a protein marker Roti-Mark STANDARD was loaded onto the third well.

3.2. Characterization of thermostability and catalytic activity of PETase and PBSase

3.2.1 NanoDSF analysis

In regards to stability, since its discovery it has been fairly obvious that PETase isn't a very thermostable enzyme, as activity could not be measured anymore above 40°C. Yoshida characterized PETase's PET-hydrolyzing activity at various different temperatures and determined the optimal temperature for its PET-degrading activity at 30°C. Joo determined PETase's melting temperature at 46.8°C, using StepOnePlus Real-Time PCR by Thermo Fisher Scientific. Austin used Differential Scanning Calorimetry to determine thermostability of PETase and reported 48.1±0.18°C for its melting temperature (Austin et al., 2018, Beckham, G., personal communication). Proper characterization of thermostability of PBSase would provide a foundation for future protein engineering and hybridization experiments. In order to characterize thermostability of both enzymes, their melting temperatures T_m were determined by Differential Scanning Fluorimetry (NanoDSF). NanoDSF describes a Differential Scanning Fluorimetry technique which is able to detect signaling of exposed tryptophane and tyrosine residues. A folded protein will have a number of these residues hidden inside the hydrophobic core of the protein, while the surface residues emit a basal fluorescent signal. Upon unfolding, all hidden residues get exposed to the aqueous solvent, which increases the fluorescent signal. The ratio of the fluorescent signal at 350nm/330nm starts off at 0.7 and increases as protein in the sample unfolds more and more. The rising level of fluorescence also increases proportional to the rising temperature. At some point however, half of all protein is unfolded, and the signal increase reaches a point at which it becomes steady, before it decreases. This point is indicated as a peak of the first derivative. A second peak might appear as a more stable sub domain of the protein eventually undergoes the same unfolding procedure, but after exposure to higher temperatures.

PBSase

Figure 23 shows the fluorescence signal of three samples of PBSase, obtained from the experiments described in chapters 3.1.3 and 3.1.4. The first derivative of the curve shows a peak at about 38°C, indicating the strongest unfolding transition, followed by one minor follow-up unfolding transition. Onset is marked at 25-27°C.

PETase

Figure 24 shows the fluorescence signal obtained from denaturing PETase, as well as the first derivative of the curve, showing a peak at 44.7°C, indicating the unfolding transition. Onset is marked at 34.7°C.

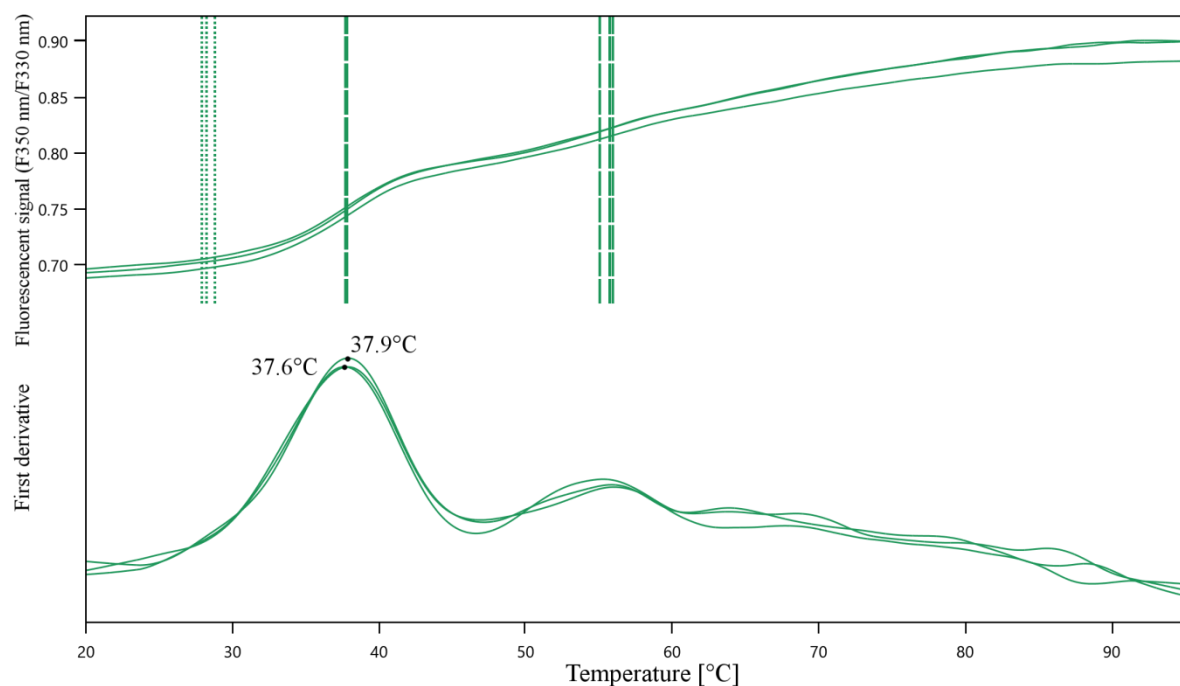


Figure 23: NanoDSF measurements following tryptophane and tyrosine fluorescence of three samples of PBSase. Fluorescence is measured in 350 nm and 330 nm and the ratio is displayed. The thinly dotted green lines represent the point of onset. The dashed lines represent a local increase maximum. The first derivative shows at which temperature the fluorescence signal has its strongest increase, indicating the point at which half of all protein has been unfolded.

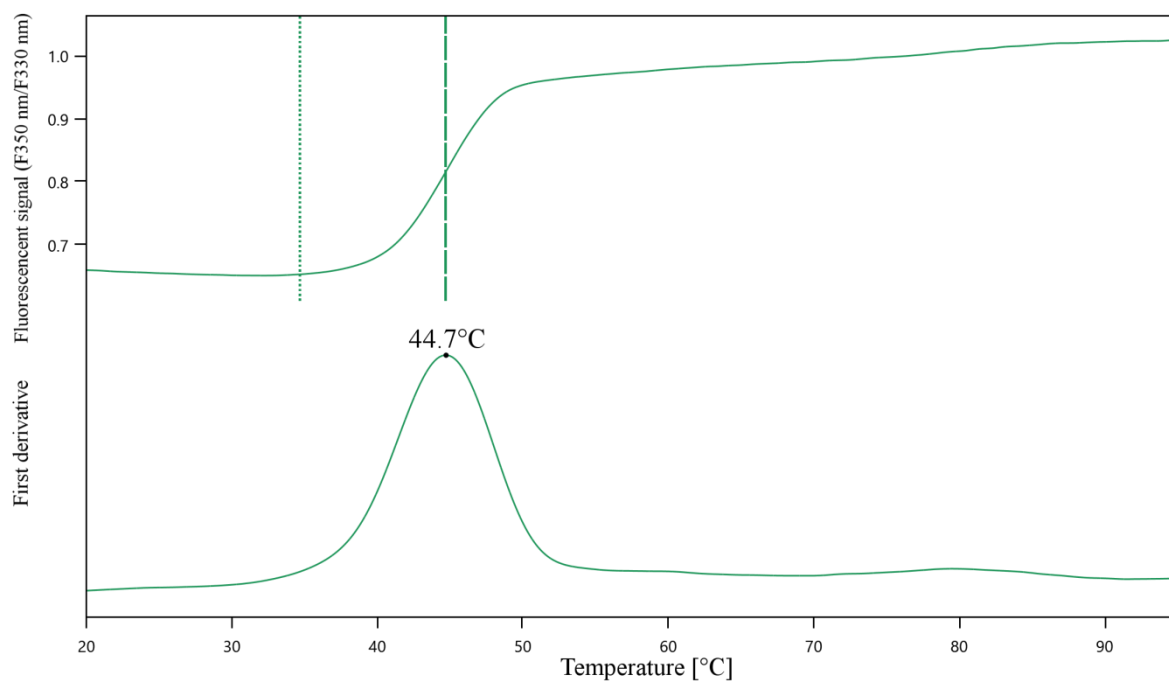


Figure 24: NanoDSF measurement of tryptophane and tyrosine fluorescence of three samples of mature PETase. Fluorescence is measured in 350 nm and 330 nm and the ratio is displayed. The thin, dotted green lines represent the point of onset. The dashed lines represent a local increase maximum. The first derivative shows at which temperature the fluorescence signal has its strongest increase, indicating the point at which half of all protein has been unfolded.

3.2.2 Turbidimetric analysis

In recent papers, experiments aimed at determining the PET-degrading activity of PETase, or PETase-derivatives, are usually performed by incubating these enzymes with a PET film of a thickness of 6 mm. Industrial applications of PETase will require the enzyme to degrade recalcitrant material like high- or low-crystallinity PET waste, since non-fiber plastic waste is abundantly present in either one of these conformations. However, a different kind of substrate could shed more light on the enzyme's inherent PET-degrading ability. Wei previously described a method of closely following the degradation of PET-nanoparticles by measuring the turbidity at 600 nm of a PET-nanoparticle solution over time to which a PET-degrading enzyme has been added (Wei et al., 2014a). A decrease in the solution's turbidity indicates surface corrosion of nanoparticles and thusly PET-degradation. Originally, this assay was developed to analyze PET-degrading activity of enzymes at higher temperatures of about 60°C. The enzyme-nanoparticle solution would be mixed with liquid agarose and solidified at low temperatures. Heating it up to 60°C would initiate the reaction. The PET-degrading enzyme would then be able to diffuse freely and access PET nanoparticles. The agarose would keep the PET-nanoparticles in suspension, eliminating a sedimentation effect, which would otherwise increase turbidity over time, resulting in inaccurate measurements. However, PETase, as well as PBSase, is not thermostable enough to withstand such high temperatures. Therefore, in this work, a derivative of Wei's turbidimetric analysis was performed. PET-nanoparticles have been produced from amorphous PET-foil. The concentration of the nanoparticles was unknown; therefore, all collected data was qualitative. Enzyme activity of PBSase and PETase was plotted over time. The PET-nanoparticle solution was added to a microtiter plate, and PBSase in sodium phosphate buffer was added to it. The reaction was started by increasing the reaction temperature to 30°C. The sedimentation effect is visualized in the negative control in Figure 25.

PBSase

IMAC-purified PBSase (obtained from expression attempt described in chapter 3.1.3) hydrolyzed *p*NPA. In order to test its catalytic activity against PET, a turbidimetric analysis was performed. Figure 23 shows changes in turbidity of a PET-nanoparticle solution containing 110 nM PBSase, relative to initial turbidity during catalysis of 3 hours at 30°C. Experiments were conducted in triplicates, using micro titer plates with 200 µl of PET-nanoparticle solution and 100 µl of full length PBSase solution per well. The undiluted nanoparticle solution reached an OD₆₀₀ of 0.3. 100 µl of pure sodium phosphate buffer served as negative control. After an initial rise in turbidity after 30 minutes, the turbidity of the enzyme solution dropped to 80% of initial turbidity. This however might not necessarily be an indicator for PET-hydrolyzing activity. The reason for this assumption is explained in detail in the discussion section.

PETase

IMAC-purified PETase, obtained from expression experiments as described in chapter 3.1.6, was split into various samples with different concentrations (40 nM, 95 nM, 190 nM, 380 nM and 950 nM). Experiments were conducted in triplicates, using microtiter plates with 200 µl of PET-nanoparticle solution and 50 µl of a PETase solution per well. 50 µl of sodium phosphate buffer served as negative control. The nanoparticle solution exhibited a faster and higher decrease in turbidity with higher concentrations of enzyme (Figure 26), but turbidity never dropped below 30% of its initial value. This can be compared to degradation of PET-nanoparticles that were produced from PET-granulate (Wei et al., 2014a), which could not be degraded by more than 80%.

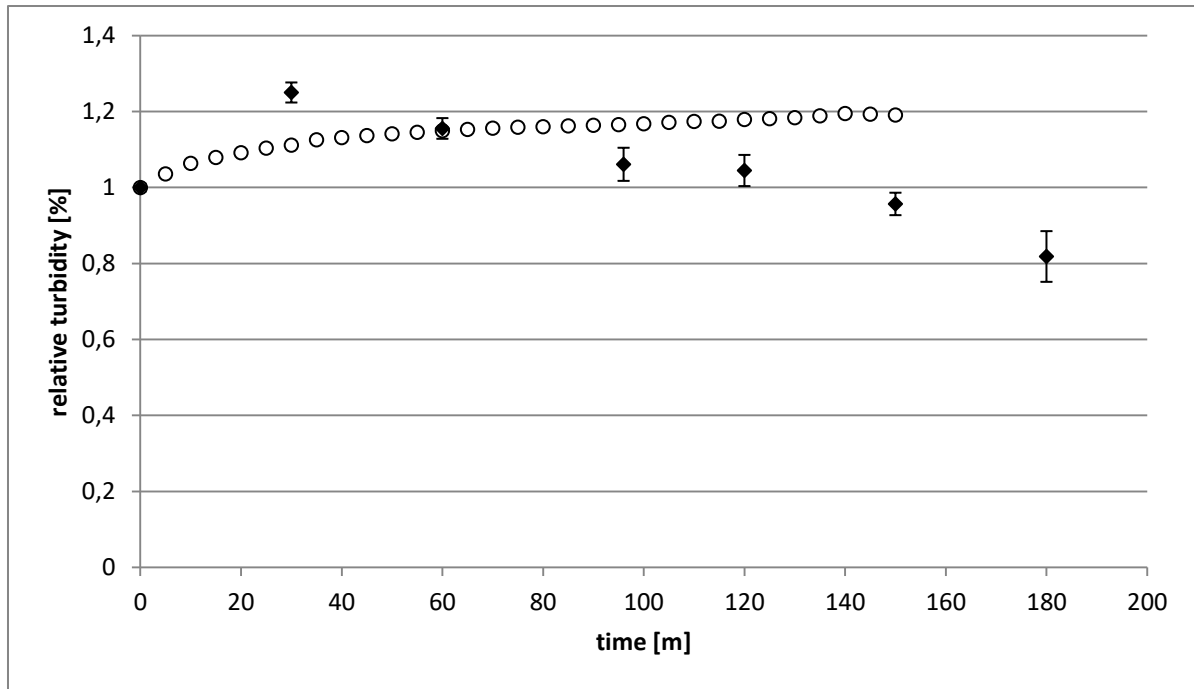


Figure 25: Qualitative analysis of PET-hydrolyzing activity of full length PBSase, based on the changes in turbidity of a PET-nanoparticle solution during three hours of reaction time at 30°C. Before measuring, the microtiter plate was shaken for 15 seconds at 2 mm amplitude, in order to remedy sedimentation. Measurements were taken every 30 minutes for full length PBSase and every 5 minutes for the negative control. Squares: full length PBSase with a protein concentration of 110 nM and a specific activity on pNPA of 0.8 U/ml. Diamonds: negative control, consisting of 100 µl of sodium phosphate buffer. OD₆₀₀ was measured and compared to the OD₆₀₀ at t₀. Error bars indicate standard deviation of triplicate measurements.

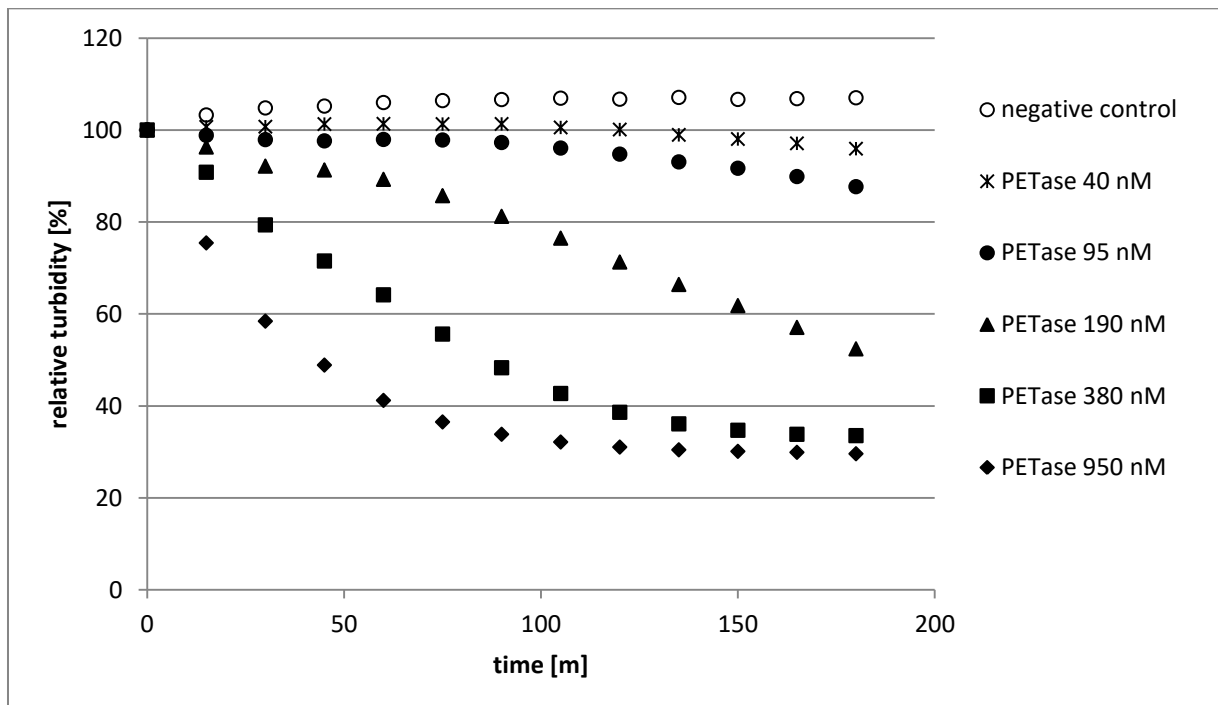


Figure 26: Qualitative analysis of PET-hydrolysing activity of mature PBSase, based on the changes in turbidity of several PET-nanoparticle solutions during 3 hours of reaction time at 30°C, using protein solutions of mature PETase with different protein concentrations. Measurements were taken every 15 minutes. The experiments were performed as triplets in a micro titerplate, using 200 µl of nanoparticle-solution and 50 µl of enzyme in Phosphate buffer. OD₆₀₀ was measured and compared to the OD₆₀₀ at t₀. Standard deviation of triplicate measurements was too low and could not be visualized by error bars.

3.2.3 *p*NPA-assay

*p*NPA, as well as the similar substrates *para*-nitrophenolbutyrate (*p*NPB) and *para*-nitrophenolelactat (*p*NPL) are commonly used for determining esterase activity of enzymes, or measuring the activity of lysates, but can also qualitatively be used to merely indicate the presence of functional esterase. However, an accurate characterization of an enzyme's catalytic activity against these substrates can be useful for engineering attempts. Therefore, both PBSase and PETase were characterized in their activity against 1 mM *p*NPA.

PBSase

Specific activity of PBSase was calculated for samples obtained from various expression experiments. Specific activity against 1mM *p*NPA, obtained from IMAC-purified PBSase ranged from 0.8 U/mg to 1 U/mg.

PETase

Specific activity of PETase against *p*NPA has been calculated in just one publication (Fecker et al., 2018), and in a thesis (Walczak, 2017). Results obtained in this work, differ greatly from these results. Fecker synthesized PETase in regular *E. coli* BL21(DE3), purified it via metal affinity chromatography, then refolded PETase by dialysis and further purified it with size-exclusion-chromatography. PETase exhibited a specific activity against 1 mM *p*NPA of about 14 U/mg, which lead to a theoretical maximum specific activity of 48 U/mg, using approximately 4 times K_m (Fecker et al., 2018), Galaz-Davison, P., personal communication). When fused to the *pelB*-sequence and synthesized in SHuffle T7 Express cells, PETase reached a specific activity against 1 mM *p*NPA of 4.77 U/mg (Walczak, 2017). In this work, PETase was produced using SHuffle T7 Express as expression host, without the use of the *pelB*-signal sequence. Specific activity against 1 mM *p*NPA was calculated to be 18.2 U/ml. This value could be further increased by further purifying PETase with methods like size exclusion chromatography.

4. Discussion

Synthesis of PBSase, using expression hosts C41(DE3), C43(DE3) and T7 Express LysY/Iq

Expression was unsuccessful in C41(DE3) and C43(DE3), as well as in T7 Express LysY/Iq, whereas Expression was successful in SHuffle T7 Express, albeit PBSase was mostly present in an insoluble form. The “Walker Strains” and T7 Express LysY/Iq are designed specifically for the production of proteins that other strains fail to synthesize due to toxicity. The inability to synthesize PBSase of C41(DE3), C43(DE3), and T7 Express LysY/Iq, all of which tightly control the transcription of the T7 RNA polymerase, combined with the ability of SHuffle T7 Express to both transcribe the PBSase gene and synthesize the enzyme, suggests presence of an unknown factor that either blocks transcription or translation of the PBSase gene in these strains, or enables them in SHuffle T7 Express.

Expression in BL21(DE3) cells, using the pelB-signal sequence also appears to have failed, as characteristic protein bands were not present in either the soluble or insoluble fraction (Figure 19A, 20A). However, it is possible that the bulk of PBSase synthesized with the pelB-sequence could have been found in the culture medium, as proteins that have been translocated with a pelB-sequence sometimes break through the outer membrane (Nikolaivits et al., 2016). Also, enzymes that exhibit a phosphatase activity, like a Cutinase from *Thermobifida fusca*, can also break through the outer membrane (Su et al., 2015). As of yet, it is unknown, if PBSase exhibits similar behavior as observed for *Thermobifida fusca* Cutinase. As of yet, no research group has expressed the PETase gene, fused to the pelB-signal sequence. In a preceding work, pelB-PETase was synthesized and very faint bands were visible on SDS-gels. The media were not investigated.

After induction of gene expression, cells that synthesized pelB-PBSase surprisingly grew to double the OD₆₀₀s of negative controls (Figure 17, 18), suggesting a growth advantage conveyed by the presence of the pelB-PBSase gene. The reason for this is unclear, but as suggested by SDS-gels on Figures 19B and 20B, PBSase was little or not at all synthesized, which implicates that cells also did not suffer metabolic stress, which usually follows overexpression of a foreign protein.

In general, better results have been obtained using TB-medium. The addition of IPTG affected cells strongly, when still in an early stage of exponential growth, while the addition of excess IPTG had little to no effect on cell growth, once the culture had reached a later stage of exponential growth. Therefore, induction of gene expression in later stages of exponential growth resulted in higher yields of protein. However, yield of functional PBSase never amounted to more than 50 µg of soluble protein.

Synthesis of PBSase with signal peptide

The signal peptide appears to have a toxic effect on the cells. This is shown in the growth and expression behavior of SHuffle T7 Express cells, attempting to synthesize full length PBSase. The strain failed to produce functional PBSase, when gene expression was induced at an OD₆₀₀ of 1, and at 33°C (data not shown), while the strain was able to produce functional PBSase, when gene expression was induced at an OD₆₀₀ of 6, and at 16°C (Figure 12B). Furthermore, when retrieving samples from overnight storage in 50 mM sodium phosphate buffer at 4°C, the cell pellets exhibit an inherent inability to suspend in 50 mM sodium phosphate buffer, and form aggregates, when withdrawn from the expression culture at least 30 minutes after induction of gene expression. The culture itself exhibits the same behavior when harvested, indicating that the lethal effect conveyed by the signal peptide affects the cells in storage, as well as during growth at 16°C. It is notable that

cells in samples taken right after induction of gene expression did not exhibit this behavior, assumingly because not enough toxic protein was synthesized at that point.

Synthesis of PBSase along with its native signal peptide had no discernible effect on PBSase folding (Figure 12A), as obtained protein yield resembled the yield of PBSase, obtained from synthesis without signal peptide. However, as the experiment was not repeated using the correct amount of IPTG, a conclusion cannot be drawn with certainty. It is furthermore unclear, whether the signal peptide broke off the protein, or got cleaved off at any point. SDS-PAGE analysis always shows bands at the same molecular size, suggesting that the signal peptide is never attached to PBSase prior to loading of the gel.

Characterizing thermostability and catalytic activity of PBSase

PBSase was outperformed by PETase in thermostability. With a melting temperature of about 38°C, PBSase is less thermostable than PETase by 6-8°C. With an onset temperature of 25.8 °C (Figure 23), the enzyme appears to be incapable of stable catalysis at 30°C. Purified PBSase reached a specific activity against *p*NPA of 0.8 U/mg. However, when performing turbidity analysis, using 110 nM of PBSase, the turbidity decreased from 100% to 80% in three hours of catalysis at 30°C (Figure 32), suggesting a much higher catalytic activity against PET than against *p*NPA. This can be explained by one of two scenarios. In the first scenario, PBSase is highly active against PET, and is able to corrode the surface of PET-nanoparticles with similar efficiency as PETase (Figure 25, 26), while exhibiting far less activity against *p*NPA (18.2 U/mg for PETase, 0.8 U/mg for PBSase). Generally, *p*NPA is easily hydrolyzed by most esterases. However, there have been reported cases of esterases that are unable to hydrolyze *p*NPA. In fact, *Ideonella sakaiensis* MHETase is an example for that (Yoshida et al., 2016, supplementary material, lines 138-141). In the second scenario, the decreasing turbidity in Figure 25 is caused by slow degradation of the enzyme itself. When conducting turbidimetric analysis, the initial OD₆₀₀ strongly depends on protein content of the added enzyme sample. The OD₆₀₀ in a negative control, consisting of PET-nanoparticles and 50 mM sodium phosphate buffer had an initial OD₆₀₀ of 0.3. When adding buffer containing enzyme, the OD₆₀₀ would rise from 0.3 to as much as 0.6. This can be caused by enzymes that presumably attach themselves to nanoparticles, increasing their absorbance. As PBSase starts to degrade at temperatures of above 25.8 °C, the decreasing amount of soluble protein might result in a slow decrease in turbidity. The mean initial turbidity of 200 µl PET nanoparticles and 100 µl of 50 mM sodium phosphate buffer, containing PBSase was at an OD₆₀₀ of 0.53. After 3 hours of catalysis at 30°C, it was at an OD₆₀₀ of 0.48, and thusly did not drop below the initial value of the negative control (OD₆₀₀ of 0.3). It is therefore possible that no, or at least very little PET-degradation happened in this experiment, and the observed increase in clarity resulted from slowly degrading enzyme.

Synthesis of PETase and characterization of thermostability and catalytic activity

As PETase has been synthesized only once before in an *E. coli* strain that expresses mutated *TrxB* and *gor* (Joo et al., 2018), it was unclear whether SHuffle T7 Express would perform adequately as expression host. Results however suggest otherwise. After IMAC purification, band intensities on an SDS-gel suggest that about half of all synthesized PETase molecules folded correctly when synthesized in SHuffle T7 Express at 16 °C (Figure 22A). IMAC-purified PETase reached a specific activity against 1 mM *p*NPA of 18.2 U/mg, which could be further enhanced if the sample was purified with additional methods, such as size exclusion chromatography. In comparison to this, Fecker et al. achieved a specific activity of 14 U/mg for their sample, which was purified once by IMAC and a second time by size exclusion chromatography, after a refolding step. This indicates an

increased efficiency for expression of the PETase gene and folding of the nascent protein, when expressed at 16°C in SHuffle T7 Express cytosol.

Regarding thermostability, research groups have previously stated a T_m of 46.8°C (Joo et al., 2018) and 48.1°C (Austin et al., 2018, Beckham, G., personal communication). In both cases, different methods were employed to determine T_m of PETase. It is therefore impossible to say, whether the difference in melting temperatures is conveyed by the employment of different expression strains, or by the use of different equipment. With a T_m of 44.7°C, measured via NanoDSF, PETase obtained in this study exhibited the lowest T_m value recorded for PETase so far. Strains that express mutated *TrxB* and *gor* provide an oxidizing environment for proteins with disulfide bonds, and have been shown to increase thermostability of their products in at least one case (Nikolaivits et al., 2016). The slight decrease in thermostability recorded in this study could represent a case in which the use of SHuffle T7 Express as expression host confers less thermostability to the product.

Turbidimetric analysis with PETase

In recent papers, attempts to characterize the activity of PETase towards PET were made (Austin et al., 2018; Fecker et al., 2018; Furukawa et al., 2018; Han et al., 2017; Joo et al., 2018; Yoshida et al., 2016). As a substrate, a 6mm PET film was always used, but the focus never was on an accurate characterization of PETase's activity. Data is presented as relative activity (Han et al., 2017; Liu et al., 2018), concentration of released products (Joo et al., 2018), or decrease in crystallinity of the PET-film (Austin et al., 2018). Also, it is notable that Yoshida mentions a decrease in released MHET and TPA upon increase of enzyme concentration when surpassing a threshold of 100 nM enzyme concentration (Yoshida et al., 2016), supplementary material). For attempts to characterize PETase's activity towards PET, the turbidimetric analysis, as described by Wei et al., 2014, could be a more reliable source. The decrease of turbidity as shown in Figure 24 supports this claim, as it shows how degradation of PET-nanoparticles was not repressed by increased amounts of enzyme. In this study, PETase in concentrations of up to 950 nM was used, and the rate by which the turbidity decreased, increased with higher molarity. These results suggest that the use of nanoparticles eliminates the phenomenon of PETase molecules negatively influencing activity of one another when highly concentrated. On a different note however, turbidity never was decreased below 30% of the initial value; this could be caused by two issues: The enzyme is not able to degrade particles that have been reduced below a certain threshold size, or is not able to access PET-chains in a certain conformation. The PET-nanoparticles used in this work originated from amorphous PET-foil. Wei performed turbidimetric analysis of PET-degradation with PET-nanoparticles of three different origins: PET-granulate, PET-film, and PET-fiber. The PET-degrading enzyme eventually decreased turbidity of nanoparticles from PET-film and PET-fiber by up to 99%. Turbidity of the solution containing nanoparticles from PET-granulate did not decrease by more than 80%, which resembles results obtained in this work. Possibly, nanoparticles from PET-granulate and PET-foil consist of PET-chains in various conformations, of which not all are accessible to PETase. An HPLC-analysis of the release products could give more information about the usefulness of nanoparticles from PET-foil.

5. Summary

Only SHuffle T7 Express was able to produce soluble PBSase, albeit in very low quantities. *E. coli* BL21(DE3) was likely able to produce pelB-PBSase, but the bulk of the enzyme, soluble or insoluble, could not be found when analyzing cell lysates. In future experiments, the expression media of such cultures should be investigated for the presence of PBSase.

The signal peptide of PBSase likely has a delayed, lethal effect on *E. coli*, stored at 4°C, or growing at 16°C.

PBSase is less thermostable than PETase by 6-10°C, with a melting point of about 38°C. Catalytic activity of PBSase against *p*NPA as well as against PET-nanoparticles remains undetermined. Likely, the catalytic activity against both substrates is weaker than the activity of PETase. One turbidimetric analysis using 110 nM of PBSase suggests that PBSase might have a strong ability to hydrolyze PET.

Using SHuffle T7 Express as expression host, PETase was synthesized as a soluble and functional protein. Specific activity against 1 mM *p*NPA was determined to be 18.2 U/mg and could be further increased by additional purification. Thermostability was determined by NanoDSF. The measured T_m of 44.7°C is less than T_m s measured by other research groups (Austin et al., 2018; Joo et al., 2018), which could be caused by use of SHuffle T7 Express as expression host.

The usefulness of turbidimetric analysis, using PET nanoparticles from amorphous PET-foil was demonstrated using various concentrations of PETase.

6. Outlook

The turbidimetric analysis of PBSase has shown a high potential PET-degrading ability. However, yield of functional protein was too low to conduct accurate measurements. Synthesizing and isolating functional PBSase proved to be very difficult, as proper intracellular folding of PBSase appears to be repressed in the environment of *E. coli* cytoplasm. To remedy this, alternative expression strains could be used for expression, like BL21(DE3) pLysSRARE (Hatahet et al., 2010), a strain similar to SHuffle T7 Express in its capability of supporting disulfide bond formation in its cytoplasm. It does this by expressing an additional gene, rather than expressing mutated versions of *TrxB* and *gor*. The use of additives can be tested, as well as the use of chaperones. Chen stated that protein solubility can be increased by performing a heatshock on the expression culture right before induction (Chen, J., Acton, T., Basu, S. K., Montelione G. T., Inouye, M., 2002, 2002). A higher yield of soluble protein could also be obtained by varying the amount of IPTG which is added to an expression culture to induce gene expression. In this work, the final concentration of IPTG was adjusted to the OD₆₀₀ of the expression culture, because expression often was not induced by lower concentrations. This could have negatively affected protein folding, especially when gene expression was induced at later stages of logarithmic growth, when cell counts were much higher. Furthermore, it is unclear whether pelB-PBSase was successfully synthesized or not. The experiment has to be repeated and the expression medium has to be investigated for the presence of PBSase. The safest way to produce high yields of functional PBSase appears to be by refolding the synthesized inclusion bodies via dialysis.

Since PBSase has a lower T_m than PETase, it is not a suitable candidate for future experiments, aimed at creating a PETase with a high melting point.

7. Materials and Methods

7.1 Materials

Equipment used throughout the study has been listed in supplementary Table S1.

7.1.1 Primers

Primer	Sequence	Use
pET22b_PBSase_fw	CGAGCACCACCACCACCACCTGAGATCC	FastCloning
pET22b_PBSase_rv	CGTACGGGTTGGTCTGGGCCATCGCCGGCTG	FastCloning
PBSase_pET22b_fw	GCCGGCGATGGCCAGACCAACCCGTACGAGC	FastCloning
PBSase_pET22b_rv	GGTGGTGGTGGTGGTGGTCTCGAGATTGCACGCCGC	FastCloning
T7_fw	TAATACGACTCACTATAGGG	Sequencing of pET-plasmids
pET24a_rv	GGGTTATGCTAGTTATTGCTCAG	Sequencing of pET-plasmids

7.1.2 Strains

Strain	Speciation	Distributor
<i>E. coli</i> T7 SHuffle Express (will be referred to as “SH”)	<i>fhuA2 lacZ::T7 gene1 [lon] ompT ahpC gal</i> <i>λatt::pNEB3-r1-cDsbC (Spec^R, lacI^q) ΔtrxB sulA11</i> <i>R(mcr-73::miniTn10--Tet^S)2 [dcm] R(zgb-210::Tn10 -- Tet^S) endA1 Δgor Δ(mcrC-mrr)114::IS10</i>	New England BioLabs GmbH (Frankfurth am Main, Deutschland)
<i>E. coli</i> BL21 (DE3)	<i>E. coli</i> str. B F ⁻ <i>ompTgaldcmlonhsdS_B(r_B⁻m_B⁻) λ(DE3 [lacI lacUV5-T7p07ind1sam7nin5]) [malB⁺]_{K-12}(λ^S)</i>	New England BioLabs GmbH (Frankfurth am Main, Deutschland)
<i>E. coli</i> BL21 (DE3) C41 OverExpress	F ⁻ <i>ompT hsdSB (rB- mB-) gal dcm</i> (DE3)	BioCat Heidelberg
<i>E. coli</i> BL21 (DE3) C43 OverExpress	F ⁻ <i>ompT hsdSB (rB- mB-) gal dcm</i> (DE3)	BioCat Heidelberg
<i>E. coli</i> T7 Express LysY/lq	MiniF lysY lacIq(Cam R)/fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10—Tet S)2 [dcm] R(zgb-210::Tn10—Tet S) endA1 delta(mcrC-mrr)114::IS10	New England BioLabs GmbH (Frankfurth am Main, Deutschland)
<i>E. coli</i> TOP 10	F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 Δ lacX74 recA1 araD139 Δ(araleu)7697 galU galK rpsL (StrR) endA1 nupG	Invitrogen Technologies (CA, USA) Life

7.1.3 Plasmids

Plasmid	contents
pET15b	(Amp ⁺)
pET21b_PBSase-SP	(Amp ⁺ , PBS-depolymerase without signalpeptide, His-tag(C-terminal))
pET21b_PBSase	(Amp ⁺ , PBS-depolymerase, His-tag(C-terminal))
pET21b_PETase-SP	(Amp ⁺ , PETase without Signal peptide, His-tag (C-terminal))

pET22b_PETase (Amp+, PETase without Signal peptide, pelB(N-terminal), His-tag (C-terminal))

7.1.4 Chemicals, Enzymes, Additives

All chemicals for the preparation of buffers and solutions as well as media and additives (including chapter 7.1.5) were achieved from Analytik Jena AG (Jena, Germany), Carl Roth GmbH & Co. KG (Karlsruhe, Germany), c-LEcta GmbH (Leipzig, Germany), Fluka (Buchs, Switzerland), Merck KGaA (Darmstadt, Germany), New England Biolabs GmbH (Frankfurt am Main, Germany), Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

Substance	Use
ampicillin (100mg/ml)	cultivation
Glycerol	Storage of cells
IPTG	Induction of protein expression
Agar (15 g / L)	LB-agar plates
DNA Ladepuffer Gel Loading Dye Purple (6 X)	Agarose-gel-electrophoresis
DNA-Marker 1 kbp DNA ladder	Agarose-gel-electrophoresis
p-nitrophenol	pNPA-assay
p-nitrophenylacetate	pNPA-assay
DMSO	pNPA-assay
Roti-Mark STANDARD (14 – 212 kDa)	SDS-PAGE
Unstained Protein Molecular Weight Marker(14,4-116 kDa)	SDS-PAGE
APS	SDS-PAGE
TEMED	SDS-PAGE
Acrylamide 30%	SDS-PAGE
TCE (2,2,2- Trichlorethanol)	SDS-PAGE, TCE-staining
InstantBlue™ Ultrafast Protein Stain	SDS-PAGE, Instant Blue staining
OptiTaQ-Polymerase	PCR
10x Pol. Puffer B	PCR
DpnI	FastCloning
Roti®garose-His/Co Beads	IMAC
EtOH 99%	Storage of columns

7.1.5 Buffers and Media

Buffer/ ingredient	Final concentration[M] / volume[L] / mass [g]	Use
TB-medium		Cultivation
TB-medium (Sigma Aldrich)	40.64 g	
Glycerol	3.2 ml	

MilliQ water	Ad 800 ml	
LB-medium		Cultivation
LB-medium (Sigma Aldrich)	20 g	
MilliQ water	Ad 800 ml	
Sodium Phosphate buffer		Washing cell pellets, desalting, IMAC, <i>p</i> NPA-assays, turbidity-analysis
Sodiumdihydrogenphosphate – monohydrate	40.6 mM	
Disodium-hydrogenphosphate – dodecahydrate	9.4 mM	
MilliQ water	Ad 1 L, pH 7.5	
IMAC-wash buffer		IMAC
TRIS-HCl (pH 7.5)	50 mM	
Sodium chloride	100 mM	
Imidazole	15 mM	
MilliQ water	Ad 1 L	
IMAC-elution buffer		IMAC
TRIS-HCl(pH 7.5)	50 mM	
Sodium chloride	100 mM	
Imidazole	250 mM	
MilliQ water	Ad 1 L	
SDS-running buffer		SDS-PAGE
TRIS	30.3 g	
Glycin	144 g	
SDS	10 g	
MilliQ water	ad 1 L	
SDS-loading buffer		SDS-PAGE
glycerole	25 %	
β-mercaptoethanol	5 %	
0,5 M TRIS- stocksolution	12,5 %	
10 % SDS-stocksolution	20 %	
0,5 % bromphenole stocksolution	2%	
		SDS-PAGE
Lower TRIS Puffer		
TRIS	182 g / L	
SDS	1,5 M, 4 g / L	
MilliQ water	pH 8,8	
Upper TRIS Puffer		SDS-PAGE
TRIS	60 g / L	

SDS 0,5 M, 4 g / L
 MilliQ water pH 6,8

Coomassie blue

Coomassie staining

Coomassie Brilliant Blue 1 g / L
 Water-free acetic acid 10 %
 Ethanole 30 %

Unstainer

Coomassie staining

Water-free acetic acid 10 %
 Ethanol 30 %

50x TAE-buffer

Agarose gel
 electrophoresis

TRIS 242 g / L
 Water-free acetic acid 57,1 mL / L
 EDTA 15,6 g / L
 MilliQ water

Agarose gel

Agarose- gel-
 electrophoresis

TAE 1x 30 ml for a small gel
 Agarose 1%
 RotiSafe Gelstain (Carl Roth, Deutschland) 1,5 µL / 30 ml of gel

7.1.6 Kits

NucleoSpin Gel and PCR Clean-up	Macherey Nagel (Düren, Germany)	gel extraction
innuPREP Plasmid Mini Kit	Analytik Jena AG (Jena, Germany)	plasmid isolation

7.1.7 Programs and webtools used

application	Name of tool	Distributor/website
Calculating ratio of insert to vector for ligation	ligation calculator	http://www.insilico.uniduesseldorf.de/Lig_Input.html
Converting protein weight to concentration	Protein weight in mol converter	http://molbiol.edu.ru/eng/scripts/01_04.html
Primer design and evaluation of sequencing results	Geneius 8.1.7.	Biomatters Ltd. (Auckland, New Zealand) http://www.geneious.com/
Image manipulation	GIMP	https://www.gimp.org
Evaluation of data	Microsoft Office	Microsoft Corporation (WA, USA)
Protein Alignment Align	Protein BLAST	National Center for Biotechnology

Sequences		Information
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7.2 Methods

7.2.1 Microbiological methods

Chemocompetent transformation

50 µl chemocompetent bacteria were taken from -80°C and put on ice to thaw. 2 µl of plasmid DNA was added to the bacteria without mixing them. After incubating the cells on ice for 20 minutes, heat shock was performed in a water bath at 42°C for 30 seconds. The bacteria were then put on ice for a minute. Then 450 µl LB-SOC was added and the tubes were incubated at 33°C at 180 rpm for an hour, for regeneration. The bacteria were then centrifuged at 17 000 g for five minutes. 300 µl of medium was discarded, and the cells were suspended in the remaining medium. The cells were then plated on LB-agar plates containing AMP and incubated at 33°C over night.

Transformation into electrocompetent cells

50 µl of electrocompetent bacteria were taken from -80°C and put on ice to thaw. 1 µl of plasmid DNA was added to the bacteria without mixing them. Electroporesis tubes were taken from ice, wiped clean and the bacteria-DNA-solution was filled into the tubes. Attention was paid to the solution touching both ends to the conducting metal. The cells were shocked with one pulse in an electroporator at 2.5 kV pulse strength and the cells were immediately filled with 200 µl LB-SOC, and the solution was transferred into a micro centrifuge tube and regenerated at 37°C for an hour, spinning at 180°C.

Overnight cultures

4 ml LB medium, with 100µg/ml ampicillin were inoculated with multiple colonies from an agar plate or with 500 µL from a glycerol stock. The cultures were grown over night at 37°C (for *E. coli* Shuffle at 33°C), shaking at 180 rpm.

Growth

Cells were grown in small (50 ml medium in a 250 ml baffled flask), mid-sized (200 ml medium in a 1 L baffled flask), and big batches (400 ml medium in a 2 L baffled flask) of either LB or TB-medium. If required for plasmid maintenance, ampicillin was added to a final concentration of 100 µg/ml. Each culture was inoculated with an amount of overnight culture in ml sufficient to immediately produce an OD₆₀₀ of 0.05.

E. coli SHuffle T7 – induction of gene expression at an OD of 1.

The cultures were grown at 33°C to an OD₆₀₀ of 1 before gene expression was induced by adding IPTG to a final concentration of 1 mM. OD₆₀₀ was measured at several points in order to observe the growth rate. Samples have been withdrawn after induction, three hours later and right before harvest. Cultivation was terminated after 23-24 hours. All harvested cells including samples were treated as described in 7.2.3. (Biochemical Methods – Sonification)

Directly after taking samples, the cells were spun down, the supernatant was discarded, the cells were suspended in cold phosphate buffer, transferred into microcentrifuge tubes, and spun down at 17 000 g and 4°C, before being stored at 4°C.

E. coli SHuffle T7 – induction of gene expression at an OD of 6.

The cultures were grown at 33°C to an OD₆₀₀ of 6 before the cultures were centrifuged at 4000 g and at 4°C for 30 minutes. The old medium was discarded and the cells were resuspended in fresh medium of the same composition. The cells were then poured back into their cultivation flasks and incubated at 16°C. OD₆₀₀ was measured and protein expression was induced by adding IPTG to a final concentration equal to the measured OD₆₀₀ in mM. OD₆₀₀ was measured at several points in order to observe the growth rate. Samples have been withdrawn after the culture reached an OD₆₀₀ of 1, after induction, and right before harvest. Cultivation was terminated after 23-24 hours. All harvested cells including samples were treated as described in 7.2.3. (Biochemical Methods – Sonification)

E. coli BL21 (DE3)

Cells were cultivated and treated the same way as described for *E. coli SHuffle T7 – induction of gene expression at an OD of 1*.

E. coli BL21 (DE3) C41

Cells were cultivated and treated the same way as described for *E. coli SHuffle T7 – induction of gene expression at an OD of 1*.

E. coli BL21 (DE3) C43

Cells were cultivated and treated the same way as described for *E. coli SHuffle T7 – induction of gene expression at an OD of 1*.

E. coli BL21 (DE3) LysY/lq – normal expression time

Cells were cultivated and treated the same way as described for *E. coli SHuffle T7 – induction of gene expression at an OD of 1*.

Storage of strains

500µl of a culture have been filled into a cryotube and 500 µl of 60% glycerol have been added. The tubes were then stored at -80°C for single use. After thawing of the culture at room temperature, the whole stock was used for cultivation. New glycerol stocks were formed from a culture and again stored at -80°C.

Only freshly transformed SHuffle cells were used for cultures.

7.2.2 Methods of Molecular Biology

Polymerase chain reaction

For amplifying the DNA-fragments of the PBSase gene and the pET22b-vector, a PCR was performed. The following recipe and program were used:

Table 3: 100 µl PCR-batches and ingredients aiming at multiplying the PBS-depolymerase gene fragment of about 900 nts (Batch 1) and the pET22b vector fragment of about 5500 nts, carrying a C-terminal histidin-tag and the pelB-sequence (Batch 2).

Ingredient	Batch 1	Batch 2
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pET22b_PBSase_fw	-	2 µl
pET22b_PBSase_rv	-	2 µl
PBSase_pET22b_fw	2 µl	-
PBSase_pET22b_rv	2 µl	-
10 X Pol Buffer C (EURx) (Gdansk, Poland))	10 µl	10 µl
dNTPs	2 µl	2 µl
pET22b-PETase	-	1 µl
pET21b-PBSase	1 µl	-
MilliQ H ₂ O	80.5 µl	80.5 µl
DMSO	0.5 µl	0.5 µl
Optitaq Polymerase (EURx (Gdansk, Poland))	1 µl	1 µl

100 µl of both batches were divided into 4 tubes and the following program was used to produce and amplify the products.

Table 4: PCR program used to create the PBS-depolymerase gene fragment of about 900 nts (Batch 1) and the pET22b vector fragment of about 5400 nts, carrying a C-terminal histidin-tag and the pelB-sequence (Batch 2). Step 1: initial separation step. Step 2: separation step. Step 3: Primer annealing step; temperature was chosen appropriately for the respective primers. Step 4: Extension step; as the Optitaq Polymerase polymerizes about 1 kb per minute, the elongation times were chosen accordingly. Steps 2-4 were repeated 30 times. Step 5: final elongation step. Step 6: Storage of PCR-products.

	Batch 1			Batch 2	
Step 1	1 minute	95 °C		1 minute	95 °C
Step 2	30 seconds	95 °C	Repeat for 30 cycles	30 seconds	95 °C
Step 3	30 seconds	55 °C		30 seconds	53 °C
Step 4	1 minute	72 °C		6 minutes	72 °C
Step 5	5 minutes 30 seconds	72 °C		5 minutes 30 seconds	72 °C
Step 6	Storage	15 °C		Storage	15 °C

After PCR has finished, 1 µl of DpnI was added to each tube, digesting the template DNA for 2 hours at 37°C. Then, the whole digest was loaded on a preparative gel.

FastCloning and DpnI-digestion

FastCloning combines ligation with transformation (Li et al., 2011). The two DNA-fragments have been produced via PCR. The methylated template DNA has been digested with DpnI, and the fragments have been loaded onto a preparative gel. The DNA was cut out and extracted from the gel using the InnuPrep Plasmid Mini Kit. The amount of insert and vector was determined photometrically via NanoDrop™. Both were transformed with a relation of insert to vector of 3:1 into chemocompetent *E. coli* Top 10 cells by inducing heatshock. The correct amount of DNA to add was calculated by the webtool “Ligation calculator”.

Agarose-gel analysis

1%-(w/v) agarose gels were prepared by dissolving 1.5 µl of RotiSafe Gelstain (Carl Roth, Germany) in 30 ml heated agarose in 1 X TAE-buffer. The mixture was poured into a form and wells were formed with combs. The gel was put into an apparatus filled with 1 X TAE-buffer. 2-10 µl of sample, mixed

1:1 with DNA-loading buffer were loaded. Additionally, 2 µl of a 1kbp DNA ladder (Carl Roth, Germany) were loaded for size comparison. The gels ran in an electrophoresis chamber at 100V, 400 mA and 150W for 30 minutes. Then, the gel was radiated with UV-light.

Sequencing

Sequencing was performed by Eurofins MWG GmbH (Ebersberg, Germany).

Gelextraction

Gel extractions were performed using the *NucleoSpin Gel and PCR Clean-up Kit* by Macherey Nagel (Düren, Germany)

Plasmid isolation

Chemical lysis and plasmid isolation was performed using the *innuPREP Plasmid Mini Kit* by Analytik Jena AG (Jena, Germany).

7.2.3 Biochemical methods

Sonication

Washing:

Directly after withdrawing samples from a culture, the cells were spun down for 10 minutes at 17 000 g and 4°C, the supernatant was discarded. The cells were then suspended in cold phosphate buffer, transferred into microcentrifuge tubes, and spun down again for 10 minutes at 17 000 g and 4°C in an ultracentrifuge. The pellets were then suspended in 500 µl cold phosphate buffer and stored at 4°C until further use.

After harvesting a culture, the cells were spun down in an appropriate centrifuge at max speed and 4°C, and the supernatant was either discarded or saved. The pellets were then resuspended in 10-15 ml of cold phosphate buffer and transferred into weighted falcon tubes. The cells were again spun down and resuspended in a standardized manner (2 ml of cold phosphate buffer for each 1 gram of cell mass) in preparation for pNPA-assays. Then, the cells were either stored at 4°C if used the next day, or stored at -20°C.

Lysis by sonication:

Time samples were lysed by sonication with a MS72 sonotrode for 1 minute at 50% power. The tubes were always kept on ice during sonication. The cells from harvested small batches were lysed for 2 minutes and 30 seconds at 50% power. Cells from medium batches were lysed using a MS73 sonotrode for 2 times 3 minutes at 50% power. Cells from big batches were lysed using a KE76 sonotrode three times for 3-5 minutes. The cells were always kept on ice.

Separation of soluble and insoluble fractions:

After sonication, the lysate was centrifuged in an ultracentrifuge at 17 000 g and at 4°C. The supernatant (called the “soluble fraction”) was saved in a new tube and stored at 4°C. The insoluble fraction was saved. The insoluble fraction of a harvested culture was discarded.

Immobilized Metal Affinity Chromatography (IMAC)

IMAC was either performed using Roti®garose-His/Co Beads in columns, or AKTA PURE. IMAC, using columns was performed using the following protocol: The column was provided in 20% EtOH. The EtOH ran through and was discarded. The column was washed three times with 10 ml wash buffer.

Afterwards, the protein sample was applied (2-8 ml) and the column was buried in ice and shaken slowly for 20 minutes. Resulting flow-through was collected and the column was washed twice with wash buffer. Both washing fractions were saved. Then, the flow-through was again poured onto the column and it was again incubated on ice, shaking slowly for about 20 minutes. The flow-through was then collected again, the column was washed twice (these washing fractions were discarded) and the protein was eluted using 8 ml of elution buffer. Elution fractions were collected in 2ml-tubes. The column was washed with 5 ml elution buffer and again with 3 times 10 ml of washing buffer, before the next use. IMAC, using ÄKTA PURE was performed using the following protocol: At first, the column was equilibrated by washing with 5 column values (CV, accounts for 5 ml each) of washing buffer. Then, the sterile filtered sample was loaded into the superloop column. Next, the column was washed with 4-6 CV of wash buffer. The flow through was collected. Then, the protein was eluted by using the elution buffer. Elution fractions were desalted using the PD-10 desalting protocol.

Desalting of purified protein, using the PD-10 desalting columns

After IMAC purification, protein solutions contained imidazole, which interferes with *p*NPA-assays. Protein solutions were desalted using the protocol for PD-10 desalting columns (GE Healthcare Bio-Sciences AB (Uppsala, Schweden)). After desalting, the proteins were stored in 50 mM sodium phosphate buffer.

Turbidimetric analysis using PET-nanoparticles

PET-hydrolyzing activity of any given enzyme can be measured by turbidimetric analysis, as previously described (Wei et al., 2014a). Instead of following the protocol described by Wei, a simplified version was performed. 200 µl of PET-nanoparticles were added to a microtiter plate and mixed with 50 µl of enzyme solution in 50 mM sodium phosphate buffer, pH 7.5. The reaction was started by exposing the mixture to 30 °C. Photometrical measurements of the mixtures at OD₆₀₀ were taken every 15 minutes. Relative turbidity was calculated using the following formula:

$$turbidity(\%) = \frac{|OD_{600}|_{tn}}{|OD_{600}|_{t0}} * 100$$

whereas *tn* represents measurements taken over the course of the reaction, while *t0* represents the starting time of the reaction.

PET-nanoparticles from amorphous PET-foil were kindly provided by Dr. Ren Wei, Institute of Biochemistry, Leipzig, Germany. Before usage, the nanoparticles were filtered with a paper filter. PET-nanoparticles were always stored at 4°C.

***p*NPA-assay**

Para-nitrophenylacetate is a substrate that is easily hydrolyzed by esterases and can therefore be used to test the functionality of most enzymes with esterase activity. The assay is fit for both qualitative and quantitative measurements of esterase activity. In this assay, *p*NPA is hydrolyzed to *para*-nitrophenol (*p*NP) and acetic acid, as shown in Figure 42.

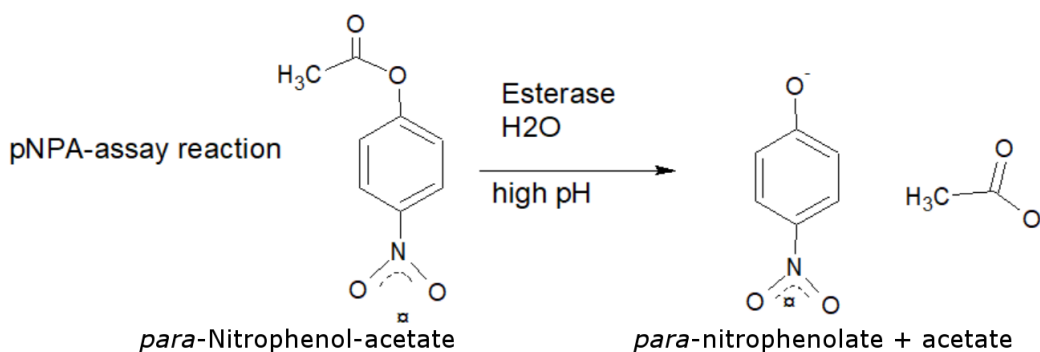


Figure 27: the catalytic mechanism of *p*NPA-hydrolyzation

Water also hydrolyzes the substrate, making blank measurements of autohydrolysis a necessity. The assay is conducted in 1 ml cuvettes, using 850 μl of phosphate buffer and adding 50 μl of enzyme solution. The reaction is started by adding 100 μl of 10 mM *p*NPA in DMSO. While the substrate solution containing *p*NPA is colorless, its product of hydrolysis, *p*NP, absorbs light at 410 nm and produces a yellow color. pH of the buffer solution has to be kept above 6. Otherwise, *p*NP would be protonated due to its pKa of 6, which would cause it to lose the yellow color. In order to calculate activity of the enzyme solution in Units per milliliter a standard curve has to be produced with different concentrations of *p*NP.

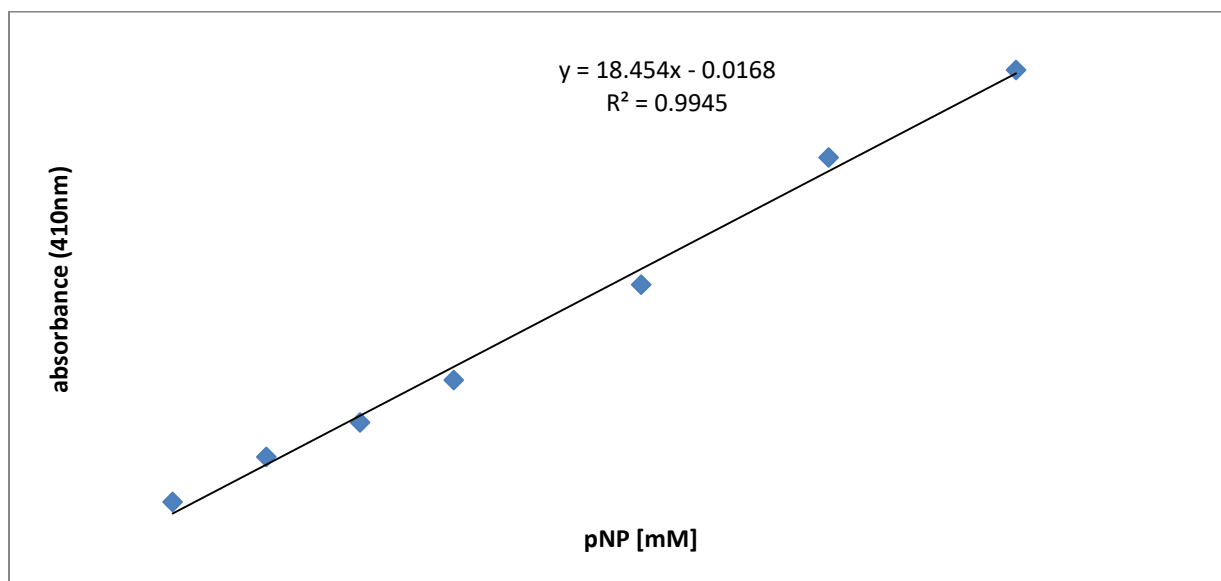


Figure 28: *p*NP-standard curve using absorbance measurements at 410 nm of 100 μl of a *p*NP solution, with rising *p*NP-concentrations in sodium phosphate buffer. Concentrations of the *p*NP-solution ranged from 0.01 mM to 0,1 mM. Each measurement was conducted in 900 μl 50 mM sodium phosphate buffer pH 7.5.

The molar absorption coefficient was calculated using the Lambert-Beer-Law:

$$A = \epsilon \cdot c \cdot d$$

Equation 1: Lambert-Beer-Law. A: measured absorbance, ϵ : molar absorption coefficient in $\text{L} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$. C: concentration of absorbing substance (mol/L), d: thickness of cuvette in cm.

The obtained molar absorption coefficient of 18.454 is comparable to data regarding molar absorptivity of *p*NP, as presented by Bowers and McComb (Bowers et al., 1980). The volumetric

activity of a reaction solution has been calculated using the following derivative equation of the Lamber-Beer-Law:

$$activity \left(\frac{\mu\text{mol}}{\text{ml} * \text{min}} \right) = \left[\frac{\frac{\Delta\text{abs}}{\Delta t(\text{min})} - \text{autohydrolysis}}{\varepsilon \left(\frac{L}{\text{mol} * \text{cm}} \right)} \right] * 20 * \text{dilution factor}$$

Equation 2: volumetric activity of 1 ml of reaction solution, containing 850 μl of 50 mM sodium phosphate buffer, 50 μl of enzyme solution in the same buffer, and 100 μl of substrate solution.

$\frac{\Delta\text{abs}}{\Delta t(\text{min})}$ is the measured change in absorbance over one minute. ε is the molar absorption coefficient. The calculated value is multiplied by the dilution factor of the enzyme solution, and an additional 20 for diluting 50 μl of enzyme solution in 1 ml of reaction mixture.

Specific activity (displayed as U/mg) of a given enzyme can be calculated by dividing the calculated activity [U/ml] of a protein solution by the protein concentration [mg/ml] of that solution.

Sodiumdodecylsulfate polyacrylamide gel electrophoresis

Using sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), denatured proteins can be separated by length as they move through a matrix of polymerized acrylamide. The proteins move from the negative pole along an electricity gradient to the positive end. Denaturation is achieved by the concerted effect of β -mercaptoethanol, SDS and a cooking step at 95°C for five to ten minutes. SDS is a detergent that works together with β -mercaptoethanol, which reduces disulfide bonds. SDS also masks the charge of proteins and turns it consistently negative. The running buffer contains Glycine, SDS and TRIS. Glycine is required as conductor, while SDS keeps the charge of the proteins negative, leading to a continuous flow of running buffer from the negatively to the positively charged pole, driving the protein through the matrix. TCE binds to tyrosine and tryptophane in proteins and emits fluorescent light under UV-light. By adding TCE, proteins in a SDS-gel can be detected via UV-light, immediately after stopping the run.

SDS-PAGE was performed using the Biorad™ system. Gels were prepared using the recipe shown in Table 12.

Table 5: ingredients used when preparing a 10% resolving gel, and a 4% stacking gel. The gels were poured immediately after addition and thorough mixing of APS and TEMED. After pouring, the acrylamide was left to polymerize for about 30 to 60 minutes.

Ingredients	Resolving Gel (10%)	Stacking Gel (4%)
Lower Buffer	2 ml	-
Upper Buffer	-	1 ml
Acrylamide, 30%	2.66 ml	0.53 ml
Ultrapure water	3.33 ml	2.47 ml
APS	40 μl	40 μl
TEMED	4 μl	4 μl
Trichloroethanol (TCE)	75 μl (optional)	

Time samples were prepared for SDS-PAGE by mixing 30 μl of soluble fraction with 10 μl of SDS-loading buffer, heating the mixture at 95°C for 5 minutes and then shortly spinning it down. 20 μl of soluble fraction were loaded. The insoluble fractions of time samples were prepared for SDS-PAGE by

adding 30 µl of SDS-loading buffer, resuspending the pellet, heating the mixture at 95°C for 10 minutes, and then spinning it down for 5 minutes at 17 000 g. 5 µl of insoluble fraction were loaded. Crude lysates of harvested cultures, were prepared by mixing 10 µl of the soluble fraction with 10 µl of SDS-loading buffer, heating the mixture at 95°C for 5 minutes, and then shortly spinning it down. 5 µl of soluble fraction were loaded. As control, 10 µl of one of two markers was loaded onto each gel.

Nanoquant – determination of protein content

Protein content of protein solutions in 50 mM sodium phosphate buffer were determined by Nanoquant assay, following the Roti®-Nanoquant manual from Carl Roth.

NanoDSF analysis

NanoDSF measurements for melting curve analysis were performed using the Prometheus NT.48 instrument (NanoTemper, Munich, Germany), following the distributor's manual. The samples were heated by constantly increasing the temperature, starting at 20°C and finishing at 95°C. The signal increase was monitored and the graph was displayed by its first derivative.

Matrix-Assisted Laser-Desorption-Ionization, using time-of-flight analysis.

MALDI-TOF was performed by the Institute of Microbiology, Department for Microbial Physiology and Molecular Biology, Greifswald University.

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9. Appendix

9.1 Zusammenfassung

Der Stamm SHuffle T7 Express war als einziger in der Lage, lösliche PBSase zu produzieren, wenn auch in sehr geringen Mengen. *E. coli* BL21(DE3) war wahrscheinlich in der Lage PelB-PBSase zu synthetisieren, aber der Großteil des Enzyms war weder im löslichen, noch im unlöslichen Teil des Zelllysates zu finden. Es ist empfehlenswert in zukünftigen Experimenten das Expressionsmedium eines solchen Stammes auf den Verbleib der PBSase zu untersuchen.

Das Signalpeptid der PBSase hat wahrscheinlich einen verzögerten, aber letalen Effekt auf *E. coli*, unabhängig ob die Zellen bei 4°C gelagert werden, oder bei 16°C weiter wachsen.

PBSase hat einen Schmelzpunkt bei etwa 38°C und ist damit um etwa 6-10°C weniger thermostabil als PETase. Die katalytische Aktivität der PBSase gegen *p*NPA und PET konnte nicht genau bestimmt werden, erscheint aber um einige Grade schwächer als die Aktivität der PETase. Eine turbidimetrische Analyse zeigte eine starke Aktivität von PBSase gegen PET-nanopartikel.

Unter Verwendung des Stammes Shuffle T7 Express konnte PETase als lösliches und funktionelles Enzym produziert werden, und zeigte eine starke Aktivität gegen PET-nanopartikel. Die spezifische Aktivität gegen 1mM *p*NPA wurde errechnet und betrug 18.2 U/mg. Dieser Wert kann noch durch weitere Aufreinigung erhöht werden. Thermostabilität wurde gemessen mittels NanoDSF und die gemessene Schmelztemperatur betrug 44.7°C, was um 2-4°C weniger ist als die Schmelzpunkte, die von anderen Gruppen gemessen wurden (Austin et al., 2018; Joo et al., 2018). Dieser leicht niedrigere Schmelzpunkt könnte durch die Verwendung von Shuffle T7 Express als Expressionsstamm zustande gekommen sein.

Die turbidimetrische Analyse unter Verwendung von PET-nanopartikeln, die aus amorpher PET-folie hergestellt wurden, wurde mit dem Einsatz von verschiedenen Konzentrationen von PETase demonstriert.

9.2 Index for abbreviations

AMP	ampicillin	PAGE	Polyacrylamide gel electrophoresis
APS	Ammoniumperoxodisulfate	PBS	Poly-butylene succinate
bp	Basepairs	PBSA	Poly-butylene succinate co-adipate
BHET	Bis-hydroxyethylterephthalate	PBSase	Polybutylene succinate depolymerase
d	Days	PE	Polyethylene
dH ₂ O	Distilled water	PET	Poly(ethylene-terephthalate)
DNA	Desoxyribonucleic acid	PET-NP	PET-nanoparticles
DMSO	Dimethylsulfoxid	PETase	Polyethylenterephthalate hydrolase
DSC	Differential Scanning Calorimetry	pNPA	<i>para</i> -nitrophenol-acetate
DSF	Differential Scanning Fluorimetry	pNP	<i>para</i> -nitrophenol
<i>E. coli</i>	<i>Escherichia coli</i>	PP	Polypropylene
EDTA	Ethylendiamintetraessigsäure	PVC	Polyvinylchloride
EG	Ethylene glycol	rpm	Revolutions per minute
g	Gram	SEC	Size exclusion chromatography
h	Hours	SDS	Sodium-dodecylsulfate
H ₂ O	Water	SN	Supernatant
IMAC	Immobilized Metal affinity chromatography	T.	<i>Thermobifida</i>
IPTG	Isopropyl β-D-1-thiogalactopyranoside	TAE	TRIS-Acetate-EDTA
kDa	Kilodalton	TB	Terrific broth
L	Liter	TCE	2,2,2-Trichlorethanol
LB	Lysogeny broth	TEMED	N,N,N',N'-tetramethylethylen-diamine.
LB-SOC	Super optimal broth with catabolite repression	TfH	<i>Thermobifida fusca</i> Hydrolase
LCC	Leaf-and-branch-compost Cutinase	TfCut	<i>Thermobifida fusca</i> Cutinase (=TfH)
m	Minute	T _g	Glass-transition temperature
MALDI-TOF	Matrix-assist Laser-Desorption-ionization using time-of-flight analysis	T _m	Melting temperature
mA	Miliampère	TPA	Terephthalic acid
mg	Miligram	TRIS	Tris-hydroxymethyl-aminomethan
MHET	Mono-(2-hydroxyethyl)terephthalate	V	Volt
mL	Mililiter	W	Watt
ng	Nanogram	μg	Microgram
OD	Optical density	μl	Microliter
P	Pellet	°C	Degree Celsius

Also, the single-letter codes and three-letter codes for aminoacids were used.

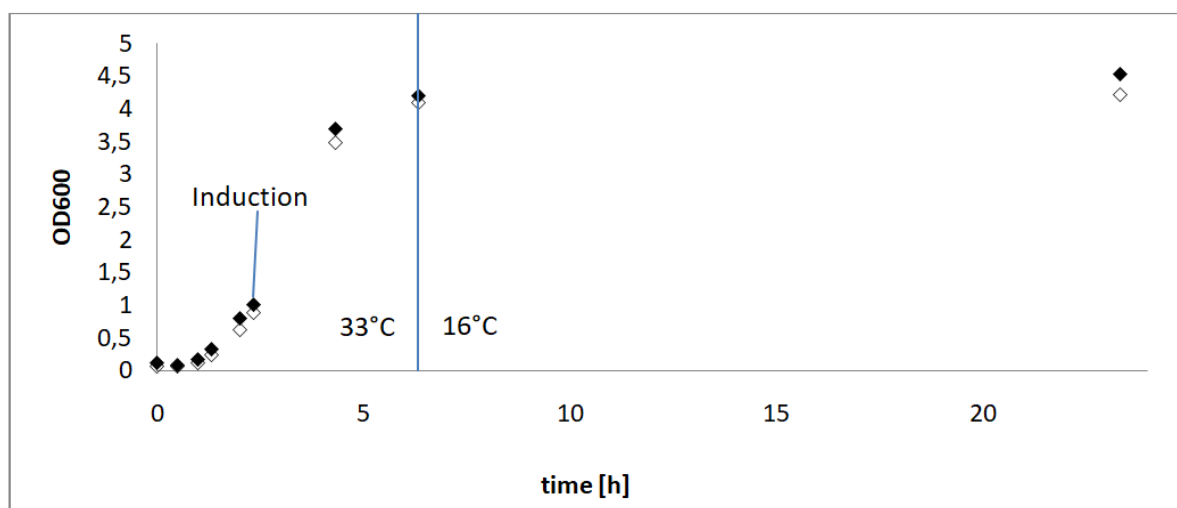
9.3 Equipment

Supplementary Table S1: Equipment used throughout the time of work.

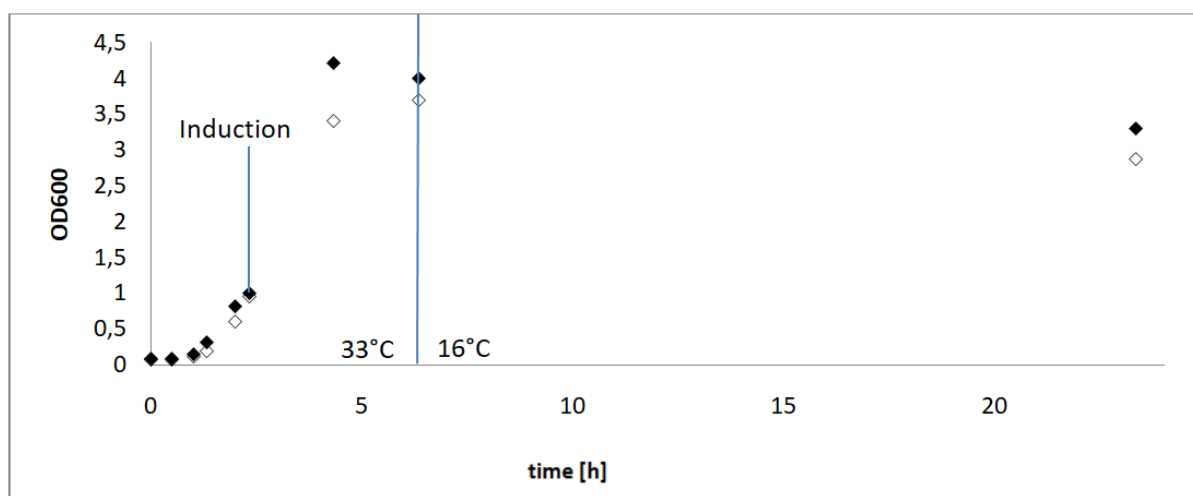
Model	Distributor	Use
V-120	Systec (Linden, Germany)	Autoclave
Laboklav 135 MS-FA	SHP Steriltechnik AG (Schloss Detzel, Germany)	Autoclave
Perfect RDS Schnell-Kochtopf	WMF (Geislingen an der Steige, Germany)	Autoclave
Heidolph MR 3001 K	Heidolph Instruments GmbH & Co. KG (Schwabach, Germany)	Magnetic stirrer
RCT Basic	IKA®-Werke GmbH & Co. KG (Staufen, Germany)	Magnetic stirrer
Mini-Sub-Cell GT	Bio-Rad Laboratories GmbH (Munich, Germany)	Agarose–gel–electrophoresis chamber
Compact XS / S	Biometra GmbH Göttingen, Germany)	Agarose–gel–electrophoresis chamber
EV231	Consort (Turnhout, Belgium)	Agarose–gel–electrophoresis power supply
EPS 301	GE Healthcare Life Sciences (Freiburg, Germany)	Agarose-gel-electrophoresis power supply
Standard Power Pack P25	Biometra GmbH (Göttingen, Germany)	Agarose-gel-electrophoresis power supply
PD-10 desalting-column	GE Healthcare Bio-Sciences AB (Uppsala, Sweden)	Desalting
Revco™ UxF -86°C-ultra low freezer	Thermo Fisher Scientific Inc. (Waltham, MA, USA)	Storage (-80°C)
Sanyo V.I.P Series MDF-U32V	Sanyo (VIP Series)	Storage (-80°C)
Eppendorf Thermomixer comfort	Eppendorf (Wesseling–Berzdorf, Germany)	Cooking samples
AKTA PRIME 25 M	GE Healthcare Life Sciences	IMAC
Incucell	MMM Medcenter Einrichtungen GmbH Munich, Germany)	Cultivation
KIMAX® baffled culture flask	Kimble Chase (Rockwood, TN, USA)	Cultivation
Minitron Infors AG (Bottmingen, Swiss)	Multitron Infors AG (Bottmingen, Swiss)	Cultivation
Unitron Infors AG (Bottmingen, Swiss)	Multitron Infors AG (Bottmingen, Swiss)	Cultivation
Minitron Infors AG (Bottmingen, Swiss)	Multitron Infors AG (Bottmingen, Swiss)	Cultivation
pH 211 Microprocessor pH Meter	HANNA Instruments Germany GmbH (Vöhringen, Germany)	Buffers
UVmini-1240	Shimadzu (Duisburg, Germany)	Photometric analysis, pNPA-assays
V-1200 Spectrophotometer	VWR International GmbH	Photometric analysis

	(Darmstadt, Germany)	
NanoDrop™ 1000	Peqlab (Erlangen, Germany)	Photometric analysis
Infinite® M200 Pro	Tecan Trading AG (Männedorf, Swiss)	Nanoquant, Turbidity-analysis
Minigel-Twin	Biometra GmbH (Göttingen, Germany)	SDS-PAGE
Mini-PROTEAN® Tetra Cell Systems	Bio-rad Laboratories GmbH (Munich, Germany)	SDS-PAGE
Herasafe™ KS15	Thermo Fisher Scientific Inc. (Waltham, MA, USA)	Sterile bench
Flexcycler2 Biometra GmbH	Biometra GmbH (Göttingen, Germany)	PCR
Sonoplus HD2070	Bandelin electronic GmbH & Co. KG (Berlin, Germany)	Sonification
Vortex-Genie® 1/2	Scientific Industries Inc. (Bohemia, NY. USA)	Vortexing Samples
Vortex-Mixer	neoLab Migge GmbH (Heidelberg, Germany)	Vortexing Samples
Explorer® E14130	Ohaus Europe GmbH (Greifensee, Swiss)	Scale
KERN EMB 600-2	KERN & SOHN GmbH (Balingen-Frommern, Germany)	Scale
KERN PCB 350-3	KERN & SOHN GmbH (Balingen-Frommern, Germany)	Scale
KERN PCB 2500-2	KERN & SOHN GmbH (Balingen-Frommern, Germany)	Scale
Sartorius AC 120 S	Sartorius AG (Göttingen, Germany)	Scale
Milli-Q Reference	Merck Millipore (Billerica, MA, USA)	Distributor of ultra-pure water
W1	Labortechnik Medingen (Arnsdorf, Germany)	Water bath
Heraeus Fresco 17 / Biofuge Fresco	Thermo Fisher Scientific Inc. (Waltham, MA, USA)	Centrifuge
Heraeus Labofuge 400 R	Thermo Fisher Scientific Inc. (Waltham, MA, USA)	Centrifuge
Heraeus Multifuge 3 S-R	Thermo Fisher Scientific Inc. (Waltham, MA, USA)	Centrifuge
Heraeus Mpico 17 / Biofuge Pico	Thermo Fisher Scientific Inc. (Waltham, MA, USA)	Centrifuge
Sprout Minizentrifuge	Biozym Scientific GmbH (Hessisch Oldendorf, Germany)	Centrifuge
Prometheus Series instrument	NanoTemper Technologies GmbH	NanoDSF

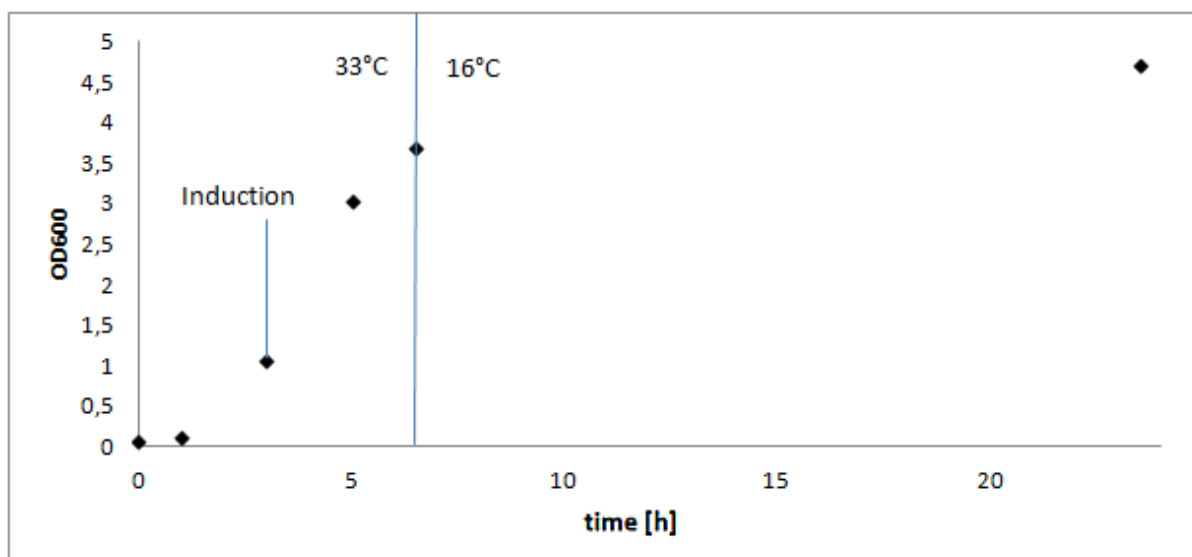
9.4 Supplementary Figures



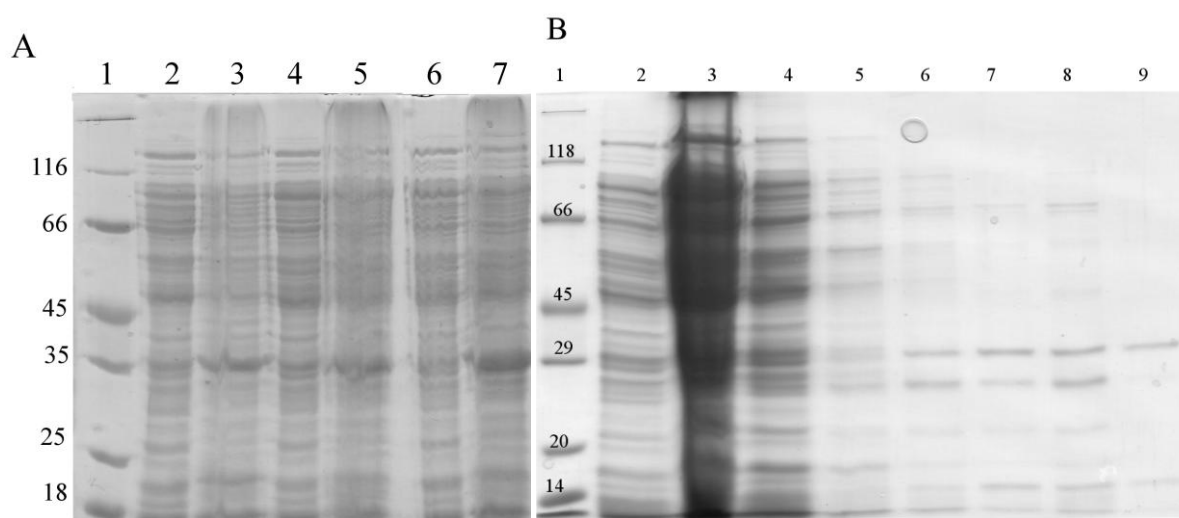
Supplementary Figure S1: Growth curve, following *E. coli* C41(DE3) cells, synthesizing mature PBSase (black symbols) after induction at an OD_{600} of 1. Growth is plotted against a culture carrying an “empty” pET15b plasmid (white symbols), grown under the same circumstances. Both cultures were set up in 50 ml LB-medium in 250 ml baffled flasks, grown continuously at 33°C for 6 hours. The cultures were then induced by adding IPTG to a final concentration of 1 mM. After 3 hours of expression at 33°C, expression temperature was decreased to 16°C over night. After 18 hours of expression at 16°C, both cultivations were terminated.



Supplementary Figure S2: Growth curve, following *E. coli* C43(DE3) cells, synthesizing mature PBSase (black symbols) after induction at an OD_{600} of 1. Growth is plotted against a culture carrying an “empty” pET15b plasmid (white symbols), grown under the same circumstances. Both cultures were set up in 50 ml LB-medium in 250 ml baffled flasks, grown continuously at 33°C for 6 hours. The cultures were then induced by adding IPTG to a final concentration of 1 mM. After 3 hours of expression at 33°C, expression temperature was decreased to 16°C over night. After 18 hours of expression at 16°C, both cultivations were terminated.



Supplementary Figure S3: Growth curve of T7 Express LysY/lq cells, synthesizing mature PBSase after induction at an OD₆₀₀ of 1. The culture was set up in 50 ml LB-medium in a 250 ml baffled flask, and grew continuously at 33°C until it reached an OD of 1, at which point the culture was induced by adding IPTG to a final concentration of 1 mM. Expression was continued for 3 hours, until expression temperature was decreased to 16°C for overnight expression. After 16 hours of expression at 16°C, the cultivation was terminated.



Supplementary Figure S4: TCE-stained SDS-gels, following the synthesis and purification of mature PBSase, synthesized from a plasmid construct that contains the shortened PBSase gene in SHuffle T7 Express cells, after induction with a final concentration of 12 mM IPTG at an OD₆₀₀ of 6. Figure S4A shows the protein contents of the soluble and insoluble fractions of crude lysates, obtained from disrupted cells of time samples. The wells show the following fractions; 2-3: soluble and insoluble fraction of t₀, taken before induction, after the culture reached an OD of 1; 4-5: soluble and insoluble fraction of t₁, taken right after induction; 6-7: soluble and insoluble fraction of t₂, taken after 16 hours of gene expression. A band is visible in insoluble fractions at a molecular size of 29 kDa. For size comparison, a protein marker Roti-Mark STANDARD was loaded onto the first well. Figure S4B shows a TCE-stained SDS-gel displaying various fractions obtained from IMAC of the soluble fraction of the culture. 2: Crude Lysate (diluted 1:10); 3: Flow-through; 4-5: Wash fractions 1 & 2. 6-10: Elution fractions 1-5. Elution fractions 1-4 were taken by eluting protein with 2 ml of Elution buffer. Elution fraction 5 was taken by eluting with 7 ml of Elution buffer. Protein bands are visible at a molecular size of 29 kDa. For size comparison, a protein marker Roti-Mark STANDARD was loaded onto the first well.