Deutsche Fassung:
Beschluss der Curricula-Kommission für Bachelor-, Master- und Diplomstudien vom 10.11.2008
Genehmigung des Senates am 1.12.2008

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommene Stellen als solche kenntlich gemacht habe.

Graz, am ……. ……..
(Unterschrift)

Englische Fassung:

STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

……. ……..
date (signature)

Technische Universität Graz

Institut für Umweltbiotechnologie

DIPLOMARBEIT

Kinetic investigation of Enzymatic Hydrolysis of Polyesters and Model Substrates

vorgelegt von

Eva Trotscha

Zur Erlangung des akademischen Grades einer

Diplomingenieurin

Betreuer:

Univ.Prof. DI Dr. Georg M. Gübitz
Institut für Umweltbiotechnologie
Technische Universität Graz

2012

ABSTRACT

Kinetic investigation of Enzymatic Hydrolysis of Polyesters and Model Substrates

Five enzymes originated from *Thermobifida fusca*, *Thermobifida cellulolysitica*, *Thermobifida alba* and *Bacillus subtilis* isolated cloned and expressed *in E.coli* BL21-Gold(DE3), were produced and investigated. In addition to the self fermented enzymes a purchased cutinase from *Humicola insolens* was explored. All enzymes were characterised according to Michaelis Menten parameters on small ester substrates, *para*-Nitrophenyl-Acetate, -Butyrate and –Palmitate and their hydrolytic activity towards the watersoluble polyester model substrate bis(benzoyloxyethyl)terephthalate (3PET). For the *Bacillus subtilits* esterase the Michaelis Menten parameter was additionally determined for the watersoluble polyester model substrate of bis(2-hydroxyethyl)terephthalate (BHET). Hydrolysis activity on Polyethyleneterephthalate (PET) of all enzymes was investigated using PET-foils. The release of degradation products was monitored by high performance liquid chromatography (HPLC) with UV-VIS detection. Enzyme hydrolysis's impact on the PET-foils was quantified by measuring changes in surface hydrophilicity using the surface investigating techniques water contact angle measurement (WCA) and fourier transform-infrared spectroscopy attenuated total reflectance (FTIR-ATR) analysis was employed to show changes in the crystallinity index on the incubated PET material. Indicated surface hydrolysis was substantiated by using fluorescence spectroscopy to quantify the amount of new formed carboxylic and hydroxyl groups on the PET foils after derivatization with 2-(bromomethyl)naphthalene.

ZUSAMMENFASSUNG

Kinetische Untersuchungen der enzymatischen Hydrolyse von Polyestern und Modellsubstraten

Es wurden fünf isolierte und klonierte Enzyme aus *Thermobifida fusca*, *Thermobifida cellulolysitica*, *Thermobifida alba* und *Bacillus subtilis*, welche in *E.coli* BL21-Gold(DE3) exprimiert wurden, produziert und untersucht. Zusätzlich zu den selbst hergestellten Enzymen wurde eine gekaufte Cutinase aus *Humicola insolens* erforscht. Alle Enzyme wurden in Bezug auf ihre Michaelis Menten Parameter der Estersubstrate *para*-Nitrophenyl-Acetat, -Butyrat und –Palmitat sowie auf ihre hydrolytische Aktivität gegen das wasserlösliche Polyester Modellsubstrat bis(benzoyloxyethyl)terephthalat (3PET) hin charakterisiert. Für die Esterase von *Bacillus subtilits* wurden zusätzlich die Michaelis Menten Parameter für das wasserlösliche Polyester Modelsubstrat bis(2-hydroxyethyl)terephthalat (BHET) bestimmt. Die Hydrolyseaktivität gegen Polyethyleneterephthalat (PET) aller Enzyme wurde unter Verwendung dünner PET-Folien erforscht. Die Freisetzung von hydrolytischen Abbauprodukten wurde mittels Hochleistungsflüssigkeitschromatographie (HPLC) unter UV-VIS Detektion überwacht. Die Auswirkungen enzymatischer Hydrolyse auf die PET-Folien wurden durch Ermittlung der Änderung in der Oberflächenhydrophobizität der Folien mit Hilfe der oberflächencharakterisierenden Methode Kontaktwinkel-Messung (WCA) nachgewiesen. Weiters wurde die Fourier-Transformations-Infrarotspektrometrie mit abgeschwächter Totalreflexion (FTIR-ATR) angewandt, um Änderungen des Kristallinitätsindex von enzymatisch behandeltem PET-Material aufzudecken. Mittels Fluoreszenzs-Spektroskopie wurde eine mögliche Hydrolyse der PET-Folien durch Quantifizierung der neu gebildeten Carboxyl- und Hydroxyl-Gruppen auf der Oberfläche nach vorheriger Derivatisierung mit 2-(bromomethyl)naphthalen nachgewiesen.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Dr. Georg Gübitz for giving me the opportunity for this work. Without his constant support and his reassuring influence this thesis would not have been possible.

Special thanks go to my thesis adviser Dr. Enrique Herrero Acero. His guidance throughout the process, his infinite patience and his ability to always keep a clear head were the key to the success of the present work.

This thesis would never have been completed without the encouragement and devotion of my beloved partner Dr. Wolfgang Belitsch. And the support of my dear friends Teresa Flock, Annemarie Marold, Stefan Engelbrecht and Katja Dzichkouvskaya.

Many thanks go to Dr. Sonja Heumann, Dr. Doris Ribitsch, Dr. Inge Eiteljörg and all the others that taught me so much about enzymes and patiently answered all my questions.

To DI Katrin Greimel, DI Alexandra Rollett and all of my co-workers, for the cooperativeness, the good atmosphere and the great time we had in the lab.

To my colleagues and superiors in my job, for giving me the opportunity to pull of my studies and showing me that a job really could be a calling.

And to my parents, Bärbel and Richard Trotscha, for their unconditional love and support.

"Previous generations have been absolutely convinced that their scientific theories were well-nigh perfect,
only for it to turn out that they had missed the point entirely.
Why should it be any different for our generation?"

T. Pratchett, The Science of Discworld 1999

TABLE OF CONTENTS

ABBREVIATIONS

BsEstB	*Bacillus subtilis* Esterase B
LB	Luria-Bertani
IPDG	Isopropyl-β-D-thio-galactoside
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
BSA	Bovine Serum Albumin
*p*NP-A, -B, -P	*para*-Nitrophenyl-Acetate, -Butyrate, -Palimate
HPLC	High Performance Liquid Chromatography
3PET	Bis(benzoyloxyethyl)terephthalate
PET	Polyethyleneterephthalate
BA	Benzoic acid
TA	Terephthalic acid
MHET	Mono-(2-hydroxyethyl)terephtalate
BHET	Bis-(2-hydroxyethyl)terephtalate
HEB	2-hydroxyethyl benzoate
WCA	Water Contact Angle
EG	Ethylene glycol

LIST OF FIGURES

LIST OF TABLES

1 INTRODUCTION

After the industrial revolution and the following emergence of chemical technologies nowadays a demand of environmentally sustainable techniques has arisen. Not only the environmental friendly production of materials but also their recycling is a field that has gained interest and around recycling of materials a whole new sector of biotechnology has come up. The so-called grey biotechnology deals with waste treatment by using microbiological systems (e.g. bacteria, fungis, enzymes, etc.) that are present in the ecosystem.
An annual growth of 4.5 % over the last eight years (Oswald, 2006) has topped in an average use of 37 kg plastics per capita in the year 2010 (Herrero Acero *et al.*, 2011). Among all plastic, Polyethyleneterephthalate (PET) with an annual production of 36 million tons (Gübitz, 2008) is one of the most widely used polymers and therefore represents a special challenge for biological waste treatment. Hence, intensive research is done to find, isolate and genetically improve organisms and/or enzymes that are capable of biotransformation of PET-materials.

Additional to the purpose of complete degradation for recycling, biological degradation of PET is expected to be deployable for improving bad surface properties of PET-materials such as fibres or foils.
PET materials are used for numerous technical applications because of their excellent physico-chemical properties but they are bearing the disadvange of being hydrophobic, which makes PET difficult to be functionalised and reduces its range of applications. Conventional alkaline hydrolysis of PET can result in material losses of up to 15 % (Brückner, 2009) implying that gently enzymatical hydrolysis would be more suitable for this application. But aromatic polyesters such as PET for a long time were considered to be non-biodegradable (Müller *et al.* 2001) and only some years ago research has demonstrated that partial enzymatic hydrolysis can generate new hydroxylic and carboxylic moieties on the PET surface without attrition of material (Gübitz, 2011). Enzymes, which were determined to be able to hydrolyse aromatic esterbonds of PET are cutinases, lipases and esterases.

In this work five new enzymes were demonstrated to hydrolyse PET. The enzymes were characterised according to different kinetic parameters and their ability to degrade PET foils was quantified. The used enzymes originated from *Thermobifida fusca, Thermobifida cellulolysitica* and *Thermobifida alba* strains and an esterase form *Bacillus subtilis.*

2 PRODUCTION OF ENZYMES

All recombinant DNA techniques, alignments and production of the required strains were done in cooperation with Dr. Doris Ribitsch and her team. Enzymes used in this work were different cutinases obtained by heterologous expression of *Thermobifida fusca, Thermobifida cellulolysitica* and *Thermobifida alba* strains and an esterase form *Bacillus subtilis* (Table 2-1).

Thermophilic actinomycetes from the *Thermobifida* genus, *Thermobifida fusca*, *Thermobifida cellulolysitica* and *Thermobifida alba*, are known to produce polymer hydrolysing enzymes. First wild types of the species have been isolated from compost in 1998 by Kleeberg *et al.* based on their ability to grow on polymer-based substrates (Kleeberg *et al.* 1998). Subsequently it was demonstrated that the enzymes produced by the organisms show hydrolase activity towards polyalkyleneterephthalates (Eberl *et al.* 2009; Gübitz and Cavaco-Paulo 2008). Müller *et al.* fully described the *Thermobifida fusca* hydrolase "BTA-hydrolase 2 CAH17554" (Müller *et al.* 2005) however *Thermobifida sp.* produces a verity of hydrolases which are not investigated yet.
Herein produced and investigated *Thermobifida sp.* enzymes have been isolated and expressed in *E.coli* BL21-Gold(DE3).

Para-nitrobenzylesterase from *Bacillus subtilis* (BsEstB) was pre-selected out of 230 bacterial strains from the TU Graz by its ability to grow on bis(benzoyloxyethyl)-terephtalate (3PET) agar plates (Ribitsch *et al.* 2011). This ability indicates a production of a 3PET-hydrolysing enzyme which was isolated and identified by ultrafiltration and tryptical digestion. After isolation of the gene region coding the particular enzyme, it was cloned into special host cells. E.*coli* BL21-Gold served as host cell for *Bacillus subtilis* para-nitrobenzylesterase (BsEstB).

To provide efficient enzyme purification, all enzymes were C-terminally fused with either a StrapTaq II or a HisTaq I affinity-taq. Furthermore the particular gene was fused with an Ampicillin or Kanamycin resistance to ashure monostrain growing in the following fermentation processes. Strains are stored on Luria-Bertani-Agar plates at 4°C.

Table 2-1 Enzymes obtained by heterologous expression in E. *coli* BL21-Gold(DE3)

Name herein	Enzyme Classification	Coding Plasmid	Source
43342_1	Cutinase	pMS_tac_43342_BTA1-His	*Thermobifida fusca*
43370_2	Cutinase	pMS_tac_43370_BTA2-His	*Thermobifida cellulolysitica*
43793_1	Cutinase	pMS_tac_43793_BTA1-His	*Thermobifida cellulolysitica*
43185_1	Cutinase	pMS_tac_43185_BTA1-His	*Thermobifida alba*
BsEstB	Para-nitrobenzyl-esterase	pMS_pnbA-Strep	*Bacillus subtilis*

Materials and Methods

Expression of BsEstB

- **Fermentation**

10 mL Luria-Bertani (LB) broth, supplemented with 100 mg L^{-1} Ampicillin were inoculated with *E.coli* cells from the LB-plates. The culture was grown overnight on a rotary shaker at 30 °C and 120 rpm. Then, 4 mL of the overnight culture were transferred to a 500 mL baffled shaking flask containing 200 mL fresh and preheated culture medium and grown at 37 °C and 120 rpm.

At an optical density (600 nm) of 0.7 – 0.8, the culture was cooled down to 20 °C to minimize inclusion body formation and induced with IPTG at a final concentration of 0.05 mM. The culture was incubated for twelve hours at 20 °C and 120 rpm.

- **Harvest**

Cells were harvested by centrifugation (4,000 rpm and 4 °C for 30 min) and stored at -20 °C.

Cell pellet derived from 50 mL culture was suspended in 3 mL 0.1 M Tris HCl, pH 7.0 and sonicated with 5 times 20 s pulses under ice cooling using a cell disruptor. Cell debris was removed by centrifugation at 13,000 rpm and 4 °C for 15 min.

After cell disruption the supernatant was cooled on ice and purified right away to avoid enzyme degrading processes like denaturation or (partial) digestion.

- **Purification**

To simplify this step, the BsEstB was C-terminally fused with a StrepTag II, allowing purification of the above attained clear supernatant by affinity chromatography using Strep-Tactin® Spin Columns (IBA, Germany). All purification steps of the recombinant BsEstB were performed according to the manufacturer's protocol with the following minor modifications. Washing and elution buffer were set to pH 7.0, protein elution was done by adding 3 times 1 mL elution buffer to result in a final volume of 3 mL pure enzyme solution.

Expression of Thermobifida sp. enzymes

- **Fermentation**

100 mL Luria-Bertani broth, supplemented with 40 mg L^{-1} Kanamycin were inoculated with cells from the LB-plates. The culture was grown overnight on a rotary shaker at 30 °C and 120 rpm. Then, a certain volume of the overnight culture is transferred to a 2 L baffled shaking flask containing 400 mL fresh and preheated culture medium and grown at 30 °C and 120 rpm. The volume of inoculums needed is depending on the growth in the overnight culture, the optical density (600nm) of the fresh inoculated main culture should be 0.1. At an optical density of 0.8, the culture is induced with IPTG at a final concentration of 0.1 mM and then incubated for another 4 hours at 30°C and 120 rpm.

- **Harvest**

Cells were harvested by centrifugation (5,000 rpm and 4 °C for 15 min) and stored at -20 °C.

Cell pellet derived from 100 mL culture was suspended in 15 mL 0.1 M Tris HCl, pH 7.0 and sonicated under ice cooling with 6 times 1 min pulses with 1 min cooling brakes in between using a cell disruptor. Cell debris was removed by centrifugation at 15,000 rpm and 4 °C for 20 min.

After cell disruption the supernatant was cooled on ice and purified right away to avoid enzyme degrading processes like denaturation or (partial) digestion.

- **Purification**

To simplify this step, the *Thermobifida* sp. enzymes were C-terminally fused with a HisTag I, allowing purification of the above attained clear supernatant by affinity chromatography using His SpinTrap™ columns (GE Healthcare). All purification steps were performed according to the manufacturer's protocol using buffers for native conditions.

Experience showed that the enzymes are stable for a longer time if a buffer exchange is performed to remove the imidazol from the enzyme solution. Therefore the produced enzyme was bound on a His Trap column and reeluted with a total volume of 3 mL imidazol free 50mM Tris HCl buffer, pH 7.0.

SDS-PAGE analysis

To confirm the purity of the enzymes they were separated on SDS gel (10% and 12%). Visualization of the protein band was done with coomassie brilliant blue or silver stain.

Chemicals

For a detailed list of all herein used chemicals and further materials vide Appendix 2 - Chemicals.

Results and Discussion

Enzyme production

A typical fermentation and purification circle following the above mentioned protocols produced 3 mL of the particular enzyme. Protein amount and concentration differed relating the quality of the fermentation. In general the *Thermobifida sp.* enzymes are expressed in much higher levels than the BsEstB, 5 mg mL^{-1} and higher compared to <1 mg mL^{-1} in successful fermentations.
Expression amount of all cultures showed very high sensitivity to their time of induction. If the BsEstB producing culture is not properly cooled down to 20 °C, before induction, enzyme is produced in very low levels or even not at all. The *Thermobifida sp.* enzymes producing cultures showed best enzyme expression when induced at OD_{600} ~0.7 while higher optical densities generally resulted in lower amounts of produced protein.

SDS-PAGE analysis of BsEstB shows a strong protein band at 55.2 kDa (Figure 2-1) (Ribitsch *et al.* 2011).

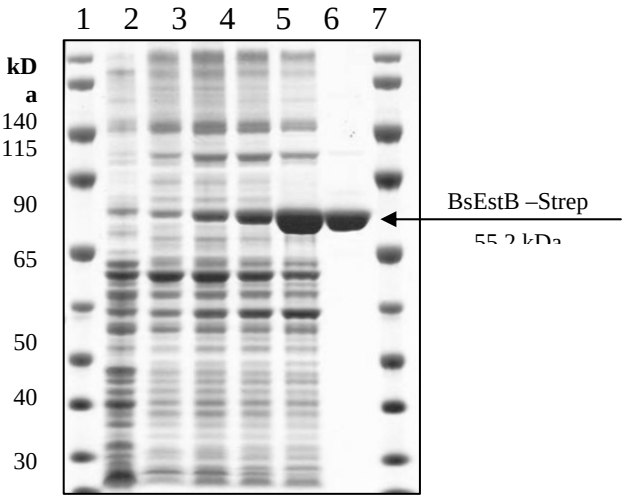


Figure 2-1 SDS-Page analysis (12 %) of BsEstB - Samples were withdrawn at different times after induction, centrifuged, disrupted and centrifuged again. Lane 1 and 8: prestained protein molecular weight marker SM0671 (Fermentas); lane 2: lysate *E.coli* BL21-Gold without plasmid; lane 3: Fermentation culture uninduced; lane 4-6: cleared lysates from induced cells after 1, 3 and 12 h of induction; lane 7: purified BsEstB. (Ribitsch *et al.* 2011)

The *Thermobifida sp.* enzymes have been found to have an average molecular mass of 29 kDa.

Table 2-2 Molecular Mass of own fermented enzymes determined by SDS-PAGE analysis

Enzyme	Molecular Mass
43342_1	29.6 kDa
43370_2	29.7 kDa
43793_1	29.4 kDa
43185_1	29.0 kDa
BsEstB	55.2 kDa

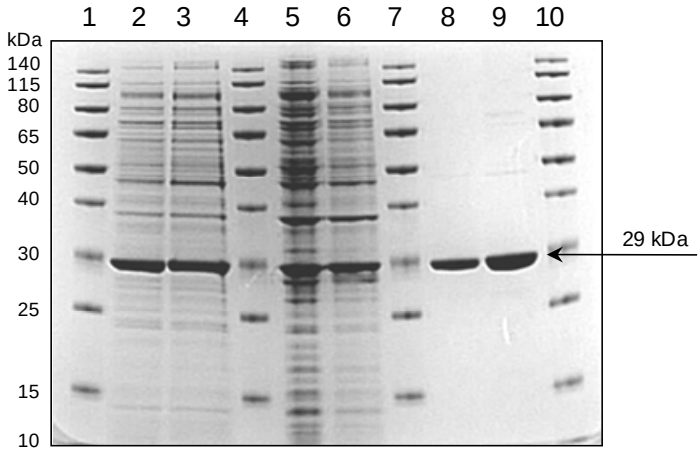


Figure 2-2 X SDS-PAGE analysis (12 %) of cutinases expressed in *E.coli* BL21-Gold(DE3) and purified by affinity-Tag - Samples were withdrawn after 4 h of induction at 30 °C, centrifuged, disrupted and centrifuged again. Lane 1, 4, 7 and 10: prestained protein molecular weight marker SM0671 (Fermentas); lane 2 and 3: soluble cell fraction of *Thermobifida sp*. enzyme (43185_1); lane 5 and 6: insoluble cell fraction of *Thermobifida sp*. enzyme (43185_1); lane 8 and 9: purified *Thermobifida sp*. enzyme (43185_1)

3 SCREENING AND CHARACTERIZATION OF ENZYMES

After the fermentation, harvested enzyme fractions were purified. In order to be able to compare the enzymes several characterisation techniques for enzyme stability and activity were applied.
The following chapters include all in-house fermented enzymes as described previously, as well as several other gifted or purchased enzymes, whichever should be screened and compared regarding their esterase activity and modes of operation.

Monitoring of the general enzyme activity was done with small esterase substrates such as *para*-Nitrophenyl-Acetate, -Butyrate and –Palmitate. For monitoring the enzyme stability during storage or incubation time the activity test with *p*-NP-Butyrate was used. This is because the procedure for *p*-NP-B has turned out to be the most stable one in daily routine.
After assuring that the respective enzyme batches show esterase activity on the small substrates, further tests were carried out to determine the particular hydrolysis activity on polyalkyleneterephthalates.

For the determination of hydrolysis potency incubations with a small synthetic monomer of Polyethyleneterephthalate (PET), bis(benzoyloxyethyl)terephtalate (3PET), were performed. Simultaneous the enzymes were also incubated with PET foils as substrate. Hydrolysis was monitored by detecting release products with HPLC/UV. Furthermore, hydrolysis indicated surface changes of the PET foils were observed. Applied techniques for surface analysis were fourier transform-infrared spectroscopy attenuated total reflectance (FTIR-ATR) and water contact angle (WCA) measurement. Simultaneously samples were sent to an external laboratory for infrared spectrosocopy after alkylation with 2-(bromomethyl)naphthalene.

Furthermore, enzyme-kinetic studies were carried out. All enzymes were characterised by their Michaelis-Menten-Parameters, K_M and v_{max}, on the three previously mentioned substrates *p*-NP-A, -B and -P.
For the Para-nitrobenzyl-esterase derived from *Bacillus subtilis* kinetic parameters were additionally determined by using bis(hydroxyethylen)terephthalate (BHET) as a substrate.

Materials and Methods

Enzymes

43342_1	Own fermented esterase from Thermobifida *fusca*
43370_2	Own fermented esterase from Thermobifida *cellulolysitica*
43793_1	Own fermented esterase from Thermobifida *cellulolysitica*
43185_1	Own fermented esterase from Thermobifida *alba*
BsEstB	Own fermented para-nitrobenzylesterase from Bacillus *subtilis*
cutinase_*H. insolens*	Cutinase from *Humicola insolens* purchased from Novozymes

All own fermented enzymes were purified by affinity chromatography as described in the chapter 2. Production of Enzymes.
Purchased cutinase_*H. insolens* was loaded onto a SDS gel to prove the absence of other enzymes. It is common for purchased enzyme solutions to contain stabilizers and different kinds of additives that might have an influence on one of the following enzyme characterization techniques. Therefore, cutinase_*H. insolens* was purified by using a VivaFlow Crossflow (Sartorius) as a dialysis device. The enzyme solution was pumped through a 100,000 Da polyethersulfon membran (Sartorius) with H_2O deion. as crossflow liquid. After this treatment the purified cutinase_*H. insolens* enzyme was stored at 4°C and used as soon as possible.

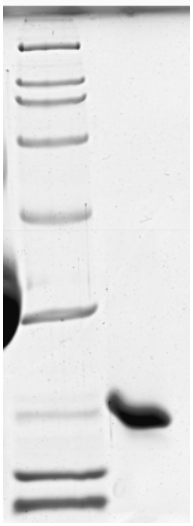


Figure 3-1 SDS-PAGE analysis (10%) of cutinase_*H. insolens* after cleaning by VivaFlow - Lane 1: Bio-Rad Broad Range Standard; lane 2: purified cutinase_*H. insolens*

Determination of protein content

Protein content of the enzyme fractions was determined using the method from Bradford (Bradford 1976). Calibrations were performed with different concentrations of bovine serum albumin in aqueous solution (see Appendix 1 - Further reading of applied methods).

Esterase activity on p-NP-Esters

Esterase activity can be determined by photometric observation of the release of *para*-Nitrophenolate (*p*-NP) from a *para*-Nitrophenyl-Ester over time.
Depending on the substrate that is linked to the *p*-NP, enzymes show different behaviour regarding their esterase-activity. Measurements were performed with substrate solutions of *p*-NP-Acetate, *p*-NP-Butyrate and to identify lipase activity on *p*-NP-Palmitate.

- **Preparation of substrate solution**

p-NP-A

A 40 mmol L^{-1} solution of *p*-Nitrophenyl-Acetate (Acros Organics, 97 %) in EtOH abs. was prepared and stored at -20 °C until use.

p-NP-B

86 µL *p*-Nitrophenyl-Butyrate (Sigma, ≥98.0 %) were dissolved in 1000 µL DMSO and stored at -20 °C until use.

p-NP-P

2 mg *p*-Nitrophenyl-Palmitate (Sigma, ≥98.0 %) were dissolved in 1000 µL i-PropOH and stored at -20 °C until use.

- **Preparation of buffer**

Further dilutions of the *p*-NP-A and *p*-NP-B assays were done with 50 mmol L^{-1} phosphate buffer.

For *p*-NP-P buffer solution 2.3 g L^{-1} Sodium taurocholate hydrate, 1.2 g L^{-1} gum arabic and 5 g L^{-1} Triton X-100 were added to a 50 mmol L^{-1} phosphate buffer.

Tests were carried out with varying pH values of the phosphate buffer. *Thermobifida sp*. enzymes were found to have the highest activity at a pH of 7.0, since the cutinase_*H. insolens* activity did not differ when using buffers with pH values ranging from 7.0 to 8.0, all further tests where carried out using pH 7.0.

- **Preparing of assay solution**

p-NP-A and *p*-NP-B

For the assay solution 1000 µL of a buffer where mixed with 10 µL of the substrate solution and kept on ice until start of the procedure.

p-NP-P

Substrate solution was diluted with the *p*-NP-P buffer 1:10 and kept on ice until start of the procedure.

- **Assay**

200 µl of the assay solution were put into a well of a 96-well-platereader plate and allowed to warm up to room temperature before continuing. Then 20 µL enzyme preparations were added and the increase of the absorbance at 405 nm was followed using a spectrophotometer type Hitachi U 2001. A blank was measured using 20 µL buffer instead of sample.

The specific activity, U mg^{-1}, was determined as the amount of *p*-Nitrophenol in µmol released per minute and mg purified enzyme.

Determination of Michaelis-Menten Parameters on p-NP-A, p-NP-B and p-NP-P

For the determination of K_M and v_{max} activity assays were carried out as mentioned above. The substrate solutions were diluted with buffer to provide different substrate concentrations. Then, the specific activity v_i in dependence of the recent substrate concentration S_i was measured and plotted in an direct Michaelis-Menten plot, v_i versus $[S]_i$.

Determination of enzyme stability

Enzyme stability tests were done with *p*-NP-B activity assay. Therefore freshly cleaned enzymes were put on stability storage at 4 °C and at their specific incubation temperature. For the *Thermobifida sp.* enzymes and BsEstB incubation temperature is 37 °C for the cutinase_*H. insolens* it is 50 °C. Approximately 1 mL enzyme was put into a 1.5 mL Eppendorf tube and put on a rotary shaker at the specific temperature. 4 °C samples were treated the same way and placed in the laboratory fridge. After a certain time samples were taken to run a *p*-NP-B activity assay as described above.

Determination of kinetic parameters on BHET

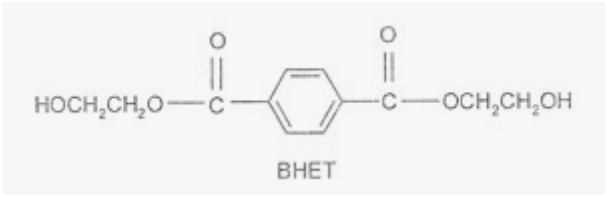


Figure 3-2 Structure of bis(2-hydroxyethyl)terephthalate

In addition to the Michaelis-Menten Parameters on the *p*-NP-Ester substrates, BsEstB was also characterised by its kinetic parameters using bis(2-hydroxyethyl)terephthalate (BHET) as a substrate. Different concentrations of BHET were properly diluted in 50 mM phosphate buffer pH 7.0 (K_2HPO_4/KH_2PO_4) using sonification, in this way providing different substrate concentrations ranging from 0.0625 mM to 1 mM. 100 µL of BHET solution were mixed with 20 µL enzyme preparation and incubated at 37 °C for different incubation times. Enzyme preparation consisted of 0.5 mg mL^{-1} purified protein in 50 mM phosphate buffer. The reaction was stopped using 1:1 (v/v) methanol abs. on ice. Hydrolysis products were determined using HPLC/UV analysis.

Hydrolysis of bis(benzoyloxyethyl)terephthalate (3PET)

Synthetic monomer 3PET

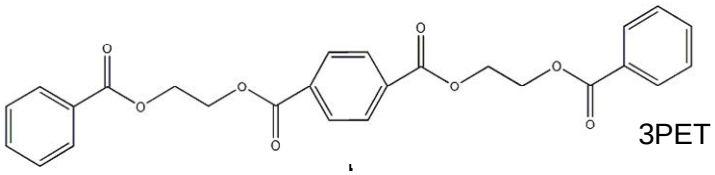


Figure 3-3 Structure of model substrate bis(benzoyloxyethyl)terephtalate

3PET was available on the institute, from a previous synthesis performed according the method published by Heumann *et al.* 2006.

For determination of 3PET hydrolysis 10 mg of the model substrate were properly dispersed in 500 µL enzyme preparation in a 2 mL Eppendorf tube and, horizontally, incubated for 5 days on a rotary shaker at 37 °C and 150 rpm. The enzyme preparation typically contained 200 µg mL^{-1} purified protein diluted with 50mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0. After incubation the samples were processed as described below and analysed via HPLC analysis.

HPLC analysis

Prior to the measurement of hydrolysis products proteins were precipitated using 1:1 (v/v) methanol abs. on ice. Samples were then centrifuged at 14,000 rpm for 15 min at 4 °C (Hettich MIKRO 200 R, Tuttlingen, Germany). 500 µL of the supernatant was brought to an HPLC vial and acidified by adding 1 µL of HCl conc. Used HPLC equipment was a DIONEX P-580 PUMP (Dionex Cooperation, Sunnyvale, USA), with an ASI-100 automated sample injector and a PDA-100 photodiode array detector. A reversed phase column RP-C18 (XTerra® RP-C18, 5 µm, 150 x 4.6 mm) was used. Analysis was carried out with 20 % acetonitrile, 20 % 10 mM sulphuric acid and 60 % (v/v) water as eluent. The flow rate was set to 1 mL min^{-1} and the column was maintained at a temperature of 25 °C. The injection volume was 10 µL. Detection of the chromophoric analytes was performed with a photodiode array detector at the wavelength of 241 nm and 228 nm (modified method from Eberl *et al.* 2008).

Hydrolysis of PET-foils

Enzymes were incubated on PET foils kindly provided by Nierstrasz. Generally, PET-foils were cut into 10 x 5 mm pieces and washed using below mentioned washing procedure. After cleaning, the foils were incubated in 2 mL Eppendorf tubes with 500 µL enzyme preparations, containing 200 µg mL^{-1} protein in 50 mM phosphate buffer pH 7.0, for 5 days on a rotary shaker at 37 °C and 150 rpm. Foils dedicated for surface analysis were cut into bigger pieces and incubated in matching glass containers, always making sure that the enzyme / surface ratio stays constant.
After incubation all foils were carefully removed and preserved for surface analysis.

Washing procedure

Adhering fat or dust could influence the hydrolysis of a polymer surface. To assure that the polymers are free from any soiling they should be properly washed before starting incubations. The used washing procedure was established by Brückner *et al.* 2009 and proven to be the most suitable for this purpose. It consists of three steps, each carried out in a fresh and clean glass flask. One washing step takes 30 min. on a rotary shaker at 50 °C, between the steps foils were rinsed with distilled water.
Step 1: 5 g L^{-1} Triton X-100
Step 2: Na_2CO_3 water free, 2 g L^{-1}
Step 3: distilled water
Since enzymes are hydrophilic molecules that might interfere with further surface analysis, also residual proteins after incubation have to be removed from the foil surface. The mentioned washing procedure has been proven, by Bückner *et al.,* to be also suitable for removing adsorbing proteins from surfaces.

HPLC analysis

After incubation the samples were processed as described above and hydrolysis products were measured by HPLC, with the same procedure as for the 3PET samples.

Water contact angle (WCA)

Contact angles of the PET foils after exposure to enzymes were measured as previously reported (Eberl *et. al.* 2008). Previous to measurement a two-step washing procedure was applied. One washing step takes 30 min on a rotary shaker at approximately 30 °C.
Step 1: Sodium Dodecyl Sulfate (SDS)
Step 2: Distilled water

Polymer films were analyzed with the Drop Shape Analysis System DSA 100 (Kruss GmbH, Hamburg, Germany) using deionized water as test liquid with a drop size of 3 µL. Contact angles were measured after 3 s and data are obtained from the averages of the measurements taken from 10 different points of the sample surface.

Fourier transform-infrared spectroscopy attenuated total reflectance (FTIR-ATR)

FTIR measurements were performed with a NEXUS Thermo Nicolet FTIR spectrometer with a Ge crystal cell (maximum depth 0.8 µm) and applying an attenuated total reflectance (ATR) accessory mod, Smart Performer. Spectra were normalized to the 1410 cm^{-1} peak before any data processing. Reported results are obtained from average spectra of at least three spectra measurements. Each measurement was carried out on a different area of the foil.

Fluorescence Spectroscopy

PET-foils for fluorescence spectroscopy measurements were cut into 100 x 10 mm pieces and washed. After cleaning, the foils were incubated in 15 mL falcon tubes with approximately 10 mL enzyme preparation, containing 200 µg mL^{-1} protein in 50 mM phosphate buffer pH 7.0, for 5 days on a rotary shaker at 37 °C and 150 rpm.

After incubation the foils were sent to an external laboratory for alkylation and measurement of fluorescence spectra.
A detailed description of the alkylation process as well as the spectroscopy can be found in Appendix 1 - Further reading of applied methods. The obtained results of surface changes are discussed below.

Results and Discussion

One goal of this work was to characterize above mentioned enzymes and to determine how their specific behaviours and activities differ from each other.
Bacillus subtilis nitrobenzylesterase cannot be compared with the *Thermobifida sp.* enzymes and is therefore discussed separately.
The hydrolysis potential of the fungal cutinase from *H. insolens* has already been proven by on various scientists (Ronkvist *et al.* 2009; IP Nagarajan 2005; IP Kellis 2002). Since the enzyme is also known to be able to hydrolyse PET materials it is used as a positive reference to the other self-fermented enzymes.

To be able to compare the substrate specificities of enzymes it is necessary to find a frame of reference which is applicably for all used enzymes. Since a high turnover on e.g. *p*-NP-B does not necessarily imply a high activity on any other substrate, a dosage based on activity (Units) is not applicable for the purpose of comparing substrate specificities. In the previous chapter is was verified by SDS-PAGE analysis that all used enzymes solutions are pure and single proteins, therefore it was decided to do the characterisation tests based on protein amount rather than activity dose.

Specific activity of enzymes was measured towards the small ester substrates *p*-NP-A, -B and -P. All tested enzymes show esterase activity. The specific activity was defined as the amount of *p*-Nitrophenol in µmol released per minute and mg purified enzyme.
Specific activities are summed in the table below.

Table 3-1 Specific activity of purified enzymes

	p-NP-A / U mg^{-1}	*p*-NP-B / U mg^{-1}	*p*-NP-P / U mg^{-1}
43370_2	3.64 ± 0.29	8.13 ± 2.43	1.72 ± 0.74
43342_1	30.79 ± 0.77	25.62 ± 12.90	2.61 ± 0.17
43793_1	57.52 ± 0.79	56.10 ± 9.49	1.73 ± 0.27
43185_1	4.97 ± 0.44	11.09 ± 1.07	1.97 ± 0.12
cutinase_*H. insolens*	44.52 ± 0.26	68.5 ± 11.75	20.83 ± 0.32

Usually enzymes show high substrate specificity and determined enzyme activities can not be compared or correlated for different substrates. Nevertheless they can give information about the way of action and preferences of a specific enzyme. Table 3-1 illustrates that all examined enzymes show a higher specific activities on the larger ester substrate *p*-NP-B. This is particularly true for 43370_2 and 43185_1 since their specific

activity on *p*-NP-B is more than 200% higher than on small compound *p*-NP-A. Activity on *p*-NP-P is significantly lower for all enzymes. This was expected since *p*-NP-P- is a typical lipase assay and, since classified as cutinases, the investigated enzymes are not expected to have special lipase properties.

Michaelis Menten Kinetik

Kinetic parameters were determined on small ester substrates *p*-NP-A, -B and –P. For *p*-NP-A and *p*-NP-B the K_M values of the four *Thermobifida sp.* cutinases were in the same range, between 127 and 213 µmol L^{-1} for *p*-NP-A and 1483 and 2133 µmol L^{-1} for *p*-NP-B. The purchased cutinase from *H. insolens* showed significantly higher K_M values on these substrates, 387 and 3667 µmol L^{-1} respectively.

For p-NP-P the K_M values of all five tested enzymes were between 177 and 327 µmol L^{-1}.

Table 3-2 Michaelis Menten Parameters of pure enzymes on the small soluble substrates

	p-NP-A		*p*-NP-B		*p*-NP-P	
	K_M / µmol L^{-1}	v_{max} / µmol min^{-1} mg^{-1}	K_M / µmol L^{-1}	v_{max} / µmol min^{-1} mg^{-1}	K_M / µmol L^{-1}	v_{max} / µmol min^{-1} mg^{-1}
43370_2	200 ± 0	4 ± 0	2133 ± 416	9 ± 2	177 ± 15	2 ± 0
43342_1	167 ± 29	32 ± 3	2100 ± 361	25 ± 7	267 ± 31	3 ± 0
43793_1	127 ± 12	63 ± 3	1483 ± 126	58 ± 8	240 ± 28	2 ± 0
43185_1	213 ± 0	5 ± 0	1933 ± 306	11 ± 1	327 ± 31	2 ± 0
cutinase_ *H. insolens*	387 ± 12	44 ± 0	3667 ± 231	80 ± 0	265 ± 5	21 ± 0

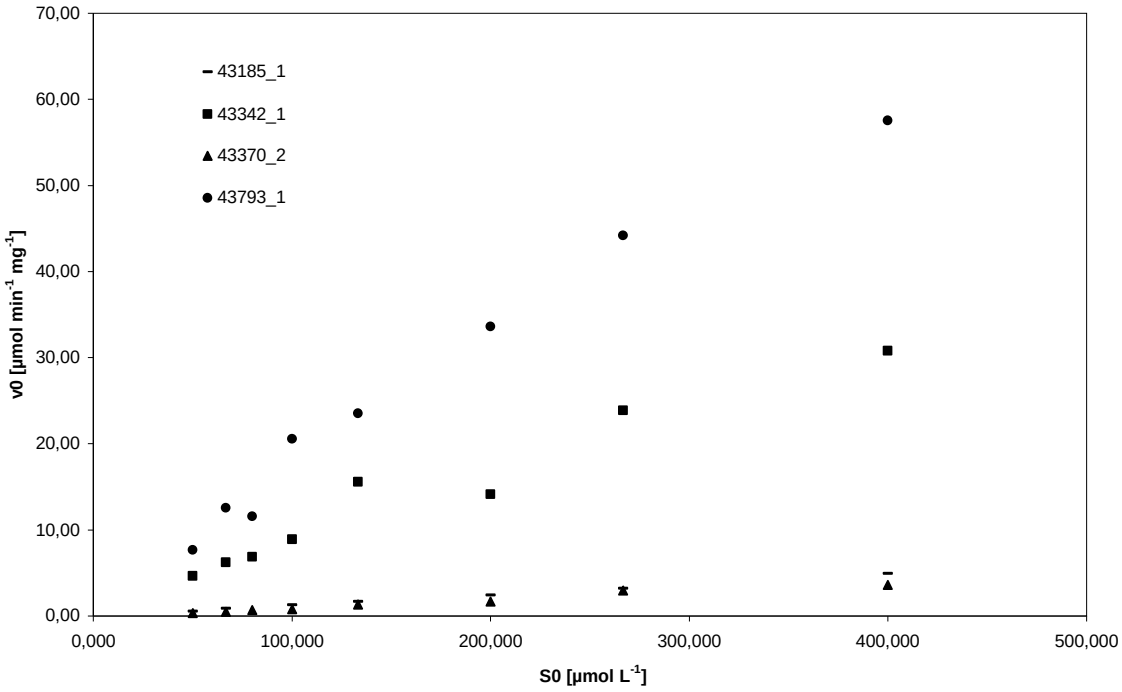


Figure 3-4 Direct Plot Michaelis Menten Kinetic on *p*-NP-A of own fermented enzymes

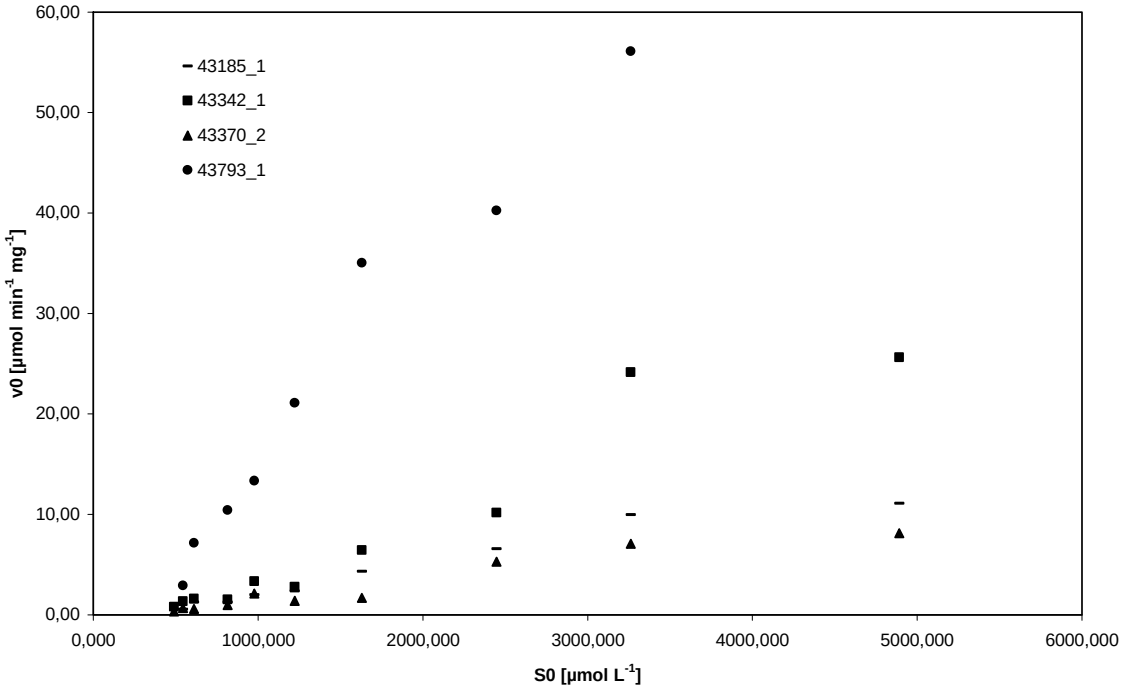


Figure 3-5 Direct Plot Michaelis Menten Kinetic on *p*-NP-B of own fermented enzymes

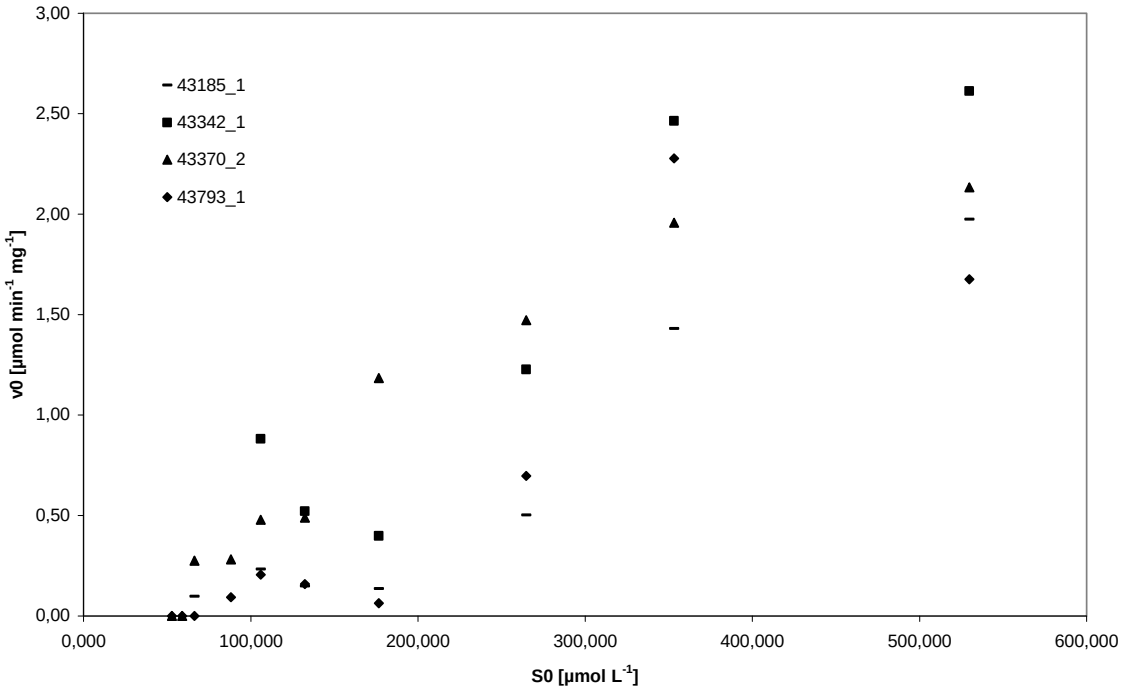


Figure 3-6 Direct Plot Michaelis Menten Kinetic on *p*-NP-P of own fermented enzymes

From the determined Michaelis Menten parameters the number of substrate molecules that the enzymes convert per second can be calculated (Table 2-3).

Table 3-3 Molecular activity (turnover number) of the pure enzymes on the small soluble substrates

	p-NP-A		*p*-NP-B		*p*-NP-P	
	K_M / µmol L^{-1}	k_{cat} / s^{-1}	K_M / µmol L^{-1}	k_{cat} / s^{-1}	K_M / µmol L^{-1}	k_{cat} / s^{-1}
43370_2	200 ± 0	2.0 ± 0.0	2133 ± 416	4.5 ± 1.0	177 ± 15	n.a.
43342_1	167 ± 29	15.8 ± 1.5	2100 ± 361	12.3 ± 1.5	267 ± 31	n.a.
43793_1	127 ± 12	30.9 ± 1.5	1483 ± 126	28.4 ± 3.9	240 ± 28	n.a.
43185_1	213 ± 0	2.0 ± 0.0	1933 ± 306	5.3 ± 0.5	327 ± 31	n.a.
cutinase_ *H. insolens*	387 ± 12	14.7 ± 0.0	3667 ± 231	26.8 ± 0.0	265 ± 5	7.0 ± 0.0

Abbreviations: n.a. – not applicable

Table 2-3 gives the turnover number of the enzymes on the small ester substrates *p*-NP-A, -B and –P. Whilst the K_M values of *Thermobifida sp.* cutinases were in the same range it can be seen that the k_{cat} values differ up to 15 times from each other, between 2.0 and 30.9 s^{-1}.

Calculation of molecular activity of the enzymes on p-NP-P was not applicable since the measured v_{max} were too small to be measured within accurate standard deviations.

Michales Menten Plots of purchases cutinase from H. *insolens*:

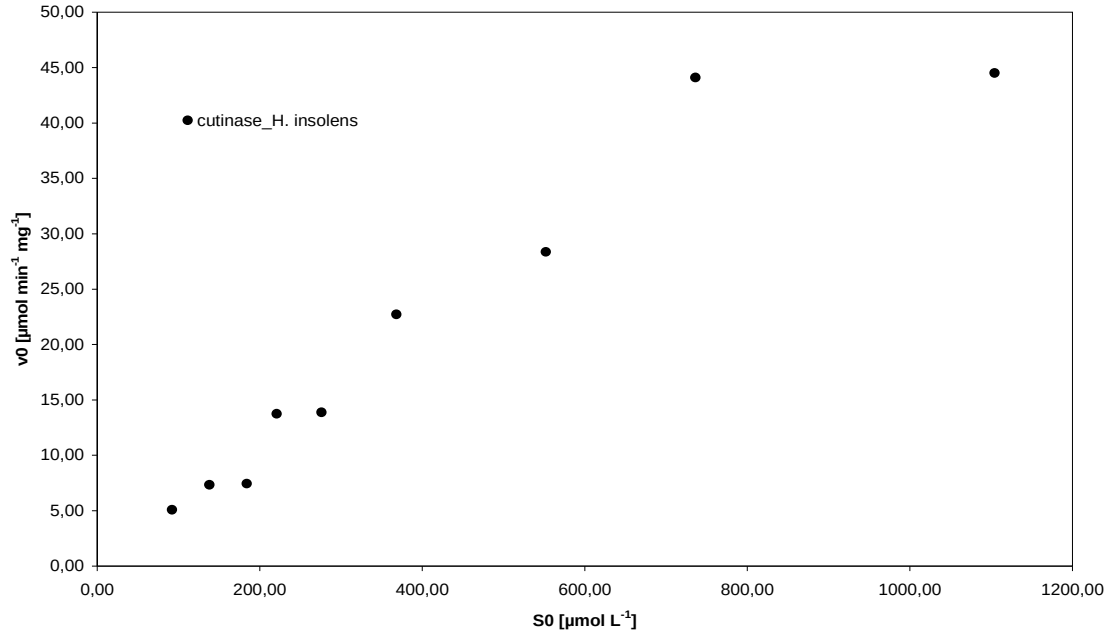


Figure 3-7 Direct Plot Michaelis Menten Kinetic on *p*-NP-A of *H. insolens* cutinase

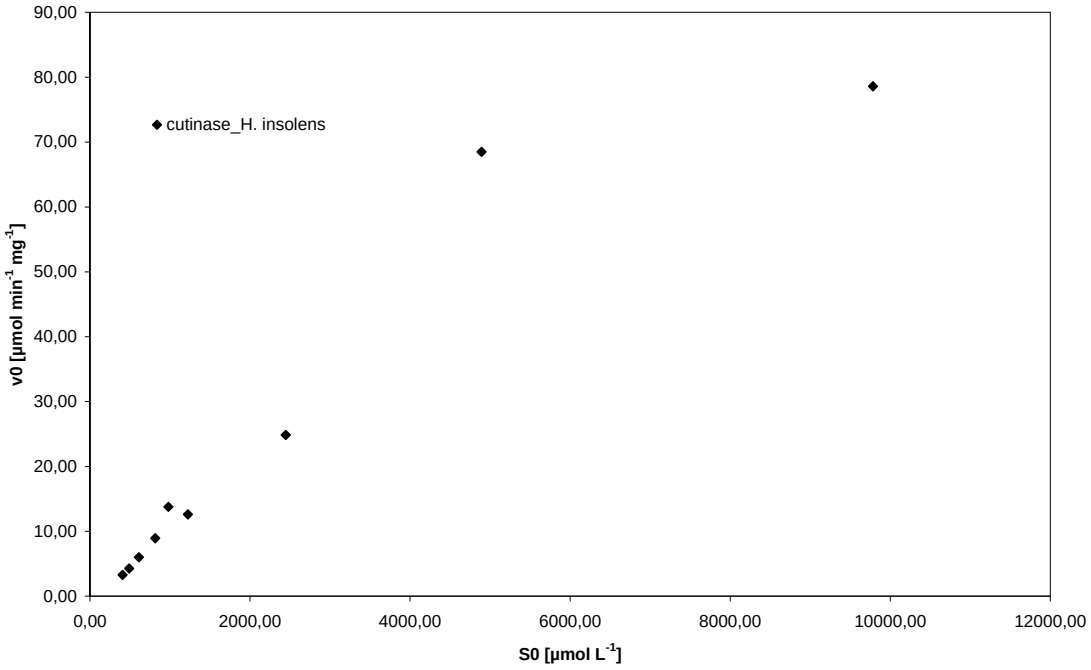


Figure 3-8 Direct Plot Michaelis Menten Kinetic on *p*-NP-B of *H. insolens* cutinase

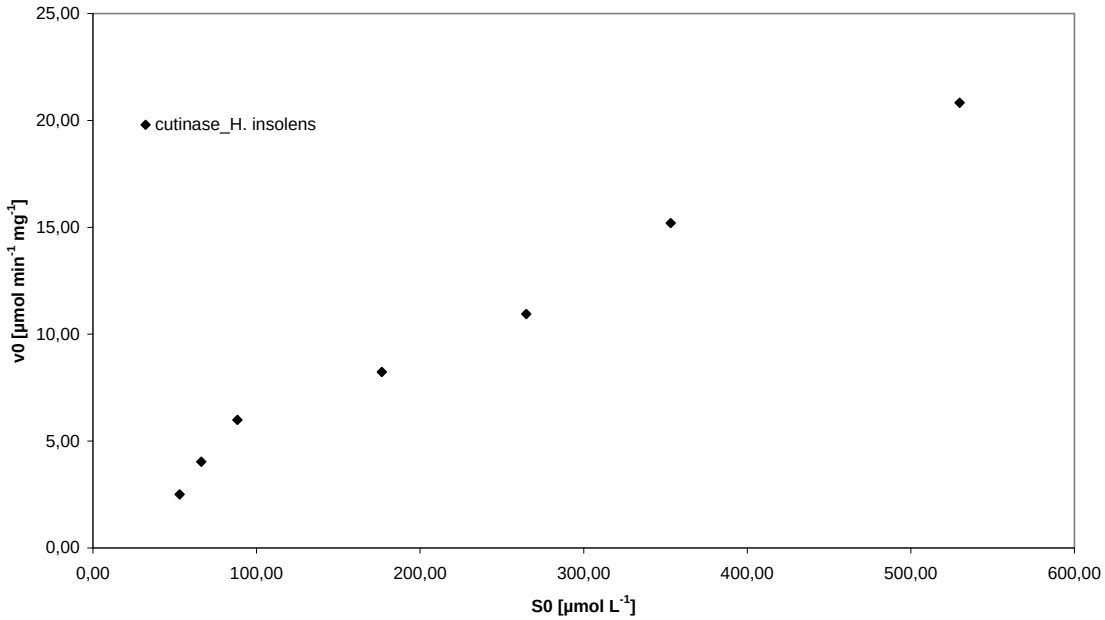


Figure 3-9 Direct Plot Michaelis Menten Kinetic on *p*-NP-P of *H. insolens* cutinase

Enzyme stability

Enzyme stability tests were done with *p*-NP-B activity assay. Therefore freshly cleaned enzymes were put on stability storage at 4 °C and at their specific incubation temperature. The enzyme activity at start was set to 100 % to see how it develops during incubation.

Table 3-4 Stability Studies monitored with p-NP-B activity assay after certain incubation time at optimum incubation temperature of *Thermobifida sp*. enzymes and Cutinase_H. insolens: 50 °C

Time / h	*Activity / %*				
	43370_2	43342_1	43793_1	43185_1	cutinase_*H. insolens*
0	100 %	100 %	100 %	100 %	100 %
3	100 %	111 %	95 %	44 %	100 %
12	68 %	40 %	58 %	42 %	95 %
36	62 %	46 %	66 %	46 %	93 %
48	11 %	6 %	n.a.	46 %	80 %
63[(1)]	6 %	15 %	46 %	34 %	62 %
120	0 %	6 %	90 %	22 %	56 %

Abbreviations: n.a. – not analysed / [(1)] for 43185_1 test point after 80h incubation instead of 63h

Investigations of enzyme stability show a high diminishment of catalytic activity within the first 12 hours of incubation for all enzymes but the one of *H. insolens*. After this first decrease the activity remains constant for the next 30 hours at a level of approximately 50 % of the starting activity.
When exceeding 40 h incubation time 43342_2 and 43370_2 have lost about 90 % of their specific activity. Whilst 43793_1 and 43185_1 show a more slowly inactivation rate, still having almost 50 % activity after 60 hours.

Determined half life times ($t_{1/2}$) of the enzymes are, 40 hours for 43370_2, 10 hours for 43342_2, 30 hours for 43185_1and almost 60 hours for 43793_1.

Given that the enzyme activity after 80 h of incubation is only ~20 % of the starting activity it can be stated, that for all herein self-fermented enzymes the previous described incubation time of 120 h can be decreased to 72 hours without losing incubation efficiency.

In contrary to the self-fermented enzymes cutinase from *H. insolens* has a higher durability to respective incubation conditions, $t_{1/2} \geq 120$ hours.

This might be caused by the fact that the enzyme´s origin is from a high resistant thermophilic fungus or that vivaflow cleaning method did not remove all enzyme-stabilizers from the purchased enzyme solution.

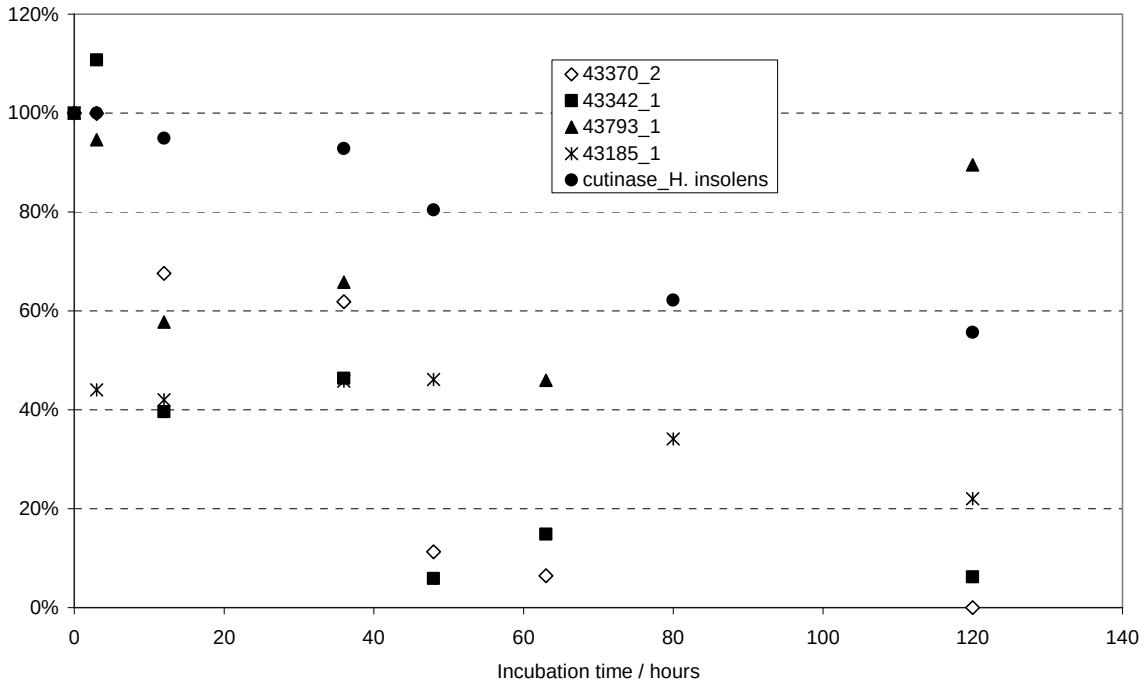


Figure 3-10 Stability Studies monitored wit p-NP-B activity assay

Hydrolysis of bis(benzoyloxyethyl)terephthalate (3PET)

In 2006 Heumann *et al.* demonstrated a correlation between the hydrolytic activity of several polyesterases when put on PET and the water insoluble PET model substrate bis(benzoyloxyethyl)-terephthalate, 3PET (Eberl *et al.* 2009; Heumann *et al.* 2006). With the model substrate a potential difference in reaction mechanism of the different enzymes is easier to evaluate, since the smaller size of the model substrate results in faster biodegradation. Therefore the water soluble degradation products after a certain time of incubation were quantified via HPLC-UV analysis. Detectable analytes are shown in Figure 3-11. Additionally to the below mentioned degradation products, it can be assumed that large amounts of UV-inactive ethylene glycol (EG) are formed through full ester cleavage of the enzymes.

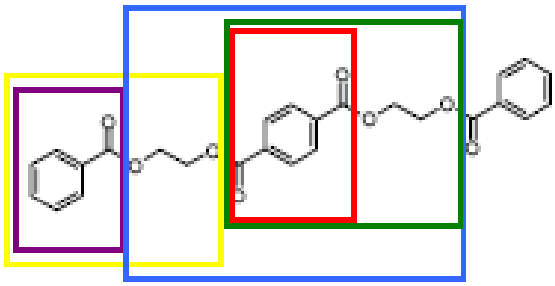


Figure 3-11 Potential biodegradation products after hydrolysis of 3PET; purple – benzoic acid (BA), yellow – hydroxyethylbenzoate (HEB), red – terephthalic acid (TA), blue – bis(2-hydroxyethyl)terephthalate (BHET), green – mono(2-hydroxyethyl)terephthalate (MHET)

All incubated enzymes were able to hydrolyse 3PET to its smallest sub-components BA and TA. The potentially formed smaller esters MHET and HEB were only intermediates, since all enzymes showed the ability to hydrolyse these too during the incubation period. Interestingly, none of the *Thermobifida sp.* enzymes produces BHET. This observation has also been made with another cutinase from *Thermobifida fusca* by A. Eberl in 2008. Like the *T. fusca* cutinase used by Eberl, the four herein used enzymes showed a rather high hydrolysis activity on the model substrate, compared to other esterases or lipases (results not shown) (Eberl et. al 2008).

Table 3-5 Total release products of 3PET after 72 h incubation (mmol per mol Enzyme)

release product / mmol mol^{-1}	43370_2	43342_1	43793_1	43185_1	cutinase_ *H. insolens*
MHET	803 ± 37	928 ± 44	261 ± 80	1134 ± 9	616 ± 23
TA	680 ± 88	430 ± 31	769 ± 59	405 ± 30	560 ± 6
BA	2030 ±147	1758 ± 13	1537 ± 549	1207 ± 53	8580 ± 30
HEB	413 ± 26	521 ± 13	410 ± 2	557 ± 3	133 ± 0
BHET	n.d.	n.d.	n.d.	n.d.	186 ± 9

Abbreviations: n.d. – not detected

To obtain more information of the mechanism of the hydrolysis process the stepwise hydrolysis after 2, 4, 6, 15, 36, 48, 72 and 120 hours were analysed.

When incubating the four *Thermobifida sp.* esterases on 3PET two generally different ways of hydrolysis can be observed. 43370_2 and 43793_1 start with releasing BA right from the beginning of the incubation while the other two enzymes first release HEB and later degrade this to BA and EG.

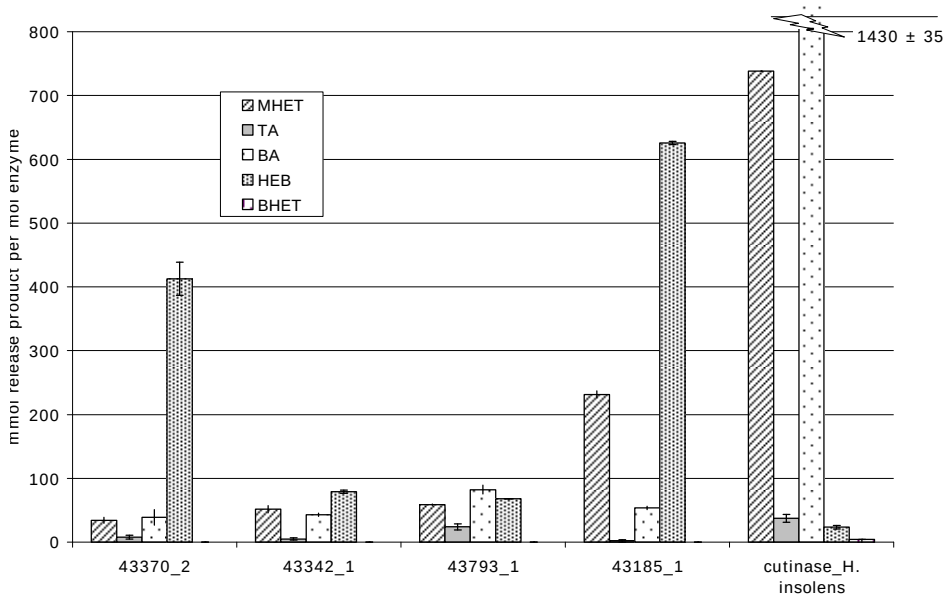


Figure 3-12 Hydrolysis products of 3PET after 2 hours incubation with Thermobifida enzymes and purchased cutinase from *H. insolens*.

Hydrolysis with 43793_1 led to a linear increase of the all degradation products within the whole investigated incubation period. It shows that after 2 days of incubation the production of degradation products is still linear but with a less ascending slope than within the first hours. Which is supported by the results of the stability investigations that illustrated that 43793_1 loses about 50% of its activity within the first 36 hours but then keeps the remaining activity up to almost 120 hours.

Furthermore, all degradation products are stoichiometrical equally abundant during the incubation period. This implies that the enzyme has no preference for hydrolysing one specific ester group of the 3PET molecule but instead cleaving the ester groups of this substrate randomly. 43793_1 was the only enzyme with the almost same specific activity on both small ester substrates (pNPA: 57.5 U mg-1, pNPB: 56.1 U mg^{-1}), again suggesting that the protein is able to hydrolyse all herein used substrates without specific preference.

Like 43793_1, 43370_2 also actively releases BA from the 3PET molecule. However no TA can be detected within the first hours of incubation. From the absence of TA it can be assumed, that the enzymatic attack of the 3PET takes place at the outer ends of the molecule, thereby releasing BA and a little HEB. After 15 hours the ration between HEB and TA changes to higher amounts of TA, while the rate of MHET production decreases, indicating that the enzyme started to degrade MHET to TA and EG. Between 36 and 48 hours incubation, the release of hydrolysis products strongly decreases and then almost stops at all. Only the amount of BA is still increasing. Since enzyme stability test show that 43370_2 is inactivated within 3 days at this conditions it is likely that the BA increase results from auto hydrolysis of former released HEB.

43342_1 and 43185_1 in contrast to the other two *Thermobifida sp.* esterases do not produce BA at in the first steps of the incubations but rather produce high amounts of HEB and MHET. Hydrolysis of theses 3PET sub-compounds and therefore generation of measurable amounts of BA just starts after more than 2 days incubation.
Especially for 43342_1 the low ratio of TA and HEB is interesting. When cleaving the inner ester groups of 3PET, and thereby releasing HEB, the enzyme is expected also to produce a certain amount of TA. But higher levels of TA can only be measured after 15 hours of incubation. A possible explanation for this might be the formation of a not detectable mono(benzoyloxyethyl)terephthalate (3PET minus HEB). An appearance of this molecule was already proposed by Brücker (2007), however its existence could not be verified within these measurements.

For 43185_1 the absolute amount of release products after 6 hours of incubation is about 10-times higher than for 43342_1 within the same time. But after the whole incubation period of release levels for both enzymes are about the same. Also results in the stability determinations showed that 43342_1 has its highest activity after a few hours at 37°C and not right at the beginning like the other enzymes.

43185_1 shows very high tendency towards HEB. At the beginning of the incubation 43185_1 releases HEB and less MHET from 3PET and like the other enzymes it is also able to resume hydrolysing these two esters. After 36 hours the ration of HEB and MHET changed to increasing level of MHET showing that the enzyme prefers HEB over MHET as substrate.

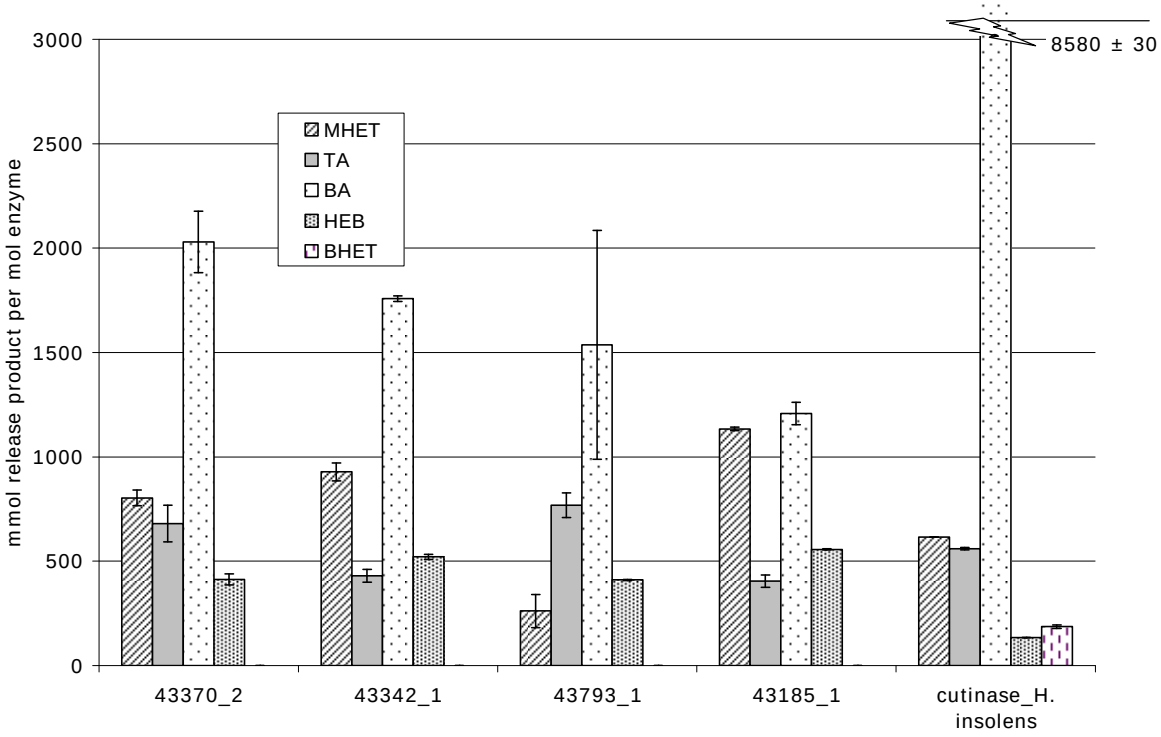


Figure 3-13 Hydrolysis products of 3PET after 72 hours of incubation with Thermobifida enzymes and purchased cutinase from *H. insolens*

Measurements of the hydrolysis products released be the cutinase from *H. insolens* showed, that the purchased enzyme acts very rapid and unspecific on all ester groups of the model substrate 3PET. No significant quantities of MHET, BHET or HEB are accumulated. If developed, these smaller ester compounds are hydrolysed immediately and almost only BA and TA can be found in the chromatograms. In comparison the absolute amount of hydrolysis products of the cutinase_*H. insolens* is approximately 6 times higher than the averaged released amounts of the other four enzymes.

Enzyme	Ability to hydrolyse 3PET and sub-compounds such as MHET and HEB	preferred ester bounds for hydrolytically attack
43370_2	Yes	exterior
43342_1	Yes	internal
43793_1	Yes	unspecific; exterior possible
43185_1	Yes; prefers HEB	internal
cutinase_ *H. insolens*	Yes	unspecific

Full data is presented in Appendix 3 - Results Hydrolysis of 3PET.

Hydrolysis of PET-foils

In the course of identifying the hydrolase activity of the enzymes on solid polymer foils of PET, the enzymes were incubated with foils of equal dimension for 5 days and the release of water soluble hydrolysis products was monitored by HPLC/UV.

Table 3-6 Total release products of PET after incubation (mmol per mol Enzyme) measured by HPLC/UV

release product / mmol mol^{-1}	43370_2	43342_1	43793_1	43185_1	cutinase_ *H. insolens*
MHET	11 ± 5	2 ± 1	2 ± 0.5	1 ± 0.1	356 ± 203
TA	5 ± 3	21 ± 4	54 ± 27	2 ± 0.2	555 ± 293
BHET	n.d.	n.d.	n.d.	n.d.	5 ± 3

Abbreviations: n.d. – not detected

Three of the five tested enzymes produce a significant amount of small water-soluble molecules that could be monitored by HPLC/UV.

The cutinase_*H. insolens* was by far the most active enzyme. The enzymes main hydrolysis product was TA, which reconfirms findings from previous studies on PET degradation by cutinase from *H. insolens (*Ronkvist *et al.* 2009). In contrast to the results from 2009, wherein no MHET was formed, Table 3-8 shows that the enzyme also produced a significant amount of MHET (356 mmol mol^{-1}).

Thermobifida alba enzyme 43185_1 does not seem to have hydrolase activity on the solid PET foil since only 2 and 1 mmol TA and MHET were produced per mol enzyme.

Highest release could be observed in incubations with the enzyme 43793_1 from *Thermobifida cellulolysitica.* The total sum of release products of 43793_1 is about twice of the second most active enzyme 43342_1. Both enzymes, 43793_1 and 43342_1, mainly produce TA and only marginal doses of MHET. This matches the results found by Eberl *et al.* where a identical enzyme, which was derived from *Thermobifida fusca* (rTfH) instead of *Thermobifida cellulolysitica,* was used to hydrolyse PET-fibres (Eberl *et al.* 2009; Eberl *et al.* 2008).

Interestingly 43370_2, in contrast to the other enzymes, released more MHET than TA. Also the total number of release products was very low. However, 43370_2 showed distinct hydrolyse activity on 3PET and the small soluble substrates.

In general the ability to hydrolyse a substrate depends on the accessibility of the substrate to the active site of the enzyme. Since the ability to hydrolyse the PET model-substrate 3PET was demonstrated above a possible conclusion could be that the enzyme´s active site is not compatible with the PET material.

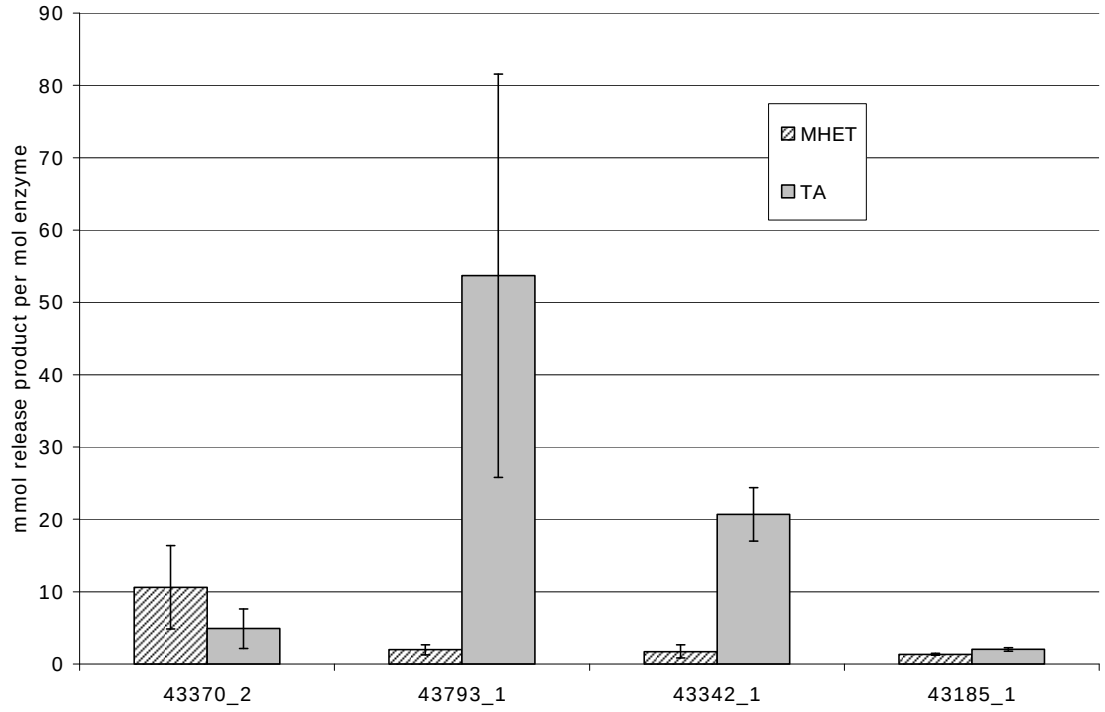


Figure 3-14 Hydrolysis of PET foil with cutinases from *Thermobifida sp.* enzymes 43370_2, 43793_1, 43342_1, 43185_1

Surface hydrolysis

HPLC analysis shows that the enzymes are capable of hydrolysing the PET material. To further observe potential hydrolysis indicated surface changes of the foils, their wettability before and after enzyme treatment was measured using the water contact angle measurements. FTIR-ATR analysis was employed to show changes in the crystallinity index on the incubated PET material. And furthermore, the surface hydrolysis was substantiated by using fluorescence spectroscopy to quantify the amount of new formed carboxylic and hydroxyl groups on the PET foils after derivatization with 2-(bromomethyl)naphthalene.

- **WCA**

The highest decrease of WCA was observed for the *Thermobifida fusca* enzyme 43793_1 followed by the *Thermobifida fusca* enzyme 43342_1. 43370_2 and *Thermobifida alba* enzyme 43185_1 only showed a very low change of the WCA of PET foil (see Table 3-7). Although increase of hydrophilicty is not necessarily linked to the amount of hydrolysis product released, it can be seen that the enzymes which produced the most water-soluble degradation products also showed the highest change in WCA.

Measuring the WCA of the PET foil after treatment with the purchased Cutinase from *H. insolens* was not possible. As already seen in the HPLC results this enzyme seems to hydrolyse the PET material very rapidly, therefore the WCA after incubation was too low to be measured within an acceptable standard deviation (WCA <10°).

Table 3-7 Water contact angles of PET foils after incubation with different enzymes

Enzyme	Water contact angle
Blank	72.3 ± 1.8
43370_2	69.5 ± 4.3
43342_1	68.5 ± 12.4
43793_1	66.0 ± 12.4
43185_1	69.1 ± 2.5
cutinase_*H. insolens*	Not accurately measurable due to low values (<10°)

- **FTIR-ATR**

When using FTIR-ATR spectroscopy the ratio between the absorption bands 1340 cm^{-1} (deriving from CH_2 wagging and marker for crystalline foil parts) and the band 1410 cm^{-1} (ring CC stretching and internal reference band) is indicator for crystallinity of foils (Donelli *et al.* 2009). Brückner *et al.* among others found, that enzymes usually prefer to hydrolyze amorphous regions of a surface. Therefore an increase of the A_{1340}/A_{1410} index would indicate a increase of crystallinity suggesting that the enzyme has degraded the amorphous parts.

To compare all self-fermented enzymes FTIR measurements were performed. For three of the four enzymes no significant changes of the crystallinity index were obtained. Only for the PET foils treated with 43342_1 an increase of their crystallinity index A_{1340}/A_{1410}, 0.290 compared to 0.260 of the blank, could be found.

- **Fluorescence spectroscopy**

Fluorescence spectroscopy after derivatization with 2-(bromomethyl)naphthalene is used to compare the amount of carboxylic and hydroxyl groups on the PET foils after incubation with and without enzyme.

As already expected from the results of FTIR-ATR and WCA analysis also fluorescence spectroscopy measurements confirmed that the esterase 43342_1 from *Thermobifida fusca* significantly alters the surface of the PET foils. After derivatization the fluorescence emissions intensity changed from 950 (Blank) to 1540 au (enzyme treated).

It was found, that the enzyme incubation alters the optical appearance of the PET foils. To get a better impression of these alterations, high resolution images of the surface were conducted by using an atomic force microscope (AFM). The images were kindly taken by colleges from the Profactor GmbH.

For the Thermobifida *sp.* enzymes almost no change could be observed but after incubation with cutinase_*H. insolens,* former clear and transparent PET foils turned opaque or milky whereas blanks incubated with buffer did not change their optical appearance.

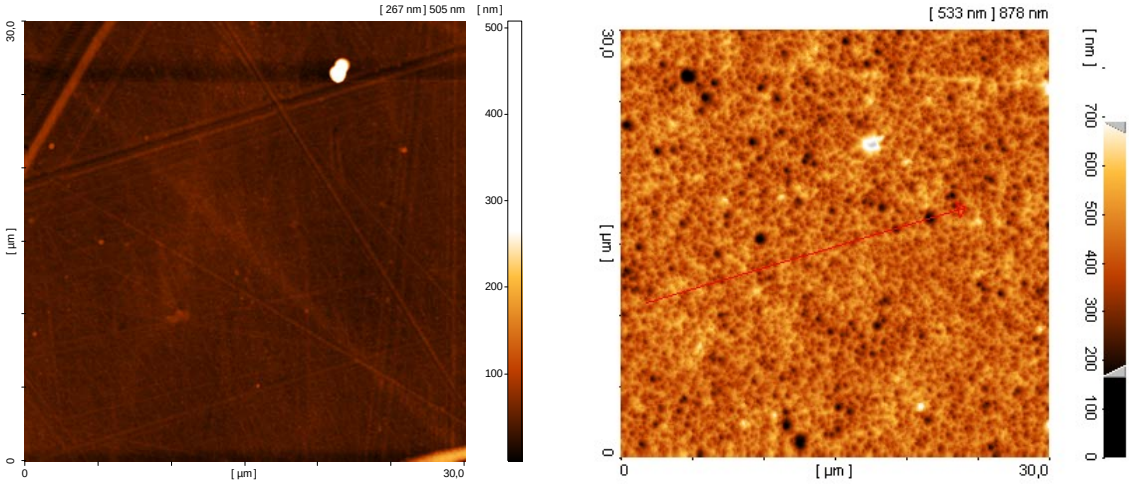


Figure 3-15 AFM images of a PET foil surface before (left) and after (right) treatment with purchased Cutinase of H. *insolens*

Bacillus subtilis nitrobenzylesterase (BsEstB)

Among bacteria several enzymes were found to have PET hydrolysing activity, most of which are either cutinases or/and or lipases. For example lipase from *Pseudomonas mendocina* (Kellis *et al.* 2001) or cutinase from *Pseudomonas simplicissimum* (Liebminger *et al.* 2007) and most recently a esterase from *Thermobifida fusca* (Oeser *et al.* 2010) were identified to have PET hydrolysing ability.

Herein produced p-nitrobenzylesterase from *Bacillus subtilis* is a classical intercellular esterase. Its pH-optimum is at pH 7.0 and the temperature optimum in terms of 3PET release was found to be 40°C. Nevertheless it was decided to perform PET incubations at 37°C hence reaction time is a key factor in PET-hydrolysis and BsEstBs stability decreases rapidly at higher temperatures to only 6h at 45°C. Enzyme stability tests showed that BsEstB keeps app. 100% of its activity for the first 36 hours then the activity slowly decreases to half of the initial value after 72 hours.

Table 3-8 and Table 3-9 give a summary of all kinetic parameters of BsEstB measured with the small ester substrates.

Table 3-8 Specific activity of purified *Bacillus subtilis* nitrobenzylesterase

	p-NP-A / U mg^{-1}	*p*-NP-B / U mg^{-1}	*p*-NP-P / U mg^{-1}
BsEstB	6.98 ± 0.36	9.85 ± 2.35	No activity

Table 3-9 Michaelis Menten Parameters and turnover number of purified Bacillus subtilis nitrobenzylesterase (Molecular Weight 55.2 kDa)

	p-NP-A			*p*-NP-B		
	K_M / µmol L^{-1}	v_{max} / µmol min^{-1} mg^{-1}	k_{cat} / s^{-1}	K_M / µmol L^{-1}	v_{max} / µmol min^{-1} mg^{-1}	k_{cat} / s^{-1}
BsEstB	190 ± 11	7 ± 0	6 ± 0	2500 ± 100	9 ± 2	8 ± 2

Additionally to *p*-NP-A and –B a Michaelis Menten kinetic was carried out using BHET as substrate.

Compared to the p-NP esters the turnover number significantly decreased with the more complex substrate to a k_{cat} of 0.14 ± 0.2 s^{-1} and a Michaelis-Menten constant of K_M = 140 ± 23 µmol L^{-1}.

In the next step the hydrolyse activity of BsEstB on the PET model substrat, 3PET was determined.

After 37 hours of incubation the maximum amount of release products was reached. One mole of BsEstB releases approximately 8 mmoles MHET, 298 mmoles TA and 750 mmoles BA whilst no HEB or BHET are formed. However, the inlet of Figure 3-16 shows that initially formed MHET seems to be degraded very fast into TA and ethyleneglycol.

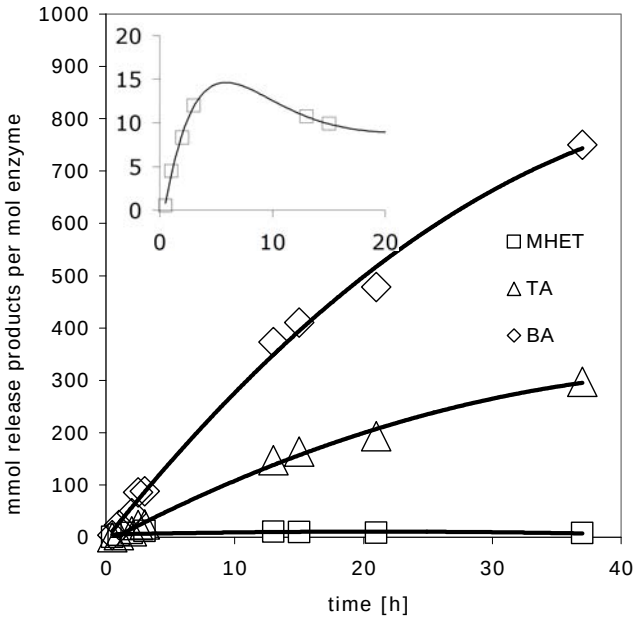


Figure 3-16 Hydrolysis of 3PET with p-nitrobenzylesterase from *B. subtilis*

Results of the small ester substrates as well as BHET already indicated that the enzyme clearly prefers less complex substrates such as *p*-NP-B. So expectedly the enzyme would prefer to first of all hydrolyse the simpler intermediates BHET and MHET. This would explain the absence of BHET in the incubations. Given that BsEstB rapidly hydrolysed the water soluble intermediates - BHET and MHET - before continuing to hydrolyse the insoluble 3PET no BHET can be detected, which leads to the proposed reaction pathway in Figure 3-17 (Ribitsch *et al.* 2011).

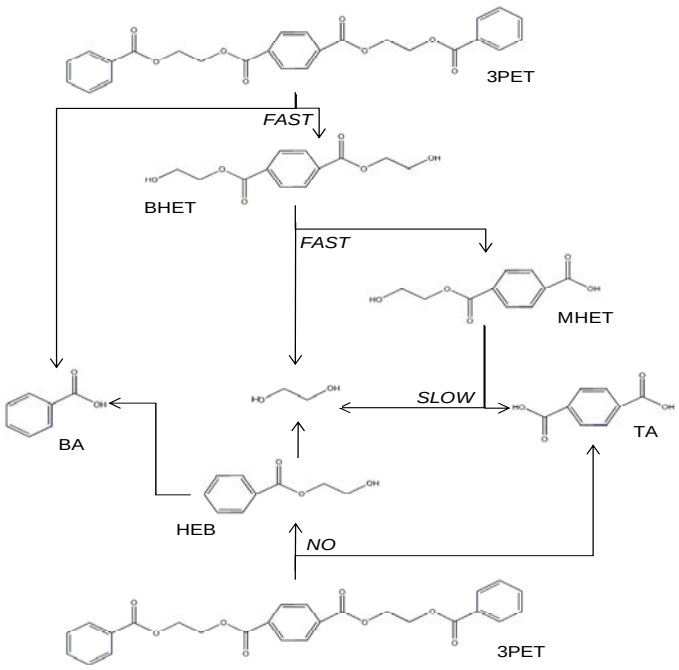


Figure 3-17 Hydrolysis of 3PET with p-nitrobenzyl-esterase from *B. subtilis* (Ribitsch *et al.* 2011)

BsEstB hydrolysis of PET foils

Although, PET is more complex than 3PET BsEstB still was able to hydrolyse PET foils.
The maximum amount of release products after 5 days of incubation were surprisingly high, with 138 mmol MHET, 254 mmol, TA and 370 mmol BA. Again no BHET was found.

The fact that the total amount of hydrolysis products of PET does not differ that much from the results obtained with 3PET indicates, that the dissolved availability of the substrate is of high significance for the enzyme in order to be able to hydrolyse. PET and 3PET are both water insoluble substrates and showed a much less degree of hydrolyzation than their water soluble model substrates.
Nevertheless, measurements of PET foils showed a clear decrease of the WCA after incubation with BsEstB. A decrease of approximately 5.6° (68.2° ± 1.7° blank, 62.6° ± 1.1° BsEstB) clearly indicates that the hydrophilicity of the foils increases during the enzyme treatment.

Compared the blank, no significant changes of the crystallinity index A_{1340}/A_{1410} of the BsEstB incubated PET foils were detected. This is most likely because of the very small amount of hydrolysed PET, so that no changes of the ratio amorphous:crystalline PET are detectable.

4 CONCLUSION AND OUTLOOK

In this work it was clearly demonstrated that three of the four herein fermented enzymes from the *Thermonbifida* species were able to hydrolyse the aromatic polyester poly(ethylene therephthalate).
The enzymes were characterized regarding their kinetic parameters. On *p*-NPA, *p*-NPB and *p*-NPP. K_M values of *Thermobifida sp.* cutinases were in the same range but the k_{cat} values differ up to 15 times from each other. The highest k_{cat} value for *p*-NPX substrates was found for 43793_1 followed by 43342_1. 43370_2 from *Thermobifida cellulosilytica* had the lowest k_{cat} value.

Upon incubation with the model substrate of PET, 3PET, 43793_1 again showed the highest release of products MEHT, TA, BA, and HEB very closely followed by 43342_1 and 43370_2. The pattern of small soluble products at different time of incubation showed, that all enzymes are able to cleave internal ester bonds of 3PET (endowise hydrolysis) however, 43370_2 seems to prefer exowise attack.

When incubating the enzymes with PET foils it could be observed that 43793_1 from *Thermobifida cellulolysitica* showed the highest hydrolase activity. Total sum of release products of 43793_1 is about twice of the second most active enzyme 43342_1. Although active on 3PET, 43185_1 showed almost none activity on PET. Interestingly 43370_2, in contrast to the other enzymes, released more MHET than TA. Also the total number of release products was very low. However, 43370_2 showed distinct hydrolysis activity on 3PET and the small soluble substrates.

Increase of surface hydrophilicity was proven by using the surface investigating techniques WCA and it was shown that the enzymes which produced the most water-soluble degradation products also showed the highest change in WCA.

It was demonstrated, that although originating from the same species the two highly similar enzymes 43793_1 and 43370_2 tremendously differed in their hydrolyse abilities. Generally it applies that an enzymes ability to hydrolyse a substrate depends on the accessibility of the substrate to the active site of the enzyme. So the next step for this work would be to create a model of the two similar enzymes and to perform a structure function comparison to elucidate why they show such different activity profiles[1].

[1] This work was done by Herrero Acero *et al.* and published in Macromolecules in 2011 (Herrero Acero, 2011)

In order to use polymers as a substrate microorganisms have to excrete extracellular enzymes since they cannot take up polymers into the cells. Scientists have found that after degrading the polymer outside the cell watersoluble intermediates are taken up and integrated into the metabolic cycle of the microorganism (Müller 2006). Therefore it was long believed that PET hydrolysis is linked to extracellular lipases or cutinases.

In this work it was demonstrated that the herein fermented intracellular *p*-Nitrobenzylesterase from *Bacillus subtilis* is capable of hydrolysing 3PET and PET up to a certain degree. Although the released amounts of soluble products was lower that for the *Thermobifida sp.* enzymes, WCA measurements of PET foils after treatment with BsEstB shoed a significant decrease indicating that the hydrophilicity of the foils increases during the enzyme treatment.

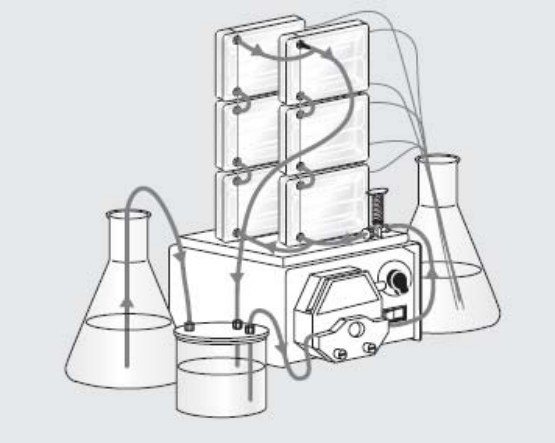
As mentioned in the beginning the grey biotechnology seeks to find microbiological systems such as bacteria, fungis or enzymes to use them in waste treatment. After identification and isolation of e.g. polyester-hydrolysing enzymes, they need to be improved with regards to their degradation abilities. Thus, the next step to a more ecological and cost efficient waste treatment is the understanding of mechanisms on the enzyme level.
Consecutive research to this work - among other things - focused on the elucidation of structure function relationships of herein identified and characterised enzymes 43793_1 and 43370_2. It was shown that the two enzymes have a very high homology of 93 % differing from each other only in 18 aminoacids. Modelling and docking revealed that also the proposed active sites of the two enzymes do not significantly differ from each other (Herrero Acero, 2011). Yet, the present work clearly exhibits tremendous differences on the hydrolysis of solid PET and also on soluble substrates. In the curse of their research Herreo Acero *et al.* stated the hypothesis, that hydrophobic and electrostatic surface properties near the active site of the enzymes are responsible for different interactions between active site and substrate (Herrero Acero, 2011).

In order to verify this hypothesis and to get more information on how the enzyme work, direct evolution of the two enzymes could be used to specifically identify the amino acids on the enzymes surface that cause an impact on the enzyme-substrate binding mechanisms. By this it might be possible to conclusive elucidate the difference in hydrolysis behaviour of the highly similar enzymes.

APPENDICES

Appendix 1 - Further reading of applied methods

Vivaflow Sartorius Setup for desalting purpose



(Reference: Sartorius VivaFlow Manual SLU6097 from http://www.sartorius-stedim.com/DE/de/Crossflow-Ultrafiltration/Vivaflow-50/oif0vjma7qw/66kwccabvv9/mp.htm?view=dl, Feb. 2011, page 5)

Strep-Tactin® Spin Columns

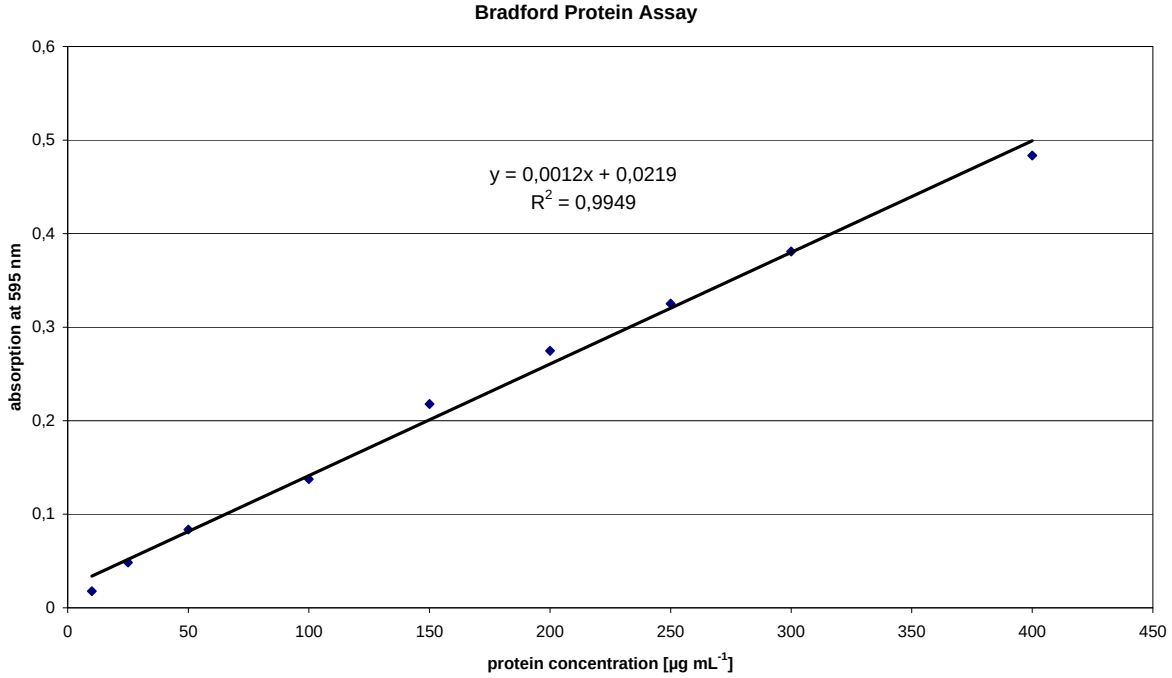
Vide Strep-Tactin® Spin Columns Purification Protocol (Version PR10-0004 IBA, Germany), Chapter 3 “Purification of Strep-tag fusion proteins using Strep-Tactin Spin Columns” purification steps 1 to 5a.

SpinTrap™ columns

Vide His SpinTrap Manual (Instructions 11-0036-91 AD, GE Healthcare), Chapter 5 “Purification protocol” purification steps 1 to 5.

Bradford Protein Assay (Bradford, 1976 & Wright *et al.*, 1996)

Bradford micro assay is carried out in 96-well microtiter plates. First, standard solutions with bovine serum albumin with protein concentrations ranging from 10 to 400 µg mL^{-1}, are prepared using H_2O purified as diluent.
After putting 200 µL diluted Bio-Rad Bradford-Reagent concentrate (1+4 with H_2O purified) into a microtiter-well, 10 µL enzyme solution are added. The preparation was carefully mixed using a clean sampler tip and incubated at room temperature for exact 15 minutes. After incubation the absorption at 595 nm is measured.



Fluorescence Spectroscopy of alkylated PET foils

Performed by Giuliano Freddi *et al.*:
PET foils of 50 x 10 mm size were alkylated in 20 mL of N,N-dimethylformamide (DMF) containing 0.05 mol L^{-1} 2-(bromomethyl)naphthalene and 0.02 mol L^{-1}potassium fluoride. Samples were incubated for 3 h at room temperature on a rotary shaker at 100 rpm. After incubation the PET foils were washed with DMF in order to remove unreacted reagents and then rinsed with distilled water. Samples were dried at room temperature and the fluorescence emission was measured using a multiplate reader (Varioskan, Thermo) with excitation and emission wavelengths of 350 and 440 nm, respectively.

Appendix 2 - Chemicals

Acetonitrile HPLC grade, VWR
Ampicillin, Sigma
BIO-RAD SDS-PAGE Molecular Weight Standards, Broad Range Catalog Number 161-0317
Bis(2-hydroxyethyl) terephthalate, Sigma
Bovine serum albumin, Neuber
Bradford reagent, Bio-Rad Concentrate
Dimethyl sulfoxide, Merck extra pure
Di-Potassium hydrogen phosphate, Roth
Ethanol abs.
Gum Arabic, Sigma
Hydrochloric acid conc., Roth
iso-Propanol, Sigma
Isopropyl β-D-1-thiogalactopyranoside, Sigma ≥99%
Kanamycin, Sigma
Methanol abs., Roth
p-Nitrophenyl-Acetate, Acros Organics 97%
p-Nitrophenyl-Butyrate, Sigma ≥ 98.0%
p-Nitrophenyl-Palmitate, Sigma ≥ 98.0%
Potassium dihydrogen phosphate, Roth
Sodium carbonate anhydrous, Roth
Sodium taurocholate hydrate, Acros Organics 98%
Sulphuric acid conc., HPLC grade
Tris (tris(hydroxymethyl)aminomethane)
Triton X-100, Roth

Appendix 3 - Results Hydrolysis of 3PET

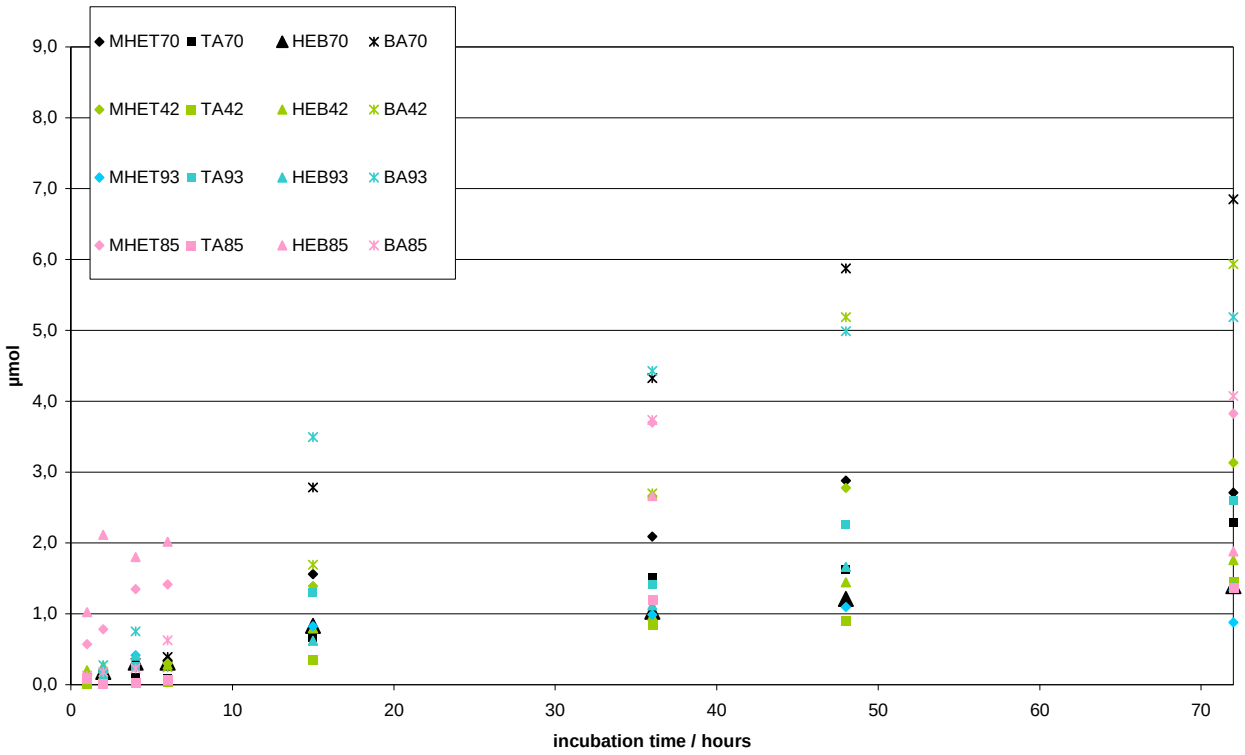


Figure 0-1 Comparison of release amounts of biodegradative products after hydrolysis of 3PET with *Thermobifida sp.* enzymes 43793_1 (blue), 43370_2 (black), 43342_1 (green) and 43185_1 (pink). Degradation products are ◇ - MHET, □ – TA, * - BA, Δ – HEB.

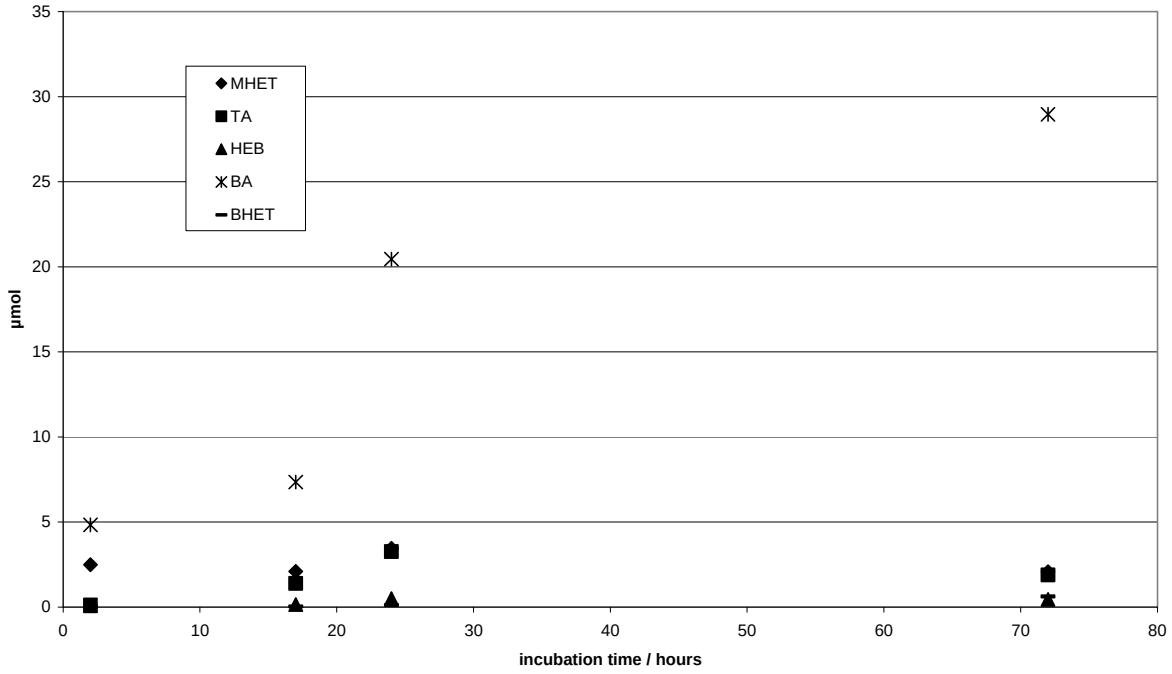
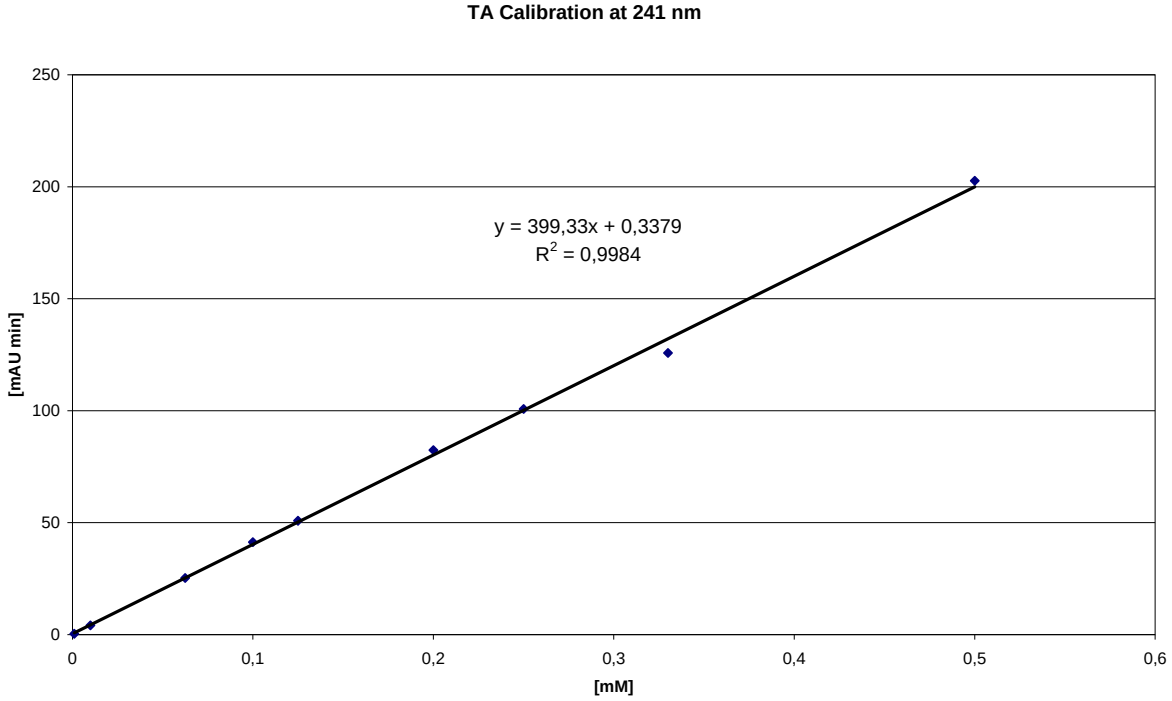
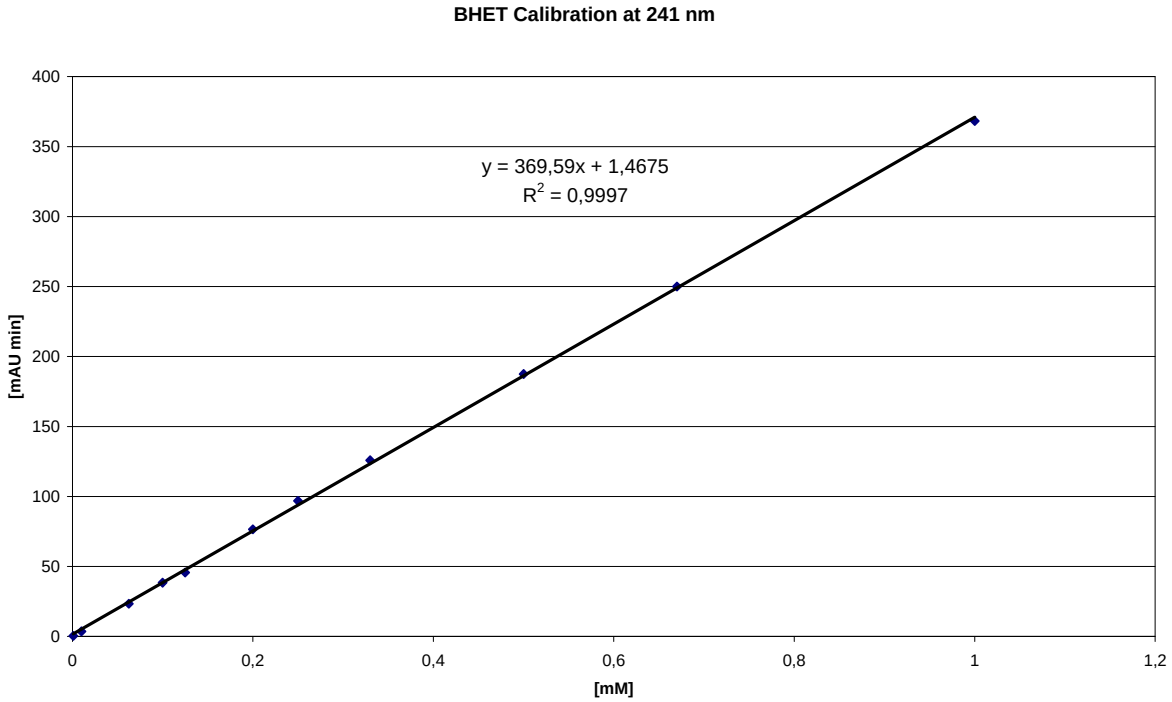
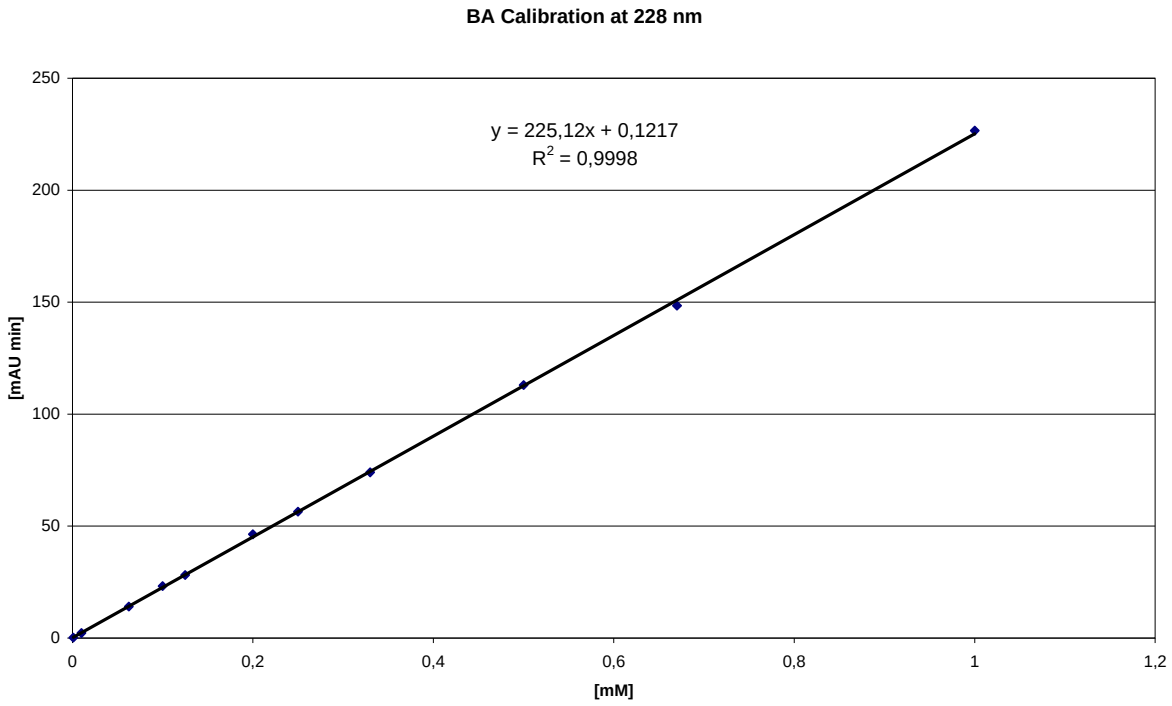


Figure 0-2 Comparison of release amounts of biodegradative products after hydrolysis of 3PET with cutinase_H. insolens. Degradation products are ◇ - MHET, □ – TA, * - BA, Δ – HEB, -- - BHET.

Appendix 4 - Calibrations of HPLC analytes






BA Calibration at 228 nm
y = 225,12x + 0,1217
R2 = 0,9998
[mAU min]
[mM]
250
200
150
100
50
0
0
0,2
0,4
0,6
0,8
1
1,2

REFERENCES

Müller, R.J., Kleeberg, I., and Deckwer, W.D. (2001) Biodegradation of polyesters containing aromatic constituents*. J. Biotechnol.* 86, 87-95.

Müller, R.J. (2006) Biological degradation of synthetic polyesters – Enzymes as potential catalysts for polyester recycling. *Proc. Biochem*. 41, 2124-2128.

Müller, R.J., Schrader, H., Profe, J., Dresler, K., Deckwer, W.D. (2005). Enzymatic degradation of poly(ethylene terephthalate): Rapid hydrolyse using a hydrolase from *T. fusca. Macromol. Rap. Comm*. 26, 1400-1405.

Vertommen, M.A.M.E., Nierstrasz, V.A., Veer, M.v.d., Warmoeskerken, M.M.C.G. (2005). Enzymatic surface modification of poly(ethylene terephthalate). *J. Biotechnol.* 120, 376-386.

Gouda, M.K., Kleeberg, I., Van den Heuvel, J., Müller, R.J., Deckwer, W.D. (2002). Production of a Polyester Degrading Extracellular Hydrolase from *Thermomonospora fusca. Biotechnol. Progr.* 18, 927-934.

Kleeberg, I., Welzel, K., Van den Heuvel, J., Müller, R.J., Deckwer, W.D. (2005). Characterization of a New Extracellular Hydrolase from Thermobifida fusca Degrading Aliphatic-Aromatic Copolyesters. *Biomacromolecul.* 6, 262-270.

Eberl, A., Heumann, S., Kotek, R., Kaufmann, F., Mitsche, S., Cavaco-Paulo, A., Gübitz, G.M. (2008). Enzymatic hydrolysis of PTT polymers and oligomers. J*ournal of Biotechnology* 135(1), 45-51.

Gübitz, G.M., Cavaco-Paulo, A. (2008). Enzymes go big: surface hydrolysis and functionalisation of synthetic polymers. *Trends Biotechnol.* 26, 32-38.

Eberl, A., Heumann, S., Brückner, T., Araujo, R., Cavaca-Paulo, A., Kaufmann, F., Kroutil, W., Gübitz G.M. (2009). Enzymatic surface hydrolysis of poly(ethylene terephthalate) and bis(benzoyloxyethyl) terephthalate by lipase and cutinase in the presence of surface active molecules. *J. Biotechnol*., 143, 207-212

Heumann, S., Eberl, A., Pobeheim, H., Liebminger, S., Fischer-Colbrie, G., Almansa, E., Cavacao-Paulo, A., Gübitz, G.M. (2006). New model substrates for enzyme hydrolysing polyethyleneterephthalate and polyamide fibres*. J. Biochem. Biophys. Meth*. 69, 89-99.

Ribitsch, D., Heumann, S., Trotscha, E., Herrero Acero, E., Greimel, K., Leber, R. (2011). Hydrolysis of Polyethyleneterephthalate by *p*-Nitrophenylesterase from *Bacillus subtilis. Biotechnol. Prog.* 27(4), 951-960.

Brückner, T., Eberl, A., Heumann, S., Rabe, M., Gübitz, G.M. (2009). Mechanistic study of enzymatic and chemical hydrolysis of poly(ethylene terephthalate) fabrics. *Journal of Biotechnology* 143, 207–212.

Ronkvist, A.M., Xie, W., Lu, W., Gross, R.A. (2009). *Macromolecules* 42(14), 5128-5138

Nagarajan, V. (2005). US patent 20050261465

Kellis, T., J. Jr., Ayrookaran, J.P., Mee-young, Y. (2002). US Patent 20020007518

Donelli, I., Freddi, G., Nierstrasz, V., Taddei, P. (2010). Surface structure and properties of poly-(ethylene terephthalate) hydrolyzed by alkali and cutinase. P. Polym. Degrad. Stab. 95(9), 1542-1550.

Kleeberg, I., Hetz, C., Kroppenstedt, R.M., Müller, R.J., Deckwer, W.D. (1998). Biodegradation of aliphatic–aromatic copolyesters by *Thermomonospora fusca* and other thermophilic compost isolates. *Appl. Environ. Microbiol*. 64(3), 1731–1735.

Liebminger, S., Eberl, A., Sousa, F., Heumann, S., Fischer-Colbrie, G., Cavacao-Paulo, A., Gübitz, G.M. (2007). Hydrolysis of PET and bis(benzoyloxyethyl) terephthalate with a new poyesterase from *Penicillium citrinum*. *Biocat. Biotrans*, 25, 171-177.

Oeser, T., Wei, R., Baumgartner, T., Billig, S., Föllner, C., Zimmermann, W., (2010). High level expression of a hydrophobic poly(ethylene terephthalate)-hydrolyzing carboxylesterase from *Thermobifida fusca* KW3 in *Escherichia coli* BL21(DE3). *J Biotechnol.,* 146, 100-104.

Herrero Acero, E., Ribitsch, D., Steinkellner, G., Gruber, K., Greimel, K., Eiteljoerg, I., Trotscha, E., Wei, R., Zimmermann, W., Zinn, M., Cavaco-Paulo, A., Freddi, G., Schwab, H., Gübitz, G., (2011). Enzymatic Surface Hydrolysis of PET: Effect of Structural Diversity on Kinetic Properties of Cutinases from *Thermobifida*. *Macromolecules* 44(12):4632-4640.

Oswald, TA., Baur, E., Brinkmann, S., Oberbach, K., Schmachtenberg, E., (2006). *International Plastics Handbook;* 4th Edition ed.; Hanser Publishers: Munich, 2006.

Gübitz, GM., (2011). Hydrolases in Polymer Chemistry: Part III: Synthesis and Limited Surface Hydrolysis of Polyesters and Other Polymers. *Enzymatic Polymerisation*, 1st Edition ed, Palmans ARA, Heise A, Eds 237.