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Polyester Degrading Enzymes from Anaerobic Sources

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Abstract

Despite the great efforts made in recycling and energy recovery of synthetic polymers, it is presently still virtually impossible to limit the pollution of our environment caused by plastic waste. Due to this situation the demand for biodegradable polymers has surged in recent years. New materials such as aliphatic-aromatic copolyesters (AAC) were invented. The commercially available ecoflex® offers excellent material properties while being readily degradable by microorganisms under aerobe conditions. In contrast to that, the anaerobe biodegradation of biodegradable polymers is still limited and many aspects remain unclear. The goal of the present thesis was to investigate the differences between aerobe and anaerobe degradation of AAC and to discover novel esterases of anaerobic origin for polyester degradation.

A mechanistic study which used a cutinase of the aerobe *Thermobifida cellulosilytica* and an esterase of the anaerobe *Clostridium botulinum* to hydrolyse various AAC model substrates revealed distinctions in the substrate specificity of the enzymes. Further experiments explored the possibility of the induction of enzyme expression using naturally occurring polymers in anaerobe sludge batches. For this purpose a novel esterase activity assay was successfully created using a fluorescein derivative as indicator. The induced sludges showed a slightly increased activity and a reduced metabolic lag phase. In an additional approach active hydrolases were isolated directly from a culture of anaerobe *Clostridia* utilizing fluorescent probes and gel electrophoresis followed by protein sequencing. The method was effectively established as a complement to existing analytical techniques.

Kurzfassung

Trotz der großen Anstrengungen die für das Recycling und die Energierückgewinnung von synthetischen Polymeren unternommen werden ist der Umweltverschmutzung durch Plastik kaum Herr zu werden. Auf Grund dieser Entwicklung ist der Bedarf an biologisch abbaubaren Polymeren in den letzten Jahren stark gestiegen. Neue Materialien wie aliphatisch-aromatische Copolyester (AAC) wurden erfunden. Das kommerziell erhältliche ecoflex® besitzt exzellente Materialeigenschaften und ist dennoch von Mikroorganismen unter aeroben Bedingungen gut abbaubar. Im Gegensatz dazu ist die anaerobe biologische Abbaubarkeit von biologisch abbaubaren Polymeren noch stark limitiert und viele Aspekte davon sind unklar. Das Ziel der vorliegenden Studie war es, die Unterschiede zwischen aerobem und anaerobem Abbau von AAC zu untersuchen und neue Esterasen anaeroben Ursprungs für den Abbau von Polyestern zu entdecken.

Eine mechanistische Studie, die eine Cutinase von der aeroben *Thermobifida cellulosilytica* und eine Esterase des aeroben *Clostridium botulinum* benutzte um AAC Modellsubstrate zu hydrolysieren, enthüllte klare Unterschiede in den Substratspezifitäten der eingesetzten Enzyme. Zusätzliche Experimente untersuchten die Möglichkeit der Induzierbarkeit der Enzymexpression in anaeroben Schlämmen mittels natürlich vorkommenden Polymeren. Zu diesem Zweck wurde ein neuartiger Esterase-Aktivitätsassay, der ein Fluoresceinderivat als Indikator benutzt, erfolgreich entwickelt. Die induzierten Schlämme zeigten eine leicht gesteigerte Aktivität und eine reduzierte metabolische Verzögerungsphase. In einem weiteren Versuchsansatz wurden aktive Hydrolasen mit Hilfe von fluoreszierenden Sonden direkt aus einer anaeroben Kultur von *Clostridia* isoliert. Diese vielverprechende Methode konnte als Ergänzung zu bereits existierenden analytischen Techniken etabliert werden.

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1 Introduction

This thesis is part of a collaboration between the Austrian Centre of Industrial Biotechnology (ACIB) and its industrial partner BASF. The main goal is to study and enhance the biodegradation of the commercially available polymer ecoflex® by using naturally occurring enzymes. Especially under anaerobic conditions, the biodegradation of polymers still faces many challenges that need to be overcome [1]. This project aims at identifying novel polyester hydrolysing enzymes from anaerobic sources, to get a better understanding of the hydrolysis process and to improve the degradability of polymers during anaerobic digestion in biogas producing plants.

The pursued strategy to accomplish this can be divided into three main parts: an *in silico* approach, an *enzymatic* approach and a *biogas* approach. *In silico*, enzymes are identified by homology through a database search and promising candidates are cloned and heterologously expressed. The *enzymatic* variant uses novel and well-studied enzymes alike in mechanistical experiments to gain more insight about the basic degradation processes. The *biogas* approach aims at inducing enzyme expression in anaerobic sludge batches to find enzymes and organisms that are able to biodegrade ecoflex.

The present study contributed to both the *biogas*- and the *enzymatic* approach. In anaerobic sludge batches an activity assay was created to assess the effectivity of inducers on enzyme expression. Furthermore, isolated enzymes from anaerobic and aerobic sources were tested on model substrates to study their hydrolysis specificity. In a final step a method to label, isolate and identify enzymes from cultures of microorganisms was established.

1.1 Plastic, waste and the environment

Synthetic polymers have become an indispensable part of our lives, and it is hardly surprising that the polymer industry is continuously growing: In 2012 the worldwide production of plastics peaked at 288 million tons, which is an all-time high and an increase of 2.8 % compared to 2011 [2].

Traditional petrochemical plastics feature a broad field of use, high cost efficiency and remarkable technical properties. However, the constant increase in durability and longevity is a double-edged sword: While the long stability, chemical inertia and therefore the resistance to environmental factors are regarded positively in respect to industrial usability, it is due to these attributes that plastics linger can pose a threat to the environment. Microorganisms have not yet had the time to adept to these new xenobiotics, and evolution still has to develop methods and enzymes to succeed with their degradation [3].

In Europe over 39 % of the yearly produced polymers are used for packaging, where they have replaced many cellulose–based products due to better physical and chemical properties [1]. Typically the materials for packaging are only used once and then discarded [4]. The enormous amounts of plastics waste that are generated every year provide a significant challenge and are attracting a lot of public and legislative attention. In general, plastics waste can be dealt with in three ways: Recycling, energy recovery and disposal, e.g. in landfills. The disposal in landfills poses the least favourable option because it hinders a resource-efficient treatment of plastic waste. However, EU-wide more than 38 % of post-consumer plastics waste is still disposed of in landfills. This has led the European Commission to draft a “Zero-Waste” programme for Europe, which proposes a total ban of plastics from landfills by the year 2025 [5]. Countries like Switzerland Germany, where at least a partial landfill ban is already in force, report a combined recycling and energy recovery rate of up to 99 % [6].

While these measures might be the solution to effectively deal with plastic waste in the longer term, a recent study [7] estimates that between 4.8 to 12.7 million metric tons of plastic waste are entering the ocean - every year. This results in gigantic floating accumulations of plastic that cause severe damage to the marine ecosystem. Through the fishing industry the plastic waste finds its way into the food chain. A solution for this pressing issue could be the use of biodegradable polymers.

1.2 Biodegradation of biodegradable polymers

Biodegradation is a process in which substances are broken down by living organisms. For water-insoluble polymers the biodegradation is a multi-step process that usually requires several different organisms to be effective [8]. The water insolubility prevents the polymers from being transported directly into the microorganisms and enforces a different strategy to be applied, of which a schematic representation can be seen in Figure 1: In the presence of synthetic or natural polymers microorganisms excrete extracellular enzymes that are able to attach to the hydrophobic polymer surface and cleave the polymer chains. The enzymes are not able to penetrate deeply into the polymer, and thus only the polymer's surface is skimmed in a surface erosion process [9]. Water soluble intermediates are generated that can be assimilated by microorganisms and metabolized, which results in the metabolic products CO_2, H_2O and CH_4 when digested anaerobically [3]. The surface erosion is normally the rate-limiting step in biodegradation.

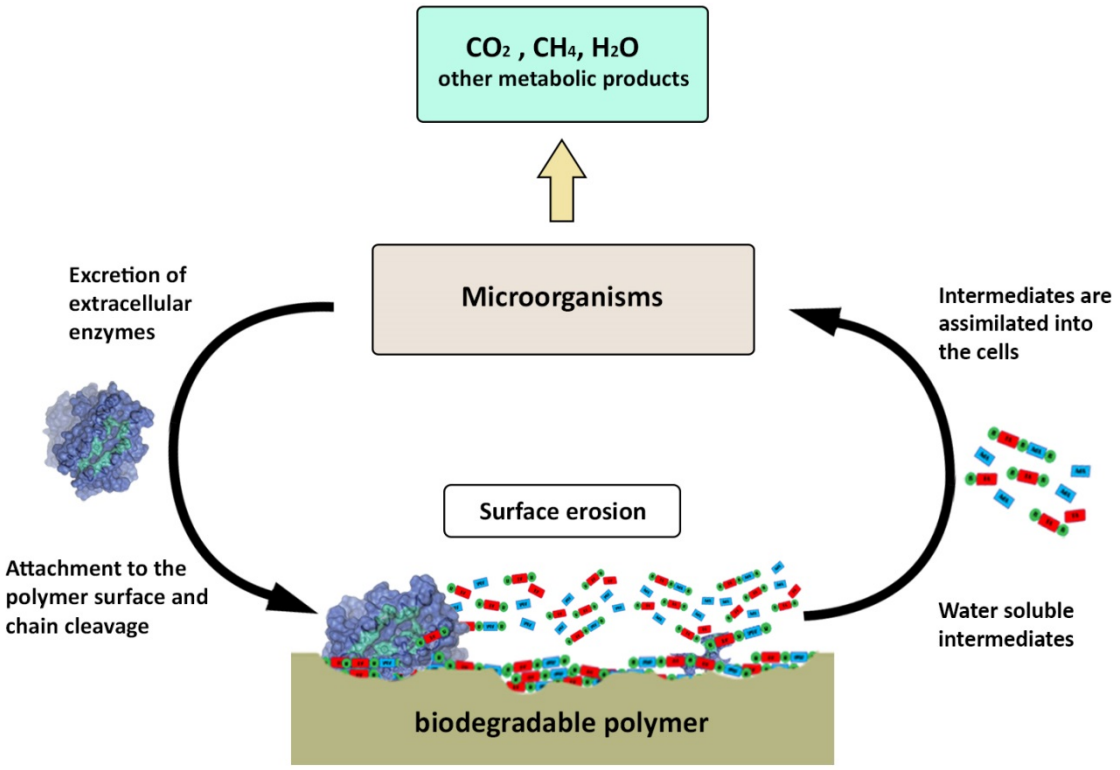


Figure 1: The process of biodegradation of biodegradable polymers. Microorganisms secrete extracellular enzymes which attach to the polymer surface and release water soluble intermediates. These degradation intermediates are then assimilated into the cells where they are metabolized to CO_2, CH_4 and H_2O. [modified from R.-J. Mueller [3]

1.3 Biodegradable polymers – aliphatic aromatic copolyesters

Polyesters possess a predominant role in the polymer world. A member of the polyester family are aliphatic polyesters. Aliphatic polyesters of natural origin are for example polyhydroxyalkanoates (PHA) that are synthesized by microorganisms and accumulate intracellular to granules. A wide variety of bacteria and fungi secrete extracellular enzymes that can process PHA that was liberated by the death and lysis of other microorganisms [10]. The biodegradability of this biopolymer was early recognised and synthetic aliphatic polyesters were created that mimicked these properties, usually derived from lactic acid. But the biodegradability of these polyesters comes at the expense of material properties and limited applications [11]. Low melting points prohibit the use at elevated temperatures, for example when in contact with hot beverages or exposed to sun light [12].

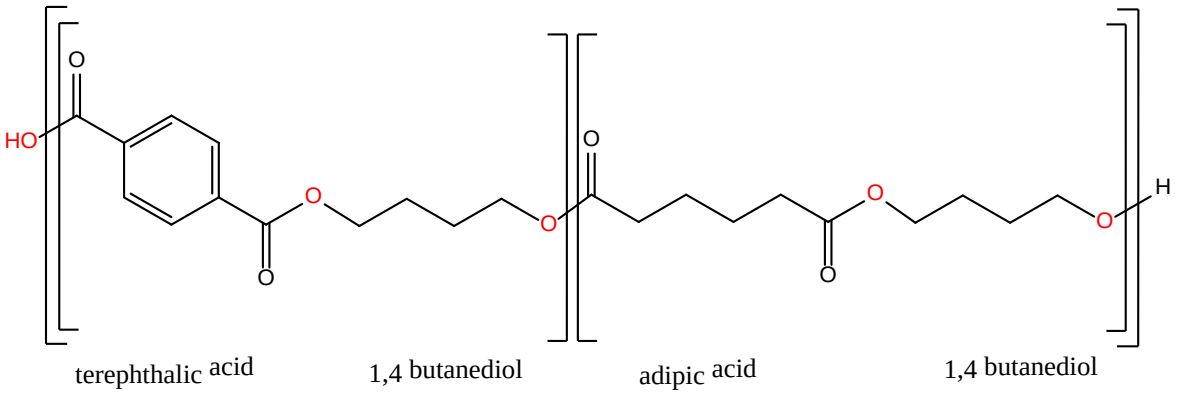
On the other side of the polymer spectrum are aromatic polyesters, a group which is best represented by polyethylenterephthalate (PET) and is a synonym for excellent mechanical and thermal characteristics. The outstanding resistance to most environmental influences is also the biggest challenge when trying to make aromatic polyesters more susceptible to microbial attacks, as they were generally considered to be virtually non-biodegradable [13].

To combine the best of both worlds, an attempt was made to mix the biodegradability of aliphatic polyesters with the favourable material properties of aromatic polyesters: Aliphatic – aromatic copolyesters (AAC) were created. Aliphatic components were introduced into the sturdy aromatic polymer chain to enhance the biodegradation process. The best results were achieved when combining aromatic terephthalic acid (Ta), aliphatic adipic acid (Ada) and 1,4 butanediol (B) to poly(butylene adipate-co-terephthalate) (PBAT) [14]. Butanediol serves as link between the aliphatic and aromatic components.

The mechanical properties can be modified by adjusting the ratio of Ta:Ada in the polymer backbone to adept the polymer for the chosen application. While biodegradability plummets when the Ta surpasses 55 % (regarding the total amounts of acid components), the material characteristics are unacceptable for Ta values below 35 % [12].

Soon commercial products were available, like ecoflex (BASF) or Eastar Bio (Eastman). The present study focussed on ecoflex as the main substrate of interest for enzymatic hydrolysis and biodegradability.

A schematic of ecoflex is depicted in Figure 2. Variants of ecoflex can additionally contain modular components such as branching agents of chain extenders [15].



Ta: $C_6H_{10}O_4$ // 146.14 g·mol^{-1} **B**: $C_4H_{10}O_2$ //90.12 g mol^{-1} **Ada**: $C_8H_6O_4$ // 166.13 g mol^{-1}

Figure 2: Structure of the aromatic aliphatic copolyester ecoflex with the ester bonds marked in red and molecular weights of terephthalic acid, 1,4 butanediol and adipic acid.

This petroleum–based polymer is certified as compostable and therefore susceptible to microbial attack, a process executed by enzymes.

1.4 Enzymes for polymer hydrolysis

Synthetic polymers are novel materials and nature has yet to develop ways to deal with these xenobiotics. However, already back in 1977 *Tokiwa* reported the hydrolysis of synthetic polyesters by isolated lipases [16]. Lipases belong to the enzyme class of hydrolases and are among the most important catalysts used in biotechnology, with many different applications. They are used in the textile and detergent industry as well as in food processing and polymer production, just to name some examples [17].

More recently another subclass of hydrolases has gained a lot of attention in polymer degradation: cutinases. The natural substrate for cutinases is cutin, a biopolymer which is part of the cuticle of plants and which is composed of hydroxy- and epoxy fatty acids [18]. Cutinases have been shown to be versatile enzymes with high activity and low specificity for cutin; hence they are promising candidates for the biodegradation of aliphatic-aromatic copolyesters [19]. In screenings, different *Thermobifida* species that express cutinases with polyesterase activity have been identified and tested on synthetic polymers [20], [21]. Cutinases are regarded as a link between esterases and lipases because they can efficiently hydrolyze soluble esters and emulsified triacylglycerols [22]. Lipases need to be activated at the hydrophobic surface. This interfacial activation causes the lipases' structure to change and allows access to the active site. While cutinases also display lipase activity, they do not exhibit this phenomenon. The absence of significant structural change upon ligand binding implies an already preformed oxyanion hole, another difference between cutinases and lipases [23].

In the meantime, with the discovery of new enzymes and the help of genetic engineering even robust polyesters like PET can be attacked by enzymes. While the complete hydrolysis of these polymers is still a long way off, current methods can at least be used as environmental friendly approaches to modify the surface properties [24]

1.5 Fluorescein derivatives as marker for enzyme activity

Fluorescein derivatives are widely used as indicators for hydrolytic activity, even in complex soil samples ([25], [26]). The present study also made use of fluorescein's favourable properties; a novel enzyme activity assay in anaerobic sludge was based on the hydrolysis of fluorescein derivatives.

The fluorescein esters are non-fluorescent, but they can be cleaved by enzymes to yield the fluorescent fluorescein. This reaction is displayed in Figure 3 for fluorescein dilaurate (FDL).

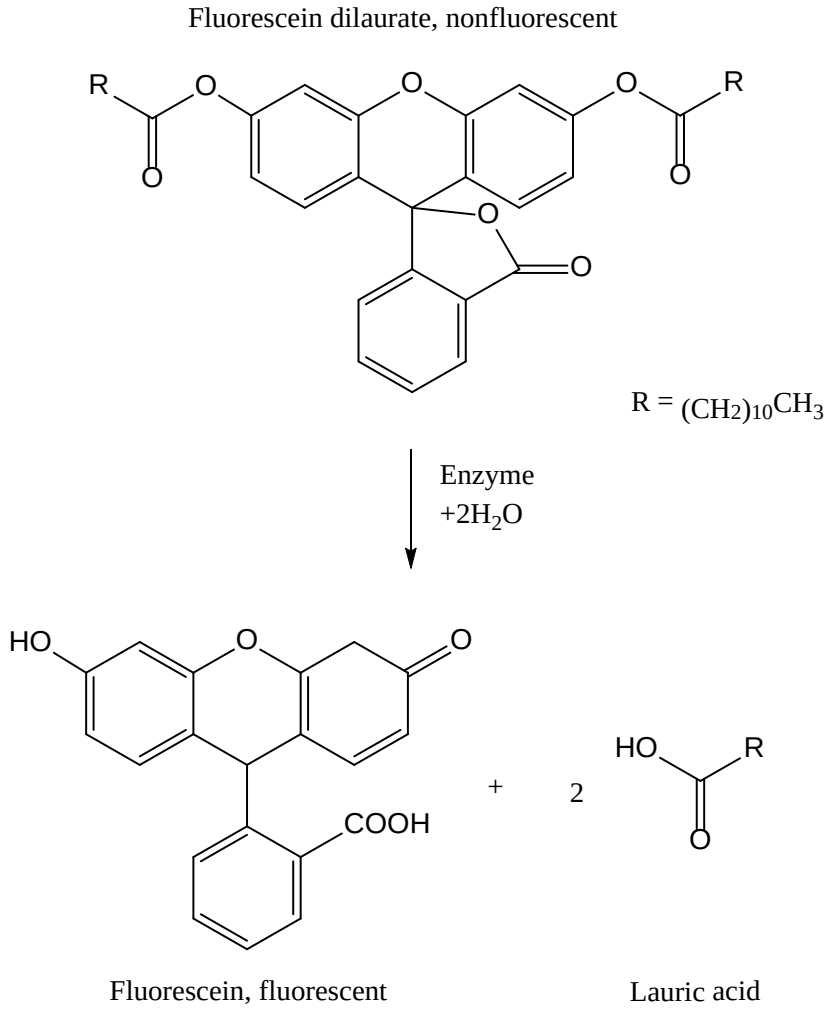


Figure 3: Enzymatic cleavage of the non-fluorescent fluorescein dilaurate to the fluorescent fluorescein.

The rate of change in the fluorescence due to production of fluorescein can be measured photometrically and correlated with the enzyme activity. Fluorescein derivatives with straight chain fatty acids have a hydrophobic nature and can be used as substrate for enzymes like lipases [27].

Fluorescein and its derivatives display a strong dependency of the pH value of their environment[28]. The absorption and emission spectra are shown in Figure 4. At acidic pH the fluorescence intensity is dramatically reduced. This is due to the fact that the di-anion of fluorescein contributes the most to the emission, and even the mono anion shows already strongly reduced intensity.

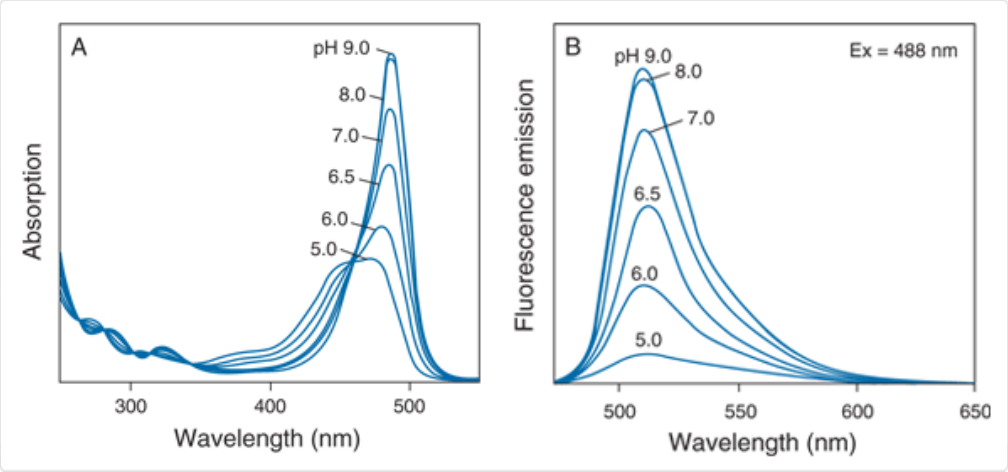


Figure 4: Absorption and fluorescence emission spectra of fluorescein at different pH values [28]**.**

2 Materials and Methods

Experimental approach

The present study is composed of three different parts which will be described consecutively.

In the beginning the mechanisms behind the degradation of PBAT were investigated: Two enzymes with esterase activity, one from aerobic *Thermobifida cellulosilytica* and the other one from the anaerobic *Clostridium botulinum*, were tested on ecoflex and PBAT model substrates of varying Ta:Ada ratio. This ratio strongly influences the material properties and has a tremendous influence on the biodegradability of the polymers. The degradation products were analysed using high pressure liquid chromatography (HPLC). The emerging patterns of monomeric and more complex hydrolysis products can provide a good insight about the substrate specificity of each enzyme.

The second part of the study consisted of a multitude of experiments involving sludge from a biogas production plant as source for enzymes of anaerobic origin:

- The effects of natural polymers as inducing agents for enzyme expression were measured with a newly developed esterase activity assay.
- Simultaneously, the degradation products of model substrates were quantified with HPLC analysis.
- Enzymes released into the sludge and other present proteins were separated by polyacrylamid gel electrophoresis (PAGE). Fluorescein derivatives and fluorescent probes were used to detect active serine hydrolases.
- Additionally the biofilm formation on polymer foils was investigated with crystal violet and LIVE/DEAD staining, and visualised via confocal laser scanning microscopy (CLSM).

Finally a method was established to extract polyesterases from a pure culture of anaerobe *Clostridia*. The culture's proteins were separated with PAGE after the active serine hydrolases were labelled. Promising bands were cut out of the gel and their amino acid sequence identified with mass spectrometry. Furthermore, the hydrolytic activity of the culture was tested on ecoflex.

Part I: Enzymatic hydrolysis of aliphatic aromatic copolyester

2.1 Enzymes

Two enzymes were chosen for the study on the degradation mechanism. The first one is the cutinase 1 of *Thermobifida cellulosilytica* (ThC_Cut1). This enzyme has shown promising hydrolytic properties on PET in previous studies ([21], [29]). It has a molecular weight (M_W) of 29400 g/mol and is filed as GenBank entry ADV92526.1.

The second enzyme was identified during a screening that revealed a number of potential hydrolytic enzymes. It was named esterase A and originates from the *Clostridium botulinum* strain ATCC 3502, hence it will be referred to as CB_EstA for the rest of this study. CB_EstA can be found under accession number CAL82416.1 and is classified as an esterase lipase with a M_W of 51700 g/mol.

2.1.1 Enzyme characterization

The enzyme concentration needed to be determined. This was achieved via the *Novagen* BCA protein assay kit. In principle, this method is based on a biuret reaction. In the presence of peptide bonds and in an alkaline solution, the reduction of Cu^{2+} to Cu^{+} occurs. Two molecules of bicinchoninic acid (BCA) then chelate a single Cu^{+} ion, which results in a purple reaction complex with strong absorbance at 562 nm. The standard microplate assay for 96 well plates was performed according to the manual. [30]

In addition to the protein concentration, kinetic parameters on a standard soluble substrate were determined. An activity assay was performed on para-nitrophenylbutyrate (pNPB). pNPB is enzymatically hydrolysed to para-nitrophenole, which can be quantified photometrically at 405 nm. The reaction is shown in Figure 5. The concentration of pNPB was ranging from 0.525 mM to 15.750 mM and the absorbance increase at 405 nm was followed for 5 minutes.

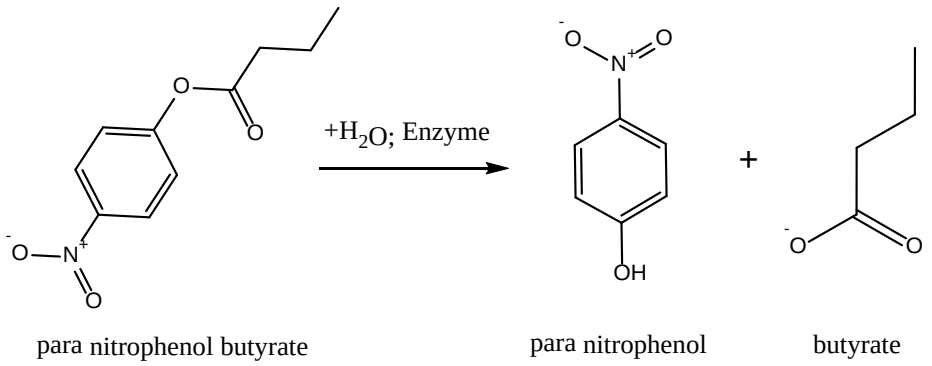


Figure 5: Enzymatic hydrolysis of pNPB to para-nitrophenol and butyrate.

2.2 ecoflex and model substrates for polyester hydrolysis

The polymer ecoflex consists of chains of randomly alternating monomers of terephthalic acid (Ta) and adipic acid (Ada), each connected through 1,4-butanediol (B). The total amount of Ta and Ada that can theoretically be set free after total substrate hydrolysis needed to be calculated. Knowing the theoretical maximum concentration of degradation products, the percentage of released products was determined.

The statistical Ta:Ada ratio of ecoflex is 47:53. An ecoflex polymer with 200 monomers therefore consists of 47 monomers Ta and 53 monomers Ada, which are linked by 100 monomers B. Based on this ratio the number of Ta and Ada molecules that can be set free after total hydrolysis can be calculated. As an example for all other model substrates, the calculated mol distribution and concentrations for 10 mg ecoflex in 2 ml buffer can be found in Table 1. One oxygen atom and two hydrogen atoms are subtracted from each monomer as they are removed in the formation of the ester bond between the monomers.

Table 1: Calculation of the approximate molar distribution of terephthalic acid (Ta) and adipic acid (Ada) in an ecoflex polymer. ecoflex' ratio of Ta:Ada is 47:53. The calculations are made for a hypothetical polymer consisting of 200 monomers. During esterification the monomers condense and one H_2O molecule separates. To take the ester bonds into account, one H_2O molecule was subtracted from each monomer and linker B (1,4 butanediol). The monomer ratios and concentrations are presented for 10 mg Ecoflex in 2 ml buffer.

ECOFLEX				total
Molecule	**Ta** -2H-1O	**Ada** -2H-1O	**B** -2H-1O	
Monomer ratio	47	53	100	
Molar mass [mg/mmol]	148,13	128,14	72,12	
Mass distribution [mg]	6962	6791	7212	20965 mg
Mass per 10 mg	3,32	3,24	3,44	
Equivalent Mol [mmol]	0,022	0,025	0,048	
Concentration in 2 ml [mol/L]	0,011	0,013	0,024	0,048 mol/L

In addition to ecoflex, several model substrates of similar structure were tested. They all consist of alternating Ta- and Ada monomers linked by B, but with different Ada:Ta ratios. A list of these polymers can be found in Table 2, including their exact Ada:Ta ratios, melting points (T_M) and molecular weight. The calculations for the respective molar ratios of Ta and Ada are presented in the appendix in App.Table 1. ecoflex as well as the model substrates were kindly provided by BASF.

Table 2: Poly(butylene adipate-co-butylene terephthalate) substrates and their respective adipic acid to terephthalic acid ratios, thermal properties and molecular weight. M_w represents the weight average, M_n the number-average.

Sample	Ada:Ta ratio	T_M [°C]	M_w [g/mol]	M_w/M_n
PBAda	100 : 0	58.8	52000	3.1
Ada90_Ta10	89.3 : 10.7	52.8	45800	2.9
Ada80_Ta20	78.9 : 21.1	47.8	32400	2.9
Ada70_Ta30	68.9 : 31:1	70.0	26700	2.8
Ada60_Ta40	58.5 : 41.5	101.4	29200	2.8
Ada50_Ta50	48.8 : 51.2	132.2	31900	2.7
ecoflex	53 : 47	125.3	65000	3.4

2.3 Enzymatic hydrolysis

The degradation was carried out with 10 mg substrate mixed with 2 ml of a 0.6 µM enzyme solution in potassium phosphate buffer (0.1 M, pH 7.0) at 50°C. The duration of the incubation was 72 hours; after each full day the reaction was stopped and the samples were measured.

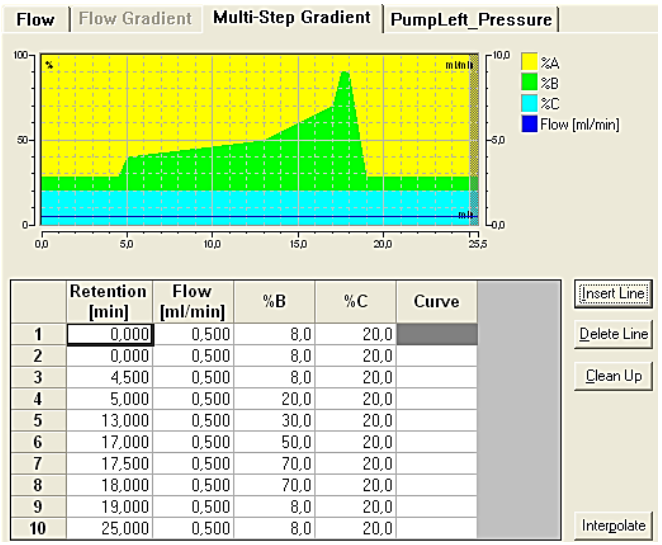
Controls for all model substrates were treated simultaneously without addition of enzymes to measure autocatalysis.

To stop the enzymatic reaction and to get rid of interfering particles, a methanol precipitation was performed. 500 µl of each sample were mixed with 500 µl of ice cold methanol and precipitated for 15 minutes on ice. After that, the samples were centrifuged at 0° C and 14000 rpm for 15 minutes. The resulting supernatants (800 µl) were mixed with 2 µl of formic acid and put in the refridgerator for 1 hour. Afterwards, samples were again centrifuged with the same parameters, and supernatants were transferred into HPLC vials for further use in the HPLC analysis.

2.4 Analysis with HPLC

The HPLC analysis is limited to soluble degradation products. The measurable degradation products of ecoflex are BTaB, BTa, Ta and Ada.

A multi-step gradient was used for the chromatography. It can be seen in detail in Figure 6. The mobile phase consisted of A: ddH_2O, B: acetonitrile and C: 0.1 % formic acid. 5 µl of the samples were injected; the flow was consistent at 0.5 ml/min; the temperature was set to 25° C. As column the XTerra RP 18 3.5 µm (waters) with precolumn was used, which is a general purpose reversed phase column. The detailed protocol as well as a list of instruments can be found in the appendix in App.Table 3.



	Retention [min]	Flow [ml/min]	%B	%C	Curve
1	0,000	0,500	8,0	20,0	
2	0,000	0,500	8,0	20,0	
3	4,500	0,500	8,0	20,0	
4	5,000	0,500	20,0	20,0	
5	13,000	0,500	30,0	20,0	
6	17,000	0,500	50,0	20,0	
7	17,500	0,500	70,0	20,0	
8	18,000	0,500	70,0	20,0	
9	19,000	0,500	8,0	20,0	
10	25,000	0,500	8,0	20,0	

Figure 6: HPLC Gradient was used for the analysis of ecoflex hydrolysis products. A: ddH_2O, B: acetonitrile and C: 0.1 % formic acid.

To be able to quantify the measured hydrolysis products, standards for BTaB, BTa, Ada and Ta were prepared. Their concentration range can be found in Table 3. The standards were dissolved in potassium phosphate buffer (0.1 M, pH 7.0) and a methanol precipitation was performed as described above.

Table 3: Concentrations of the standards prepared for the analysis byHPLC

Standard	Concentration range
Ta	0.0125 – 1.0 mM
BTaB	0.025 – 2.5 mM
BTa	0.025 – 2.5 mM
Ada	1.5 - 50 mM

Part II: Inducing enzyme expression in anaerobic sludge batches with polymers

2.5 Anaerobic batch cultures

An overview of the experimental setup for the sludges can be found in Figure 8 on the next page.

For the following batch experiments, two different kinds of anaerobic sludge were used. Both originated from the same biogas plant. Over the next months, however, they were set on divergent diets. One fraction of the sludge was long term mono digesting maize silage, while the other fraction was fed with naturally occurring polyesters (PHA and cutina), as well as model substrates (similar to those in 2.5.2) as part of an experiment to induce the expression of polyester degrading enzymes.

As inoculum for the batches a mixture of both sludge-types was used. In 1000 ml Duran flasks, 50 ml of the maize-sludge were mixed with 120 ml of the polyester-sludge and solved in 330 ml minimal medium (described in 2.5.1) to a total volume of 500 ml. The minimal medium provides nutrients and buffering capacity. As carbon source, one model substrate was added per batch (see Table 5 for details). Additionally, as the model substrates could be rather difficult to digest and endanger the survival of the batch, 0.5 g PHA were added. This was done directly at the beginning of the experiment and every 30 days thereafter.

Abiotic batches were incubated as controls. They contained minimal medium and the respective model substrates but no sludge. To hinder microbial growth, sodium azide (NaN_3) was added to a concentration of 0.2 % w/V.

All batches were kept at mesophilic temperature at 30° C.

Furthermore, two batches were launched using the maize-digesting-sludge to be able to assess the effect of PHA on enzyme expression. 120 ml maize sludge were mixed with 380 ml minimal medium. Instead of PHA, 0.5 g maize silage was used. The polymer-carbon source was the polymer ecoflex.

Figure 7: Experimental setup of the sludge batches with different model substrates for induction of enzyme expression.

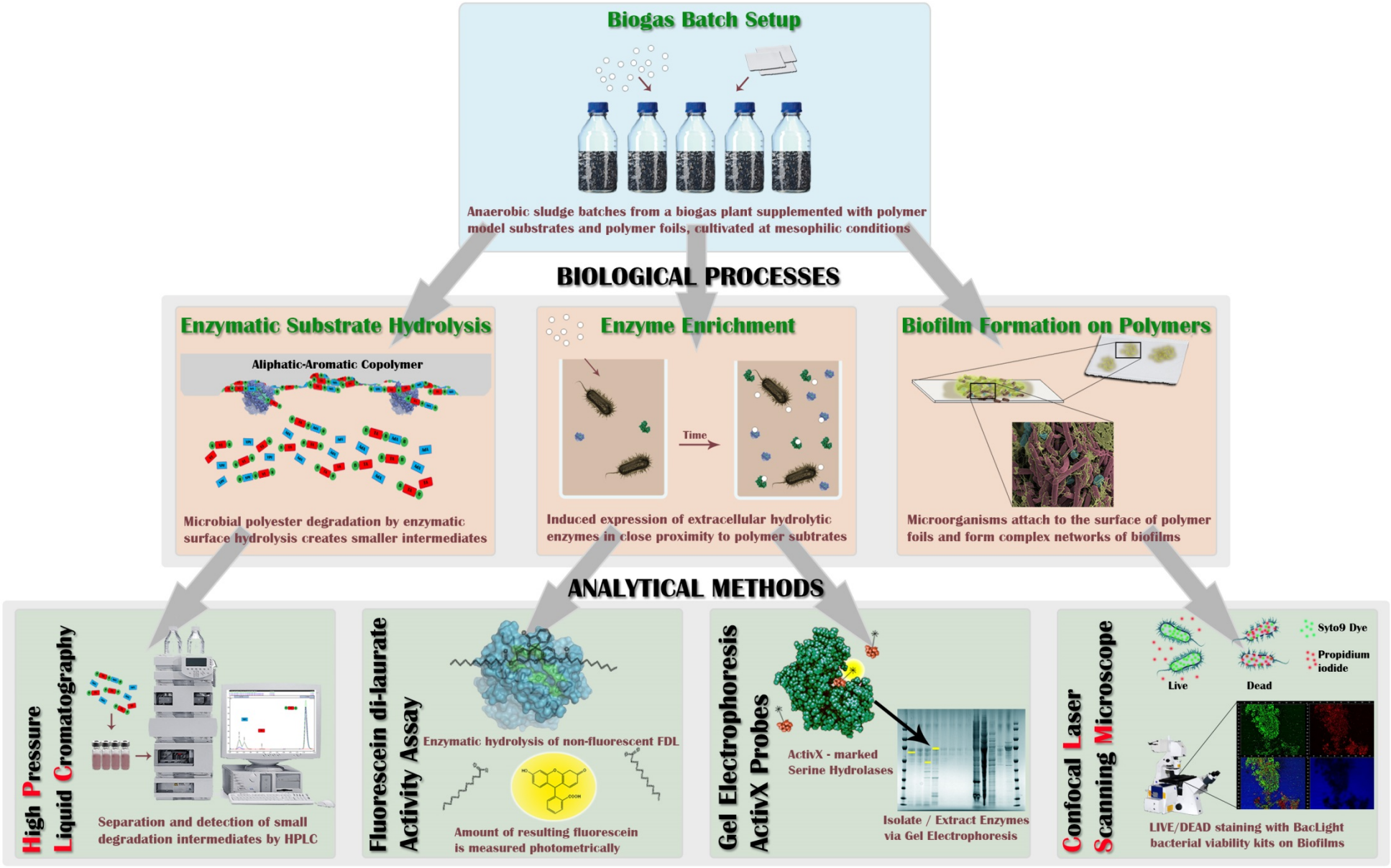


Figure 8: Overview about the setup, biological processes and detection methods involved in the anaerobic biogas sludge batch experiment. Polymer model substrates and polymer foils were used to induce expression of polymer hydrolysing enzymes. The hydrolysis products, enriched enzymes and the biofilm formation were analysed: The hydrolysis products by HPLC, the enzyme enrichment with an activity assay and PAGE, and the biofilm formation was observed with confocal microscopy.

2.5.1 Minimal medium and trace element solution for anaerobic cultivation

The minimal medium was prepared as described in ISO 14853:2005 [31]. After dissolving the compounds in ddH_2O, the medium was purged with nitrogen for 20 min per liter to create oxygen-free conditions. The O_2 indicator resazurin was added (1 mg/l), turning from blue to pink, depending on the O_2 concentration. The pH was adjusted to 7.0 with HCl (concentrated). One millilitre of the supplemental trace element solution described by Schlegel [32] was added. Both the ingredients for the minimal medium as well as for the trace element solution can be found in Table 4.

Table 4: Ingredients of the synthetic minimal medium and the trace element solution.

Minimal medium components	[g] / l	Trace element solution	[mg] / l
KH_2PO_4	0.27	$ZnCl_2$	70
$Na_2HPO_4*12H_2O$	1.12	$MnCl_2*4H_2O$	100
NH_4Cl	0.53	$CoCl_2*6H_2O$	200
$CaCl_2*2H_2O$	0.075	$NiCl_2*6H_2O$	100
$MgCl_2*6H_2O$	0.1	$CuCl_2*2H_2O$	20
$FeCl_2*4H_2O$	0.02	$NaMoO_4*2H_2O$	50
Resazurin	0.001	$Na_2SeO_3*5H_2O$	26
ddH_2O	1 L	HCl (25%ig)	1 ml
Na_2S*1H_2O	0.1	ddH_2O	1 l

2.5.2 Tested model substrates

Several model substrates were used as carbon source addition for the minimal medium. These model substrates were also used to monitor the hydrolytic activity in the sludges. Three of the substances – BTaBTaB, BTaB, and BaBTaBBa – were smaller molecules, mimicking degradation fragments of aromatic polymers. Their chemical structure and properties are shown in Figure 9. The other two tested materials were ecoflex and oligobutyrate-adipate-terephthalate (OBAT), which are aromatic-aliphatic copolyester.

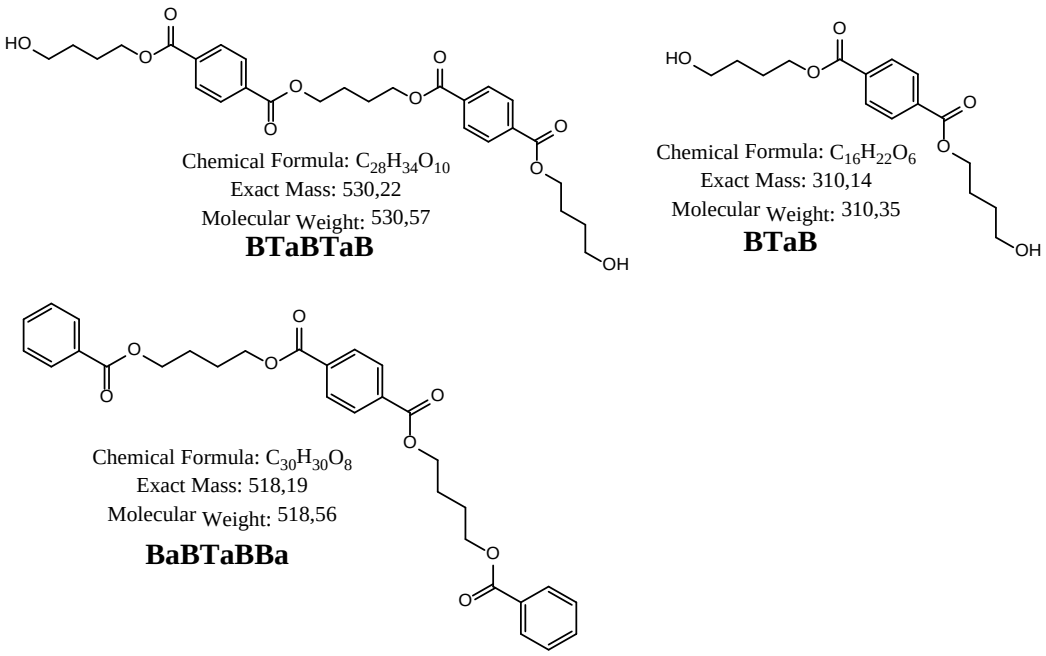


Figure 9: Three of the tested model substrates. In addition to these three, ecoflex and OBAT (the oligomeric form of ecoflex) were incubated in the anaerobic batches. B: butanediol, Ta: terephthalic acid, Ba: benzoic acid.

For each batch, only one model substrate was used. 1 g of each substance was weighed in to reach a final concentration of 0.2 % w/V, with the exception of the "Standards" batch, which contained different amounts of Ta, Ada and benzoic acid (Ba). The tested substances and their final concentrations in the batches can be found in Table 5. Abiotic batches used minimal medium without sludge.

Table 5: A list of the model substrates used in the sludge batches and abiotic batches. The maximum detectable concentration represents the highest amount of hydrolysis products that could theoretically be detected in the batch.

				Maximum detectable conc. in [mM]				
model substrate		**M_W [g/mol]**	**Conc. [mM]**	**BTaB**	**Bta**	**Ta**	**Ba**	**Ada**
Sludge batches	BTaBTaB	530.22	3.8	3.8	7.5	7.5	-	-
	BTaB	310.14	6.4	6.4	6.4	6.4	-	-
	BaBTaBBa	518.19	3.9	3.9	3.9	3.9	7.7	-
	OBAT	-	n.a.	4.5	4.5	4.5	-	5.1
	ecoflex	-	n.a.	4.5	4.5	4.5	-	5.1
	Standards	varies	varies	-	-	6.4	6.5	6.5
Abiotic batches	BTaBTaB	530.22	3.8	3.8	7.5	7.5	-	-
	BTaB	310.14	6.4	6.4	6.4	6.4	-	-
	BaBTaBBa	518.19	3.9	3.9	3.9	3.9	7.7	-
	OBAT	-	n.a.	4.5	4.5	4.5	-	5.1
	ecoflex	-	n.a.	4.5	4.5	4.5	-	5.1
	Standards	varies	varies	-	-	6.7	6.4	6.6

2.6 Development of an esterase activity assay in anaerobic sludge

The enzyme activity assay developed in this study is an adapted method of existing assays by *Green et al.* [25], ISO 14853 [31] and mainly an ACIB intern standard operating procedure (SOP) which was created in 2010 by Clemens Gamerith. Two potential substrates were used to develop an assay for enzymatic hydrolysis of esters: fluorescein diacetate (M_W = 416.38 g/mol, abbreviated FDA) and fluorescein dilaurate (M_W = 696.91 g/mol, abbreviated FDL).

A number of pre-tests were performed to find the optimal conditions and parameters. The hydrolytic activity on FDA and FDL of enzymes present in the sludge was studied in detail. The properties that were examined included substrate concentration, substrate stability, temperature of reaction and incubation, reproducibility and numerous variations of the experimental settings of the spectrophotometer (Tecan Infinite 200). The individual components of the assay were tested separately, leaving the other factors as they were in the preliminary method. During all stages of method development the samples were run in triplicates. The following aspects were investigated in detail:

- *Substrate stability*: Respectively 20 µl of 400 µM stock solutions (in acetone) of FDA and FDL were mixed with 180 µl buffer ($NaPO_4$, 0.1 M, pH 8.0) and monitored spectrophotometrically over 140 minutes to assess the spontaneous hydrolysis of fluorescein esters.
- *Optimal substrate concentration*: various FDA and FDL stock solutions of different concentrations were tested in sludge. The concentration range of the stock solutions was between 100 µM – 1 mM.
- *Addition of enzymes*: To test the effects of the complex sludge matrix as environment for released enzymes, sludge was spiked with defined amounts of Cutinase 1 of *Humicola insolens* (HiC, received from Novozymes). At first, HiC's hydrolytic activity on FDA and FDL was tested in buffer (sodium phosphate, 0.1 M, pH 8.0) and then compared to its activity when added to sludge. The background sludge activity was subtracted.
- *Sample preparation*: The effects of centrifugation, vortexing, pre-heating the samples to reaction temperature and different dilutions were investigated closely. Furthermore, the settings for the spectrophotometer such as shaking, duration, number of reads and method of measuring were examined.

Esterase activity is given as units of enzyme activity per milliliter (U / ml), with the enzyme unit being defined as the conversion of 1 µmol substrate per minute.

2.7 Final enzyme activity assay

The final version of the newly developed activity assay was applied as a tool to measure the change in hydrolytic activity in all sludge batches over time.

At first, the sludge samples were diluted 1:5 with buffer (sodium phosphate, 0.1 M, pH 8.0) and centrifuged (Eppendorf MiniSpin, 12000 g, 10 min) to remove solid particles and decrease turbidity. 180 µl of the supernatant were pre-heated to 37°C in a thermomixer (Eppendorf). After five minutes, 20 µl of a fluorescein dilaurate stock solution (200 µM in acetone, M_W = 696.91 g / mol) were added to start the reaction. The change in fluorescence was measured on a spectrophotometer over 90 minutes. The protocol can be found in the appendix (App.Table 4). Blanks were prepared with FDL in buffer.

2.7.1 Preparation of fluorescein standards

Fluorescein sodium salt (M = 376.27 g / mol) was dissolved in ddH_2O. The following dilutions were prepared: 25 µM, 20 µM, 15 µM, 10 µM, 5 µM and a blank without fluorescein. The standards were added to the sludge samples instead of the FDL stock solution. Unlike FDL, the fluorescein standard is rather instable and should be prepared freshly. With the standards, the activity can be quantified. When calculating the hydrolytic activity it is important to take into account that for every fluorescein produced, two esters need to be hydrolyzed.

2.8 High performance liquid chromatography analysis

In the HPLC analysis soluble degradation products can be measured and quantified. In our experimental setup the measurable hydrolysis products of ecoflex are BTaB, BTa, Ta and Ada.

Prior to HPLC analysis, the batch samples were centrifuged (Eppendorf MiniSpin, 12000 g, 10 min) and a Carrez clarification [33] was performed. The Carrez clarification is widely used in analytics and was used to eliminate turbidity by precipitation of proteins and fatty acids. First, 200 µl of each sludge were diluted with 780 µl of ddH_2O. The precipitation was carried out with two solutions: *C1* (1.06 g $K_4[Fe(CN)_6]$* 3 H_2O in 10 ml ddH_2O) and *C2* (2.88 g $ZnSO_4$ * 7 H_2O in 10 ml ddH_2O). 10 µl of C1 were added to the diluted samples and mixed thoroughly by vortexing. After one minute of incubation 10 µl of C2 were added and again vortexed. Following 20 minutes of incubation, the samples were centrifuged (14000 g, 0°C, 30 min, Biofuge Primo, Heraeus). 400 µl of the supernatant were transferred respectively to a fresh Eppendorf reaction tube, mixed with 400 µl of ice cooled methanol and acidified by adding 2 µl of concentrated formic acid. After an incubation of one hour at 4°C the samples were centrifuged again (14000 g, 0°C, 15 min) and 600 µl of the supernatants were transferred to HPLC vials to be analysed by means of HPLC.

To quantify the hydrolysis products BTaB, Ba, Ada and Ta, standards were prepared. Their concentration range can be found in Table 6. Standards were dissolved in potassium phosphate buffer (0.1 M, pH 7.0) and a Carrez clarification was performed as described above.

Table 6: Concentrations of the standards prepared for HPLC analysis.

Standard	Concentration range
Ta	0.0125 – 0.4 mM
BTaB	0.025 - 1 mM
Ba	0.125 - 5 mM
Ada	0.1 - 20 mM

2.9 Extraction of Ta

The solubility of Ta in an aqueous surrounding is very low, which could result in undetectable precipitates of Ta in the sludge batches. Additionally, Ta could adhere to cells and avoid detection. To be able to safely quantify all Ta, an extraction with dimethyl sulfoxide (DMSO) was performed.

40 ml of each biogas batch were centrifuged (20 min, 4348 g, room temperature) and the supernatant was saved. Pellets were then resuspended in 10 ml DMSO by carefully mixing and rotating for 15 min at 30 rpm. Then the samples were centrifuged using the same settings, and the supernatant was again kept for analysis. Eventually, an ultrasonic lysis was performed to get access to Ta that might have already been taken up by the cells. To do so, 20 ml of each batch were centrifuged (20 min, 7000 g) to get hold of the supernatant. The resulting pellets were then resuspended in 45 ml buffer (potassium phosphate, 0.1 M, pH 7.0) and a lysate was created with the use of ultrasound: 6 minute runtime, duty cycle 80%, and output 7-8. In a second centrifugation step (30 min, 20000 rpm) the remaining cell debris was removed. The resulting pellet was mixed with 1 ml potassium phosphate buffer.

As control, batches were spiked with 5 mM Ta and treated like the other batches.

The samples were analysed by HPLC as described above, with new standards to match the added DMSO.

2.10 Separating extracellular proteins by PAGE and detection of hydrolases with FDL

The proteins in sludge were separated for identification using PAGE. Native PAGE was done using self-poured gels; an example for their composition is given in Table 7.

Table 7: Concentrations of the components for two 0.75 mm strong gels with 8 % acrylamide. For acrylamid (AA) gels of different concentrations, the AA amount was changed accordingly.

	Separating gel 8 % [ml]	Stacking gel 4 % [ml]
40 % acrylamide, bis	2	1.0
Tris HCl; 1.5 M; pH 8.8	2.5	2.5
ddH_2O	5.4	6.5
10 % Ammonium persulfate	0.05	0.05
Tetramethylethylendiamin	0.012	0.012

The experimental conditions were widely varied to increase the sharpness of the protein bands. The main parameters that were investigated were the following:

- AA concentration in the gel: ranging from 5 % to 8 % AA
- Use of commercially available gels instead of self-made ones: Novex® NuPAGE 4-12 % Bis-Tris Gel
- Removing interfering material using Vivaspin columns (*Sartorius stedin biotech*, 30000 MW)
- Varying electrophoresis settings: 60 – 100 volt, run time 1.5 – 4 hours
- Spiking sludge with enzymes (HiC)
- Denaturating PAGE instead of native PAGE

For the detection of active enzymes with hydrolytic activity, fluorescein derivatives were used. 2 mg FDA and 3.35 mg FDL were solved in 100 µl acetone. 50 µl were then mixed with 50 ml buffer (sodium phosphate, pH 8.0, 0.1 M). The gels were put in the solutions and images of the gels were taken with the Universal Hood III (Bio-Rad). The emission filter used for the fluorescence was the 605DF50 at Cy3 settings for excitation / emission.

Additionally, all proteins were stained using a KANG solution [34]. After washing the gels in ddH_2O they were stained for 12 hours in the KANG solution. This was followed by another washing step with ddH_2O to remove the excess staining fluid. The ingredients for the KANG solution as well as for the loading buffers and running buffers can be found in Table 8 .

Table 8: Composition of the loading buffers, the KANG solution and the running buffer used for PAGE.

KANG solution	
Coomassie blue	0.2 g
aluminium sulfate 18 hydrate	50 g
ethanol (96%)	100 ml
ortho-phosphoric acid (100 %)	20 g
ddH_2O	900 ml
Loading buffer native 4x	
Glycerole	4 ml
0.25 M Tris HCl buffer pH 6.8	5 ml
Bromophenol blue	0.015 %
ddH_2O	1 ml
Loading buffer denaturating (Laemmli [35]**) 4x**	
0.25 M Tris HCl buffer pH 6.8	5 ml
Glycerole	4 ml
Bromophenol blue	0.004 %
ddH_2O	1 ml
Sodium dodecyl sulfate (SDS)	8 %
Running buffer 5x	
Glycine	72 g
Tris	15 g
ddH_2O	To 1000 ml

For the denaturating PAGE with the Laemmli buffer, the samples were cooked prior to analysis at 95° C for 7 minutes (Thermomixer comfort, *Eppendorf*). When working with denaturated samples, no ActivX probes were used and the samples were KANG stained.

2.11 Visualisation of biofilms on polymer foils

Crystal violet staining

Crystal violet is widely used for staining, especially in histology and the Gram's Method [36] of classifying bacteria. The experiment consisted of two parts:

In the first step, polymer foils were incubated with bacteria that are able and likely to produce biofilms to show the possibility of biofilm formation on the polymers listed in Table 9.

For the second part, pieces of the polymer foils were incubated in biogas sludge and then stained with crystal violet, to check for biofilm formation on the samples.

2.11.1 Polymer foils

Table 9 shows a list of all available polymer films for testing, produced by BASF. All of them were used in the biogas sludge part of the experiment, whereas for the biofilm formation assay (described in 2.11.2) only the 400 µm ecovio and the 50 µm ecoflex polymers were used.

Table 9: List of the available polymer foils, their thickness in µm and their composition. PLA: poly(lactic acid), PBS: poly (butylene succinate)

Thickness [µm]	Material
520	ecovio: 80 % PLA, 20 % ecoflex C2308
400	ecovio: 80 %PLA, 20 % ecoflex C2308
50	ecoflex F Blend A1200
30	ecoflex F Blend A1200
25	Bionolle: 70 % PBS / 30 % PHBH
15	ecoflex F Blend A1200

All polymer foils were cut into 1x1 cm pieces and washed with Triton X (5 g/l), Na_2CO_3 (2 g/l) and ddH_2O for 30 min respectively before being incubated in the biogas sludge or used in the biofilm formation assay.

2.11.2 Biofilm formation with *E.coli* XL1 and *S. aureus* "Praktikum"

Cultures of biofilm forming bacteria (*Escherichia coli* XL1 and *Staphylococcus aureus* "Praktikum") were grown in tryptic soy broth (TSB, 30 g / L), which was supplemented with 0.75 % glucose to a final concentration of 1 % (in the following named TSB+). The cultures were incubated for 8 hours at 37°C and 130 rpm in 50 ml flasks. The 1x1 cm polymer foil pieces were attached to the bottom of sterile 12 well plates using silicon glue. The silicon glue did not show any reaction with the crystal violet. Into each well, 1.8 ml fresh TSB+ medium was put, together with 200 µl of bacterial solution (1:10 dilution). After 16 hours incubation at 37°C without shaking, the plates were washed three

times with ddH_2O by dunking them carefully into water. The biofilm formation assay was performed as described in 2.11.4

2.11.3 Biofilm formation during incubation of polymer foils in sludge

The biogas sludge used for incubating the polymer film pieces was the same that was used in the previous experiment (see 2.5 for details).

All of the available variants of polymer foil pieces were incubated in the sludge for 38 days before they were removed and washed three times with ddH_2O by dunking them carefully into water with tweezers. The biofilm formation assay was performed as described in 2.11.2.

LIVE/DEAD Bac*Light *Bacterial Viability Kit for CLSM analysis

The LIVE/DEAD BacLight Bacterial Viability Kit [LifeTechnologies] allows the distinction between alive and dead bacteria. It is based on a two-dye system: The first dye (SYTO9) is a green fluorescent nucleic acid stain that is able to dye all bacteria, whether their membrane is intact or not. The second dye is propidium iodide, which is a red fluorescent nucleic acid stain that can only penetrate damaged membranes and acts as a competitor for SYTO9 in dead cells. Due to this competition and with a surplus of propidium iodide, live cells are green- and dead cells are red-fluorescent.

For this experiment the same sludge as described in 2.11.3 was used. Polymer foil pieces were obtained as described in 2.11.1.

2.11.4 Biofilm formation assay and crystal violet staining

Special thanks to *Barbara Thallinger*, who adapted this assay based on *Kwasny* and *Opperman* [1].

The biofilms were fixed on the plastic foils for 60 minutes at 60°C. 500 µl of a 0.06 % crystal violet solution were added to each well to stain the biofilms. After 5 – 10 minutes they were again washed by dunking them into ddH_2O, until the water remained clear. The foils were each put into a 2 ml Eppendorf tube. 1 ml of 30 % acetic acid was added to dissolve the crystal violet under strong vortexing. The absorbance at 595 nm of the solutions was then measured on a plate reader.

2.11.5 Staining of cells in the sludge

The LIVE/DEAD Kit was first used to check the amount of living cells in the biogas sludge that was used to incubate the plastic foil pieces. To reduce the mobility of the bacteria, microscope slides were covered with a thin layer of gelatin. To achieve that 0.36 g gelatin were dissolved in 10 ml H_2O with the help of a microwave oven and then put on the slides with a toothpick. The staining itself was then done according to the manual [37], page 3: “Staining bacteria in suspension”.

2.11.6 Staining of biofilms on polymer film pieces after incubation in sludge

After 21 days of incubation, the plastic foil pieces were removed from the sludge and put into 2 ml Eppendorf tubes. Foils with strengths of 25 µm (Bionolle) and 50 µm (ecoflex) were used, and a 50 µm (ecoflex) negative control. For the staining, 2.25 µl of component A were mixed with 2.25 µl of component B (see [37]) in 1.5 ml ddH_2O and filled in the Eppendorf tubes. The plastic film pieces were afterwards put on a microscope slide (without gelatin) and the glass lid sealed with nail polish.

2.11.7 Confocal laser scanning microscopy

The marked microorganisms were observed with a Leica TCS SPE confocal microscope (Leica Microsystems, Germany). Three confocal light channels were observed simultaneously and the emitted light was detected at following ranges: Red (600 nm – 654 nm); green (505 nm – 552 nm) and blue (420 nm – 474 nm). The objectives ACS APO 63.0 x 1.30 oil and ACS APO 40.0 x 1.15 oil were used to get the confocal stacks. Up to 20 stacks were made per sample with a step size of 0.55 µm.

Part III: Enzyme isolation from *Clostridium hathewayi*

2.12 Culture and growth media

Four cultures of *C. hathewayi sp*. (DSM 13479) were provided by BASF. In the following, they are referred to as Cultures A, B, C and D. The *C. hathewayi* cultures were grown under anaerobic conditions in a PYX – Medium. The PYX – Medium consists of 5 g trypticase peptone, 5 g peptone from meat, 10 g yeast extract, 5 g glucose, 1 mg resazurin, 40 ml salt solution, 0.5 g cysteine-HCl x H_2O and 1000 ml H_2O. For the cultures C and D, no glucose was used and the amount of yeast extract and peptone in the PYX Medium was reduced and substituted with PBAT. PBAT was added in an attempt to induce the expression of hydrolytic enzymes.

2.13 Preparation of supernatant, resuspended pellets and lysates

20 ml of each culture were centrifuged (20 min, 7000 g) to get hold of the supernatant. The resulting pellets were then resuspended in 45 ml buffer (potassium phosphate, 0.1 M, pH 7.0). 15 ml resuspended whole cells were kept for further analysis and with the remaining cells a lysate was created with the use of ultrasound [Vibra Cell, Sonics & Materials]: 6 minute runtime, duty cycle 80 %, output 7-8. In a second centrifugation step (30 min, 20000 rpm) the remaining cell debris was removed. The resulting pellet was mixed with 1 ml potassium phosphate buffer.

2.14 PAGE and ActivX TAMRA-FP Serine hydrolase probe labelling

The culture's supernatants, their lysates (after ultrasonification, see 2.9) and the resuspended pellets after the ultrasonification were analyzed. All samples were first labeled with the ActivX TAMRA-FP Serine hydrolase probes (*Thermo Scientific*) as described in the manufacturer's manual [38] These probes consist of a tag linked to a fluorophosphanate group that can covalently label serines in enzymatically active serine hydrolases [39].

The PAGE was done with the commercially available Novex® NuPAGE 4-12 % Bis-Tris Gel. Sample preparation and electrophoresis were performed according to the manual [40].

The ActivX probes bind only to active serine hydrolases and can be detected on the gel as the probes are fluorescent. The gels were first analyzed under fluorescent light and later KANG–stained to mark all protein bands. The bands that were marked by the ActivX probes were cut out and sent to an external lab. There they were in-gel digested with trypsin and their sequence was analyzed. Peptide sequences were then run against the full NCBI database.

The concentration of proteins in the first PAGE gel was very low; therefore a methanol-chloroform precipitation method was used to increase the protein concentration.

To 150 µl sample, 600 µl of methanol and 150 µl chloroform were added. After vortexing and mixing with 450 µl ddH_2O the samples were vortexed again and centrifuged (1 min, 12000 g). The upper organic phase was removed without disturbing the interphase which contains the proteins and collected for disposal. Another 450 µl methanol were added; the samples were vortexed and again centrifuged (1 min, 12000 g). The supernatant was completely removed; the remaining pellets were air dried. The samples were then mixed with 10 µl buffer and 0.2 µl ActivX probe solution and analysed via PAGE as described before.

2.15 Enzymatic hydrolysis of ecoflex and BTaB

To test the culture's ability to hydrolyze polyesters, two substrates for the enzymatic digestion were selected: ecoflex and BTaB. 10 mg of milled ecoflex or 4.25 mg BTaB were incubated at 37° C and 100 rpm in a volume of 2 ml for 7 days. Again the supernatants, lysates and resuspended whole cells of all four cultures were analyzed. As blank reactions, samples were incubated without addition of substrates; buffer and medium were incubated with and without substrates. Blank reactions were performed simultaneously.

Details for the sample preparation prior to the HPLC analysis can be found in 2.3. The HPLC analysis itself was carried out as described in 2.4.

2.16 Sequencing of *C.hathewayi* cultures

A sequencing of each *C.hathewayi* culture was performed to confirm the results of the protein analysis.

Clostridia tend to sporulation, so the DNA needed to be extracted with the Bacterial/Fungal DNA Kit (Precellys) which utilizes glass beads for cell lysis. Lysis and DNA extraction were performed according to the manual [41]. The purity of the isolated DNA was checked photometrically (NanoDrop).

The primers used for the PCR can be seen in Table 10. They amplify the DNA region coding for 16S rRNA, a universally conserved region in bacteria [42].

Table 10: Primers used in the PCR amplification. The forward primer f27 and the reverse primer r1492 target a highly conserved region coding for 16S rRNA.

Primer	Sequence 5' – 3'
f27	AGAGTTTGATC(A/C)TGGCTCAG
r1492	TACGG(C/T)TACCTTGTTACGACTT

The PCR protocol for DNA amplification is shown in Table 11, together with the amounts of DNA and primers used.

Table 11: Amount of DNA and primers used and the PCR protocol for amplification.

DNA	H_2O	10 µM 27f	10 µM 1492r	Taq&Go	
1 µl [for 100 ng / µL]	22.4	0.3	0.3	6	µl

Temperature	Time	Cycles
95	5 min	
95	30 sec	
57	30 sec	n = 30
72	90 sec	
72	5 min	
15	D	

A control gel was performed. PCR products were purified using the *Wizard Plus SV Minipreps DNA – Purification System* from *Promega.* The purified PCR products were mixed with primers and sent to LGC Genomics for sequencing.

3 Results and Discussion

Part I: Enzymatic hydrolysis of aliphatic aromatic copolyester

3.1 Enzyme Characterization

The protein concentrations of both enzymes, ThC_Cut1 and CB_EstA, were determined using the BCA protein assay kit. Additionally, the enzyme activity on pNPB was measured. The results of the BCA protein assay and the pNPB activity assay are listed in Table 12. The activity was calculated according to the following formula:

$$\frac{U}{ml} = \frac{\Delta E405\ [abs\ min^{-1}]}{11.920\ [abs\ mM^{-1}cm^{-1}] * 0.6509\ [cm]} * \frac{0.22\ [ml]}{0.02\ [ml]} * dilution\ factor$$

$\frac{U}{ml}$ … volumetric enzyme activity [µmol min^{-1} mL^{-1}]

11.920 … molar extinction coefficient of pNPB at pH 7.0 in potassium phosphate buffer [abs mM^{-1} cm^{-1}]

$\frac{0.22\ ml}{0.02\ ml}$ … total reaction volume per volume of enzyme solution

ΔE405 … change of absorbance per minute [abs min^{-1}]

0.6509 … path length of the light beam [cm]

Both enzymes showed activity on the soluble substrate pNPB. However, the specific activity of ThC_Cut1 was 450 U/mg and thus 7.5 times higher than the CB_EstA's specific activity, which was 60 U/mg. The results are summarized in Table 12. Taking into account the larger molecular weight of the CB_EstA (M_W = 51700 g/mol) compared to the ThC_Cut1 (M_W = 29400 g/mol), the activity of ThC_Cut1 on an equimolar basis is still 4 times higher.

Table 12: Summary of the enzymatic characterization of CB_EstA and Thc_Cut1.

Enzyme	Concentration	Units/ml	Units/mg	kcat [s^{-1}]
CB_EstA	10.5 mg/ml	630	60	52
ThC_Cut1	3.1 mg/ml	1400	450	221

While there are no reference values available for CB_EstA, ThC_Cut1's performance is similar to results described in literature. Acero *et al.* [21] reported a turnover number of kcat = 195 s^{-1} and Ribitsch *et al* [29] measured kcat = 325 s^{-1}. By fusing a binding module to the cutinase, this number could even be increased to kcat = 342 s^{-1}.

3.2 Hydrolysis of ecoflex and model substrates by ThC_Cut1 and CB_EstA

The aliphatic-aromatic copolyester ecoflex and several polymeric ecoflex model substrates with variations in Ta : Ada ratios were enzymatically hydrolysed. The esterase A from the anaerobic strain *C. botulinum* and the cutinase 1 from *Thermobifida cellulosilytica* were tested for their ability to hydrolyse the ester bonds in the polymeric substrates and the hydrolysis products were measured by HPLC. Both enzymes were incubated with the polyesters in the same concentration (0.6 µM) and at the same temperature (50° C). The degree of degradation was assessed by analysis of the intermediates formed in the degradation process. The hydrolysis products Ada, Ta, BTa and BTaB were quantified by means of HPLC analysis. Figure 10 shows a schematic example of the hydrolysis

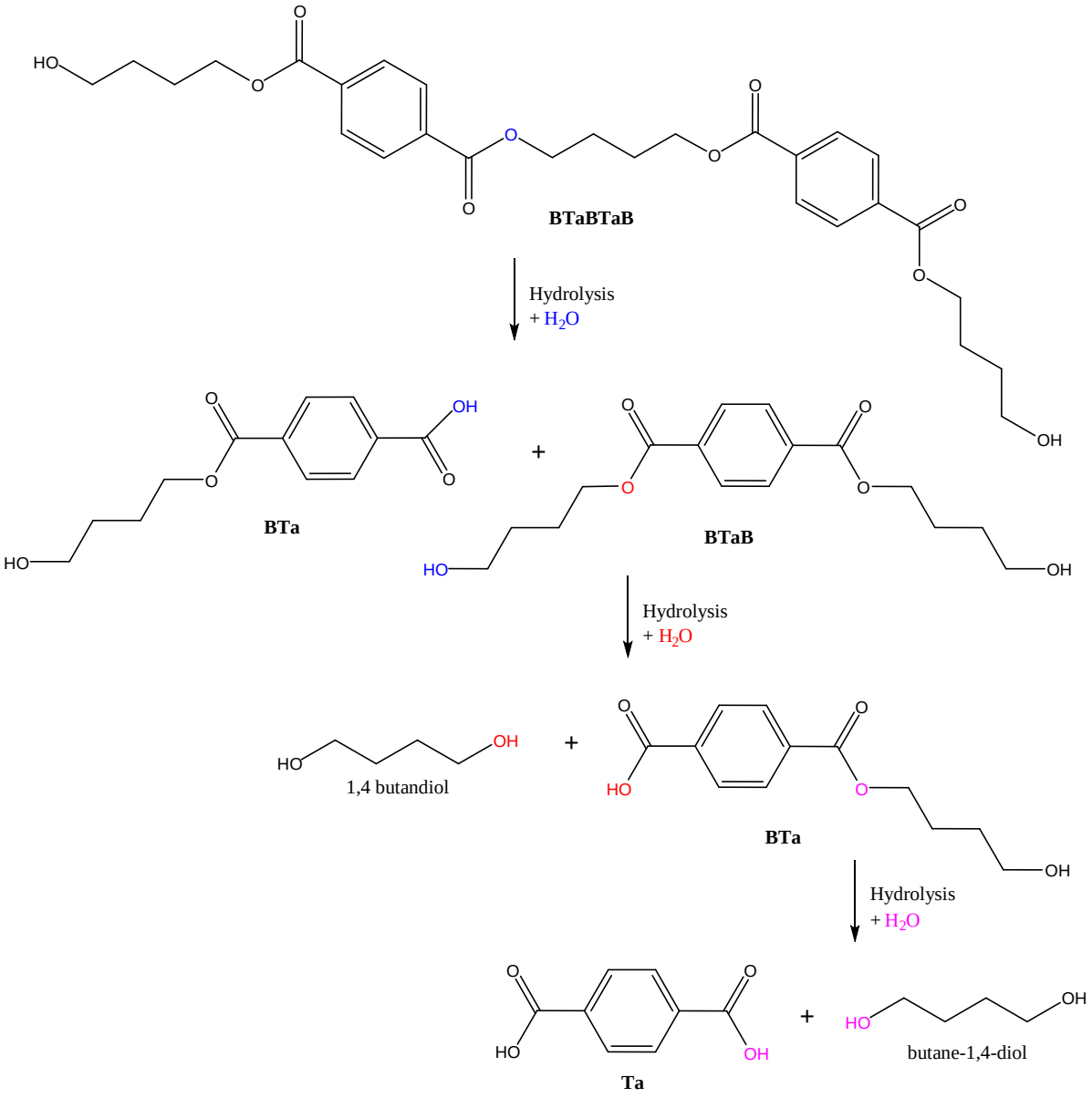


Figure 10: Schematic representation of the hydrolysis of a possible ecoflex degradation intermediate. The ester bonds are enzymatically hydrolysed and smaller products emerge which can be quantified by HPLC.

process, in which a larger degradation intermediate is enzymatically cleaved into the monomeric units.

3.2.1 Degradation with ThC_Cut1

ThC_Cut1 showed remarkable hydrolytic activity on all substrates. An overview of the formed degradation products is given in Figure 11.

As a main trend an increase in the hydrolysis rate was observed with Ada : Ta ratios towards more Ada, the only exception being Ada80_Ta20. The analysis also shows that ThC_Cut1 seems to be cutting the polymers exo-wise: BTaB and presumably also larger degradation intermediates were not identified. This leads to the conclusion that ThC_Cut1 cleaves the ester bonds from the outside of the polymer chains rather than from within, since with an endo-wise hydrolysis pattern bigger intermediates should be detectable.

BTa is present in all samples in a decreasing concentration over time. After 3 days of degradation, mainly the monomers of the copolyester were detected. The percentage of Ta set free of the total available Ta in the model substrates ranged from 21.5 % (Ada80_Ta20) up to 89.0 % (Ada_90_Ta10). Even ecoflex and the copolyester with a high Ta content showed a high degree of degradation. On ecoflex, 32.7 % of total Ta was released; this is equal to 6577 mol Ta per mol enzyme.

The percentages of Ada that were set free were comparably lower in all polymers than the amount of Ta that was released. Up to 52 % of the total amount were measured in the copolymers, the highest being Ada90_Ta10, which translates to 18600 mole Ada per mole enzyme. In the polymer that consisted of only the aliphatic compound, PBAda, 60.8% of Ada was detected as hydrolysis product, which is equivalently 24150 mole Ada per mole enzyme.

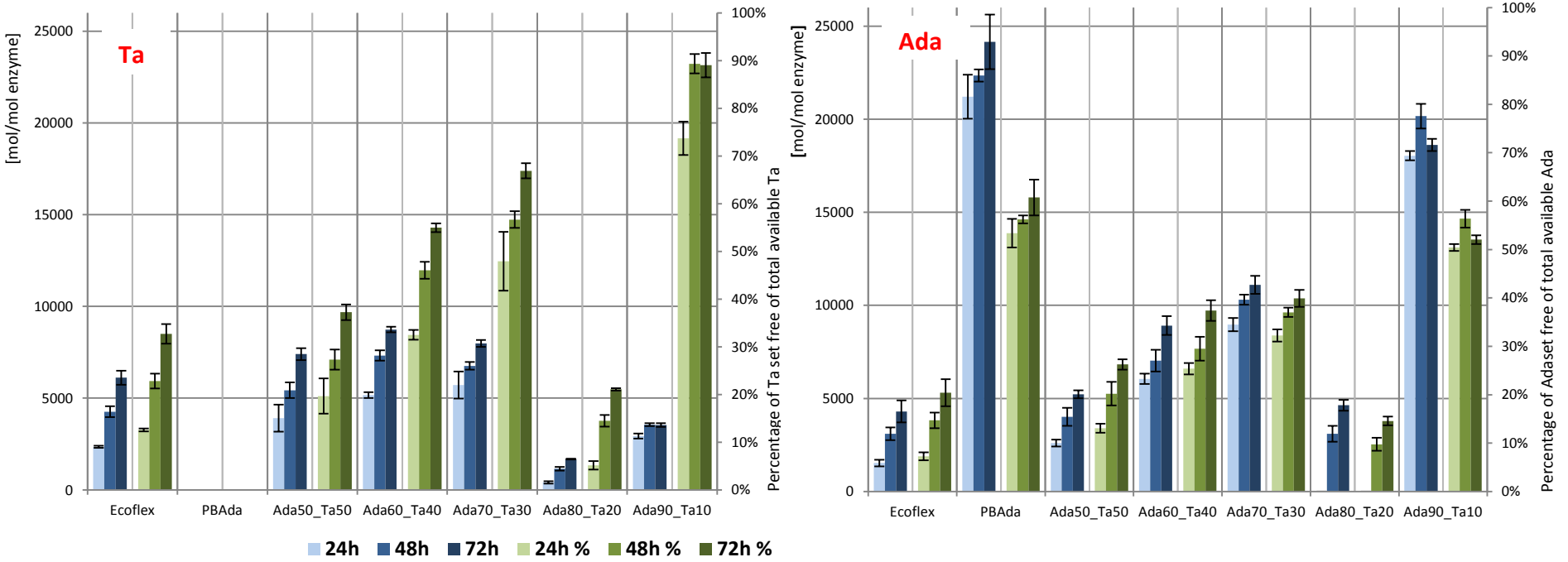
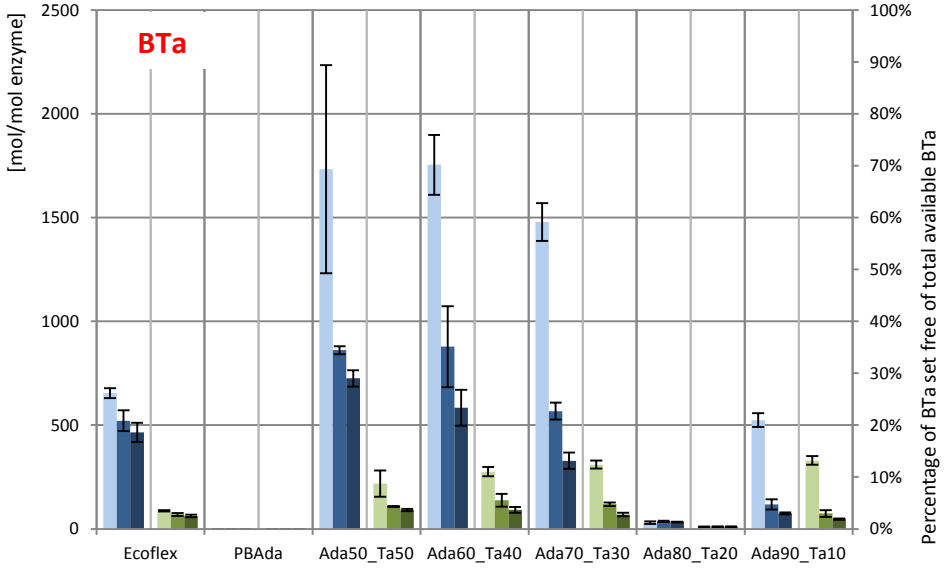


Figure 11: Hydrolysis products of different model substrates released by ThC_Cut1. On the primary vertical axis, the mole products that are released per mole enzyme are displayed. On the secondary vertical axis, the percentage that is enzymatically set free of the total available amount is depicted. The degradation was carried out with an enzyme concentration of 0.6 µM at 50° C. The reaction was stopped after 24h, 48h and 72h to show the progression of the degradation.



3.2.2 Degradation with CB_EstA

CB_EstA displayed the ability to cleave ester bonds on all tested substrates, including ecoflex. The results of the degradation for all polymers are shown in Figure 12. Overall, the more the compositions of the copolyesters shift towards Ada, the higher the hydrolytic activity. Larger degradation intermediates (BTa and BTaB) are predominant throughout all samples.

The monomer Ta is released in low quantities, ranging from 0.02 % (ecoflex) to 0.3 % (Ada90_Ta10). In mole Ta released per mole enzyme, the amounts ranged from 4.33 (ecoflex) to 17.81 (Ada80_Ta20). The quantities for Ada were higher, albeit for ecoflex, Ada50_Ta50 and Ada60_Ta40 no Ada could be detected. PBAda underwent a total degradation of 15 %, which equals 5900 mol/mol enzyme released.

More interesting are the larger intermediates BTaB and BTa. Both were measured in much higher concentrations than the monomeric components. Again ecoflex shows the smallest amount of intermediates with 0.2 % BTaB and 0.4 % BTa. The numbers increase with rising Ada amounts and reach their maximum at Ada90_Ta10 with 17.4 % BTaB and 22.0 % BTa. While the quantities of BTa steadily continued to rise over the whole 72 h, the concentrations for BTaB were declining on most substrates after 24 h. Only for Ada80_Ta20 and Ada90_Ta10 the BTaB pinnacle is not reached until 48 h.

3.3 Discussion: comparison of both enzymes and their hydrolysis specificity

The comparison of the hydrolysis behaviour of the tested enzymes is very interesting, as they originate from quite different environments: *Thermobifida cellulosilytica* is an aerobic organism, while *Clostridium botulinum* has an obligate anaerobic metabolism and seems to be a promising candidate for the search for polyesterases from anaerobic sources. In this degradation experiment, the ThC_Cut1 seems to outperform the CB_EstA on all tested substrates; however, the hydrolysis patterns are quite diverse and can give insight into the underlying mechanism and the parameters controlling polyester degradation. The quantity and type of degradation products that are created by enzymatic hydrolysis are depending on many factors – mainly the type of enzyme, their substrate specificity and the composition of the substrate.

3.3.1 Size and amount of hydrolysis intermediates

The majority of products formed by hydrolysis with ThC_Cut1 are the monomers Ta and Ada, while only low concentrations of BTa were released. In contrast to that, enzymatic cleavage by CB_EstA yielded primarily in BTaB and BTa and only small fractions of Ta.

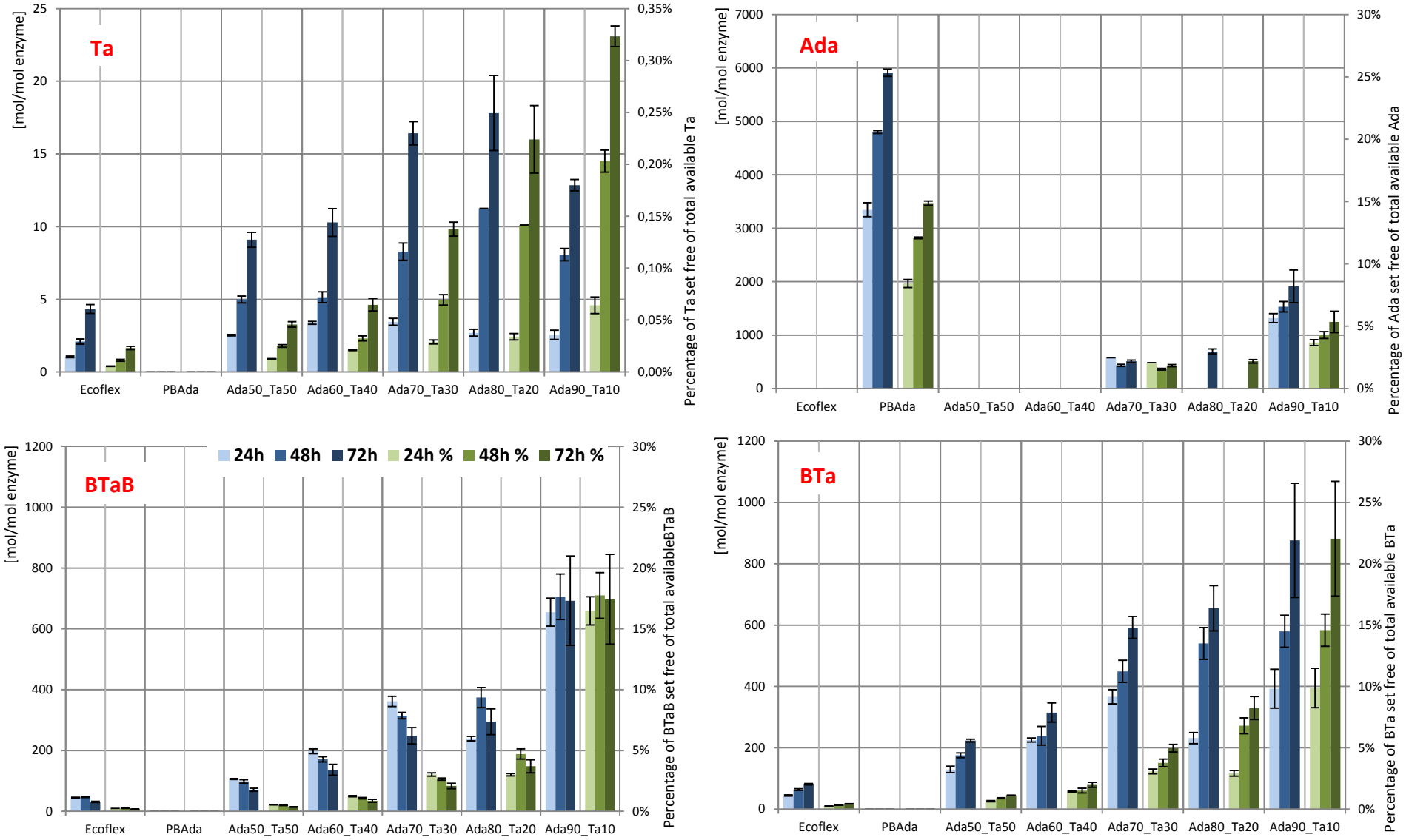
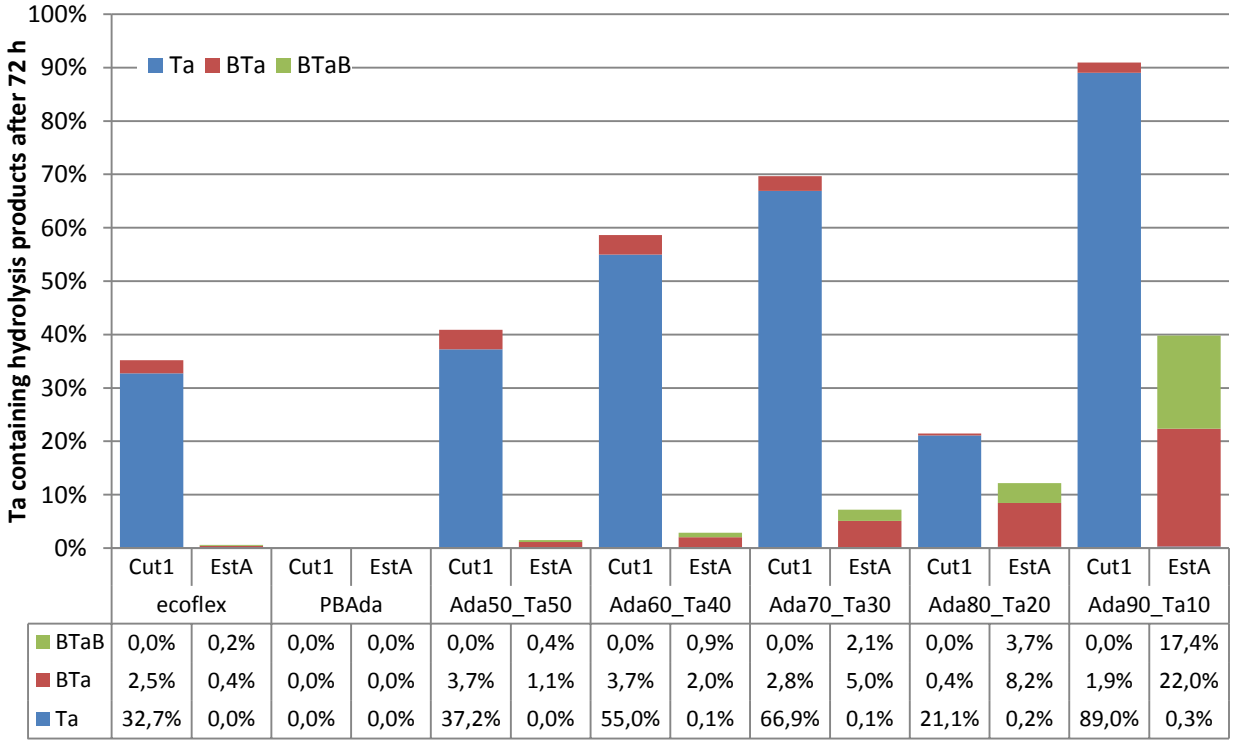


Figure 12: Hydrolysis products of different model substrates released by CB_EstA. On the primary vertical axis, the mol product that are released per mol enzyme are displayed. On the secondary vertical axis, the percentage that is enzymatically set free of the total available amount is shown. The degradation was carried out with an enzyme concentration of 0.6 µM at 50° C. The reaction was stopped after 24h, 48h and 72h to show the progression of the degradation.

This difference is visualised in Figure 13, where it is also clearly evident that the percentage of released products is by far greater for ThC_Cut1 for all tested copolyester. It has to be mentioned that the main focus of the analysis was on components containing Ta, since for Ada only the monomeric form could be quantified – no standards for larger intermediates like BAda, BAdaB are currently available.



	Cut1	EstA	Cut1	EstA	Cut1	EstA	Cut1	EstA	Cut1	EstA	Cut1	EstA	Cut1	EstA
	ecoflex		PBAda		Ada50_Ta50		Ada60_Ta40		Ada70_Ta30		Ada80_Ta20		Ada90_Ta10	
BTaB	0,0%	0,2%	0,0%	0,0%	0,0%	0,4%	0,0%	0,9%	0,0%	2,1%	0,0%	3,7%	0,0%	17,4%
BTa	2,5%	0,4%	0,0%	0,0%	3,7%	1,1%	3,7%	2,0%	2,8%	5,0%	0,4%	8,2%	1,9%	22,0%
Ta	32,7%	0,0%	0,0%	0,0%	37,2%	0,0%	55,0%	0,1%	66,9%	0,1%	21,1%	0,2%	89,0%	0,3%

Figure 13: Concentration of detected Ta containing hydrolysis products released by ThC_Cut1 and CB_EstA after 72 h of incubation.

To be able to release the monomeric elements of the copolyester, the enzymes need to hydrolyse the ester bonds between B and Ta as well as the ones between B and Ada. This is only possible if the active site allows small ligands to bind, which can be hard to achieve if the active site lies in a deep cavity inside the enzyme.

Cutinases in general are in a unique position, they serve as a connecting link between lipases and esterases [22]. While lipases require interfacial activation on hydrophobic surfaces to open a lid over the active site and form the oxyanion hole, the catalytic serine of cutinases is not buried under these surface loops. Additionally, the oxyanion hole is already preformed [23]. This leads to an easier accessible active site for solvent and substrates. Dissolved esters and oligomers formed during random cleavage of polymer chains might not be accessible for lipases, which leaves larger degradation intermediates, for example soluble oligomers, with intact ester bonds. This results in plateaus of less than 100 % cleavage because of the solubility of the smaller ligands [43]. Esterases and cutinases on the other hand have the ability to hydrolyse dissolved esters of these water soluble

intermediates. A hydrolase from *Thermobifida fusca*, which could be labelled a cutinase, has shown total hydrolysis of aliphatic-aromatic copolyester [3]. That is only possible if the active site remains accessible without the presence of a hydrophobic polymer.

In the present study, CB_EstA is displaying more of a lipase-like behaviour, as hardly any intermediates are totally hydrolysed to Ta. In contrast to this assumption, CB_EstA showed activity on the soluble substrate pNPB in the activity assay. Taking this into consideration, it is possible that the lack of total hydrolysis is due to the overall lower enzymatic activity.

3.3.2 The influence of melting points and chain mobility on biodegradability

Beside the varying hydrolysis intermediates, the other result that catches the eye is the increase in degradation with rising Ada content in the copolyesters. Pure aliphatic polyesters are easily biodegradable, while aromatic polyesters like PET are known for their formidable material characteristics [9]. The combination of both brings forth copolyesters that can be biodegraded and are yet also great to process and durable. The ratio of aliphatic to aromatic components is the factor that has the highest influence on the characteristics of the polymers.

Concerning copolyesters composed of Ta and Ada linked by 1,4 butanediol, studies suggest the optimal content of Ta being between 35 % - 55 % in the di-acid component [12]. Below 35 % the material properties decline rapidly, and above 55 % the biodegradability plummets. It was demonstrated that structural differences of the polymer chain play only a secondary role: A steric hindrance due to ester bonds in proximity to bulky aromatic groups was ruled out to be the predominant controlling factor in polyester hydrolysis [9]. The primary influence on biodegradation is the mobility of the polymer chain and therefore the ability of parts of the chain to temporarily escape the embedding crystal for a certain distance to form a kind of loop and penetrate into the active site of hydrolytic enzymes [43]. This mobility of the polyester chains is directly linked to the temperature difference between the melting point of the polymer (T_M) and the temperature at which the degradation takes place (in the following this difference is abbreviated ΔT_{MT}). ΔT_{MT} lower than 30° C seems to guarantee high degradation rates as the polyester chains in the crystalline domains are mobile enough to access the active site of the enzyme.

In App.Table 2 in the appendix the data gathered in the enzymatic degradation experiment is summarized together with a list of all substrates and their corresponding T_M values. The correlation between ΔT_{MT} and the percentage of Ta and Ada released of total is visualised in Figure 14. Our data is in agreement with results presented in other studies ([3], [12]), although one anomaly was detected: The model substrate Ada80_Ta20, which has a melting point slightly below the reaction temperature of 50 °C (T_M = 47.8° C) showed low degradation rates with both enzymes. Crossing the

melting point causes the polymer to melt, which could lead to a decreased surface area – and consequently make the polymer less susceptible to enzymatic hydrolysis.

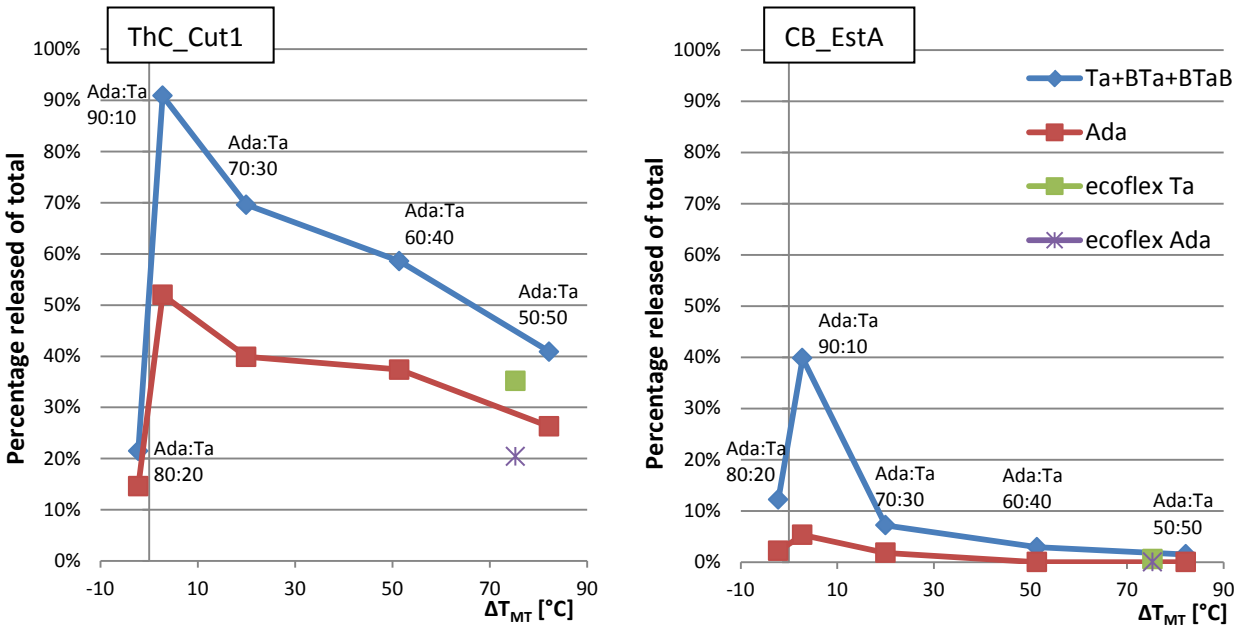


Figure 14: Correlation between ΔT_{MT} enzymatic hydrolysis rates for ecoflex (green and violet) and all tested polymeric ecoflex model substrates (red and blue). ΔT_{MT} is the difference between the melting point and the reaction temperature. ecoflex is shown separately. A negative ΔT_{MT} value was reached for Ada80_Ta20, as the reaction temperature of 50 °C was above the T_M. Released Ta, BTa and BTaB concentrations were added for each polymer to represent the total amount of aromatic components released by enzymatic hydrolysis.

The copolyester ecoflex was presented separately in Figure 14 as it is our main substrate of interest. Its proportion of Ada : Ta ratio to the melting point matches those of the other copolyesters: Ada : Ta of 53 : 47 with T_M = 125.3° C. However, ecoflex' M_W is 65000 g/mol, which is much higher than the M_W of the model substrates (Ada50_Ta50 M_W = 31900 g/mol; Ada60_Ta40 M_W = 29200 g/mol). This distinction could be responsible for the lower enzymatic hydrolysis of ecoflex when compared to the model substrates.

Part II: Inducing enzyme expression in anaerobic sludge batches with polymers

3.4 Development of an esterase activity assay based on fluorescein derivatives

Numerous pre-tests were necessary to develop a reliable esterase activity assay in sludge. At first, a substrate of choice was determined. A comparison of FDA and FDL can be seen in Figure 15. Both substrates where monitored over time – first only in buffer, then in sludge. In buffer the stability and autocatalysis of each substrate was examined. In the sludge the substrates were additionally hydrolysed enzymatically.

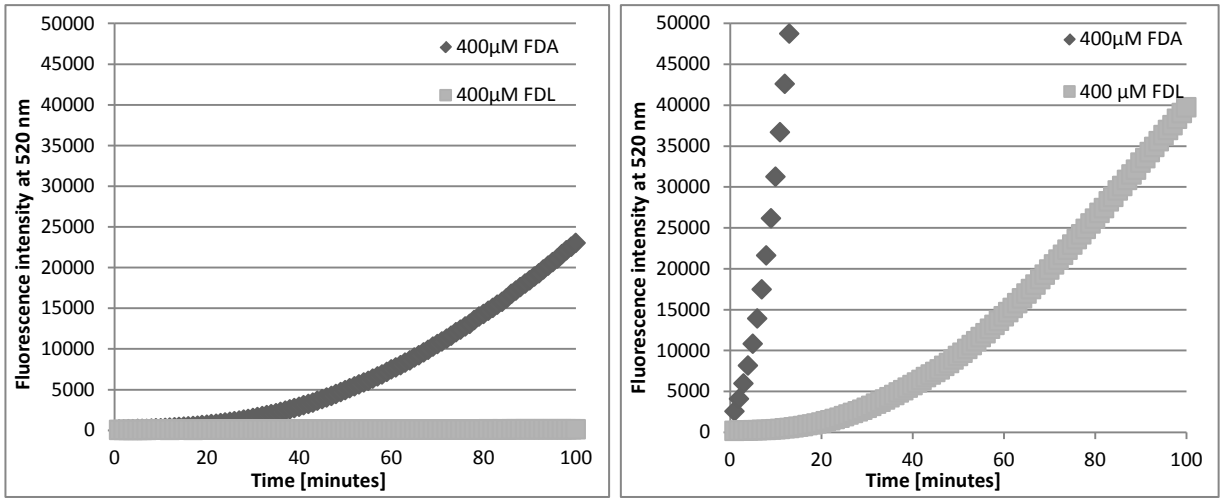


Figure 15: Left side: Autocatalysis of 400 µM FDL or FDA in $NaPO_3$ buffer pH 7.0. Right side: Enzymatic hydrolysis of 400 µM FDA or FDL in sludge.

The autocatalysis of FDL and FDA was observed spectrophotometrically via the fluorescence increase. FDL was more stable over long periods of time when compared to FDA. FDA showed a higher autohydrolysis rate in buffer at moderate conditions – pH 7.0, 37° C – than FDL. When performing the same test in a sludge matrix, FDA was hydrolysed even faster, which can be attributed to its easier accessibility for enzymes leading to higher hydrolysis rates. *Ge et al.* [44] came to the same results when they synthesized and examined several fluorescein esters of straight chain fatty acids: The spontaneous hydrolysis of esters with longer chains proceeded slower than the hydrolysis of shorter chains. The larger, bulkier fluorescein derivatives were more stable, but also proved to be more difficult to hydrolyse by enzymes. However, *Ge et al.* stated that FDL was the best substrate for measuring enzyme activity; it possesses great stability and the highest lipase hydrolysis efficiency in a series of fluorescein esters. Due to these above presented results, FDL was chosen as substrate for the activity assay. In a next step, the optimal FDL concentration needed to be determined. Best results were achieved with a substrate stock solution at 200 µM (data not shown).

Results and Discussion

It is difficult to assess the effects of the substrate concentration on all enzymes in the sludge, as every enzyme behaves differently regarding substrate inhibition. *Green et al.* created a similar hydrolysis assay for soil samples and observed a low correlation between substrate concentration and hydrolytic activity in most soils, with the exception of highly active soil [25]. Additionally, FDA forms a precipitate at concentrations higher than 47.6 mM.

To test the reproducibility and reliability of the assay, sludge was spiked with HiC prior to performing the assay. This test resulted in deviations of up to ±8 % when spiking sludge with different concentrations of the enzyme (data not shown). When subtracting the background enzymatic activity of the sludge, the measured activity represented the added enzyme very accurately.

Interestingly, all samples showed an enzymatic lag phase of several minutes. This was also observed when performing the assay without additional enzymes. By keeping the samples at 37° C during the preparation of the assay, this lag phase could only be reduced slightly. The linear range of the reaction started after 15 minutes. For the determination of the activity only the linear part was taken into account.

Regarding the preparation of suitable standards, the main obstacle was that the fluorescence of fluorescein is very sensitive to the pH, as the intensity decreases drastically in acidic regions [28]. In anaerobic batches cultures, it is not possible to maintain completely stable pH values over the course of the whole experiment. Therefore, fluorescein standards needed to be prepared for every single measurement, which is rather time consuming when handling numerous samples. Additionally, centrifugation of the sludge samples proved to be necessary to remove the solid fraction.

The development of the esterase activity assay for sludge samples was successful. FDL was proven to be the ideal substrate for measuring esterase activities in complex matrices like biogas sludge and the results are reliable. However, the sludge matrix can be difficult to handle, as it is rather inhomogeneous.

3.5 Induction of enzyme expression in anaerobic batch cultures

The change in esterase activity in the sludge samples was measured over a period of 114 days and turned out to be quite similar for all anaerobic batches. An overview of the esterase activities in the different cultures is given in Figure 16. Using model substrates as carbon source for anaerobic digestion apparently influences the production of extracellular enzymes to a varying degree; the relative increase in activity from day one to day 35 is listed in Table 13. In the first 35 days the enzyme activity on FDL increases by 161 % to 395 %, in the case of BTaBTaB it even reaches 662 %. This equals an increase from 2.5 U/ml to 19 U/ml esterase activity in the sludge. The two substrates that were only made of butanediol and terephthalate - BTaBTaB and BTaB - had the highest effect on the enzyme activity, followed by OBAT and ecoflex, which both contain adipic acid. Addition of the monomers Ada, Ta and Ba as carbon source had the lowest impact on esterase activity. The endpoint measurement on day 114 shows that esterase activity declined in all samples. However, the measured activities stayed above the levels of the starting day.

Table 13: Esterase activity measured on the substrate FDL, in units per milliliter. The tested anaerobic batch cultures are sorted ascending from highest relative increase in activity to lowest.

Name of anaerobic batch	Addition of PHA/cutina to induce esterase expression	Start [U/ml]	Day 35 [U/ml]	Day 114 [U/ml]	Relative increase from the start to day 35
BTaBTaB	yes	2.5 ± 0.5	19 ± 0.7	4.9 ± 0.2	+662 %
BTaB	yes	2.5 ± 0.5	12 ± 2.2	5.1 ± 0.3	+395 %
OBTA	yes	2.5 ± 0.5	10 ± 1.9	6.8 ± 0.7	+311 %
ecoflex	yes	2.5 ± 0.5	8.9 ± 0.7	4.1 ± 0.6	**+258 %**
eco n.i. b	no	0.6 ± 0.01	2.0 ± 0.1	1.4 ± 0.07	**+257 %**
BaBTaBBa	yes	2.5 ± 0.5	8.3 ±	7.8 ± 0.1	+234 %
Standards	yes	2.2 ± 0.8	6.2 ± 0.2	4.4 ± 0.2	+175 %
eco n.i. a	no	0.9 ± 0.1	2.4 ± 0.2	1.3 ± 0.1	**+161 %**

The sludges used for batches *eco n.i.a* and *eco n.i.b* differed from the others as they had never been in contact with PHA or cutina before and during the experiment. Using natural polyesters such as PHA might cause the microorganisms to adapt to this carbon source and produce an excess of extracellular enzymes, which could allow the bacteria to deal with synthetic polyesters like ecoflex more efficiently. Looking at our data, the enzyme activity on FDL at the start of the experiment was more than twice as high for the sludges that had been fed with PHA or cutina than for those which had been fed with maize silage. However, the relative increase in activity over 35 days with ecoflex as substrate was less conclusive: for one of the batches fed with maize we observed the same relative increase in activity as for the PHA fed sludge, while for the second one the activity increase was much lower.

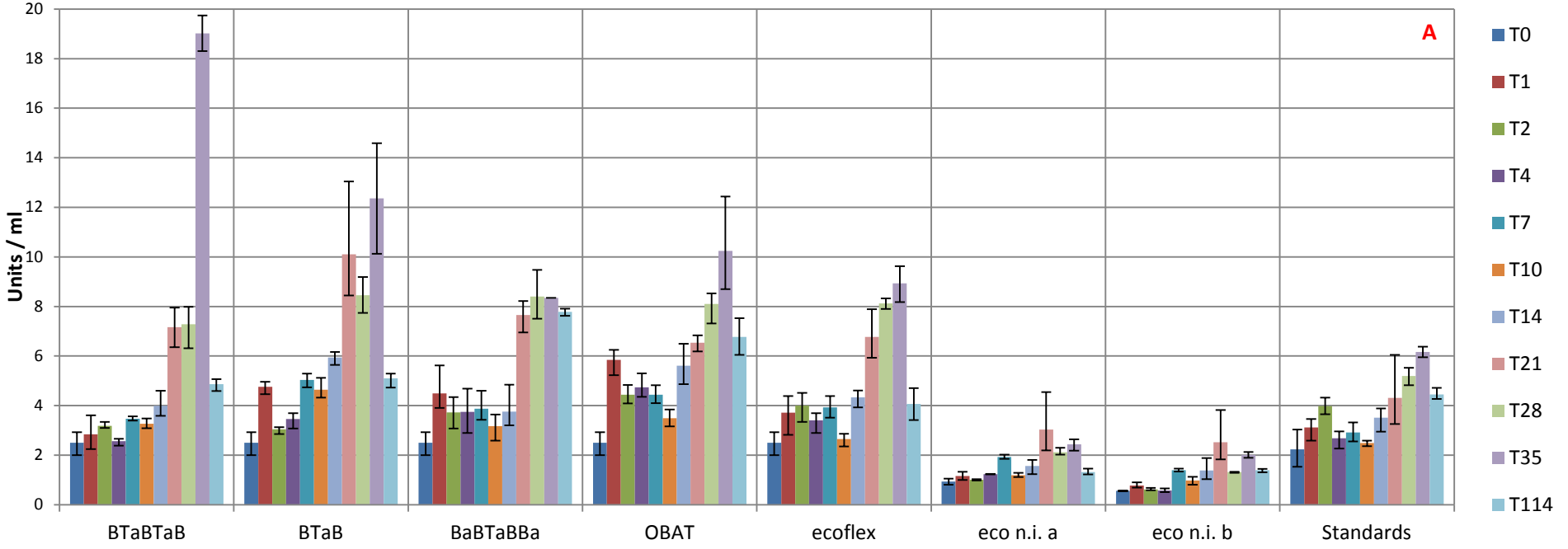
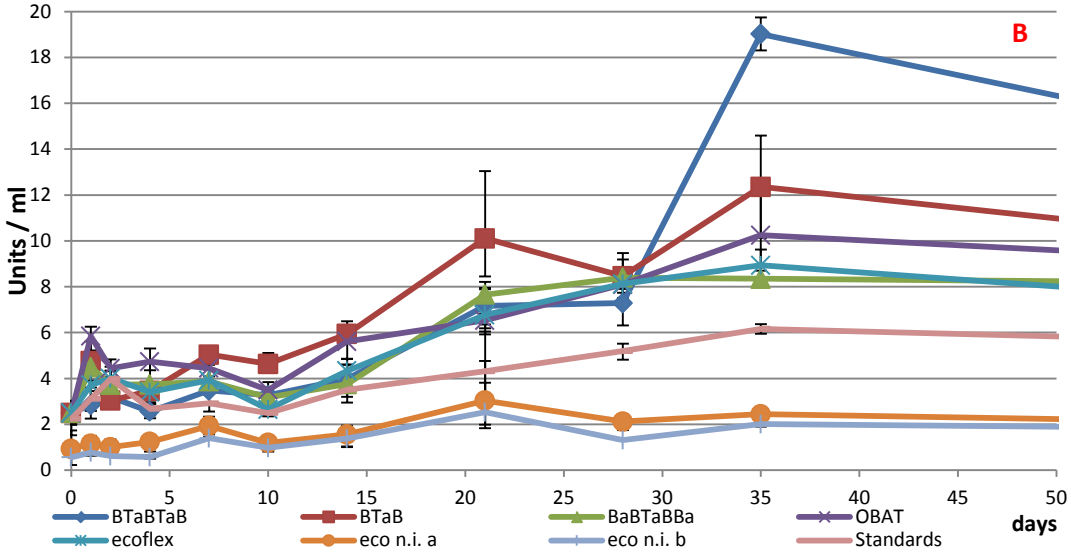


Figure 16: Summary of the esterase activity on FDL in the different sludges obtained from anaerobic batches. "T" is the time passed in days. Diagrams A and B represent the same data, with a correctly proportioned timeline in diagram B. Most of the change in activity was expected in the first week, therefore more measurements were performed in the first 10 days. Afterwards the activity was monitored weekly until day 35. After 114 days the experiment was stopped (not shown in B).



Plausible explanations for different behaviour in the batches are inhomogeneities in the sludge matrix. Examples are wet or dry zones or polymer particles that precipitate and are not available for metabolism.

All abiotic control batches displayed no degradation of the model substrates.

Kleerebezem et al. stated that the increase in degradation can either be attributed to growth of a specific organism originally present in the inoculum, or time dependent adaption of organisms to the substrate [45]. Therefore the activity assay could be further enhanced by simultaneously monitoring the increase in biomass and the increase in enzyme activity. Without knowledge of the amount of present microorganisms, the increase in enzymatic activity could also be attributed to higher numbers of bacteria rather than an induced enzyme expression. Simple methods to address this problem are a cell viability assay (see [46]) or counting the cells (as described in [47]). These methods could further enhance the accuracy and reliability of the developed assay.

3.6 HPLC analysis of hydrolysis products

Parallel to the activity measurements, the degradation products were monitored by HPLC to gain more insight about the degradation process.

Of all detected hydrolysis products observing Ta proved to be most useful, as it was the only degradation product that could be detected in all sludge batches. The results for Ta are displayed below in Figure 17.

All batches showed an increase in Ta concentration up to day 21. After that the concentration started to decline until no Ta could be detected. This process was nicely displayed in the Standards batch: the high starting concentration of 6.7 mM began to decrease after 21 days.

The possibility of Ta being accumulated and absorbed within the sludge rather than being mineralized was reported by *Macarie et al*. [48] In contrast to that *Kleerebezem et al.* [49] came to the conclusion that Ta would not be adsorbed to lipidic membranes of sludge biomass as it does not contain hydrophobic groups like methyl. To determine whether Ta was masked in the sludge due to precipitation or adsorption an extraction with DMSO was performed. The results were clear: After sample preparation and extraction with DMSO, no Ta was found in any sample – neither in the supernatant, nor the resuspended whole cells or the lysates (data not shown). Only in the control group, which was spiked with Ta, Ta could be detected. This confirms the mineralization of Ta and the other monomers, as none of them were detected either.

The concentration of Ada, Ba, BTa and BTaB detected in the batches can be found in the appendix: BTaB in App.Figure 3, Ba in App.Figure 4, Ada in App.Figure 5 and BTa in App.Figure 6.

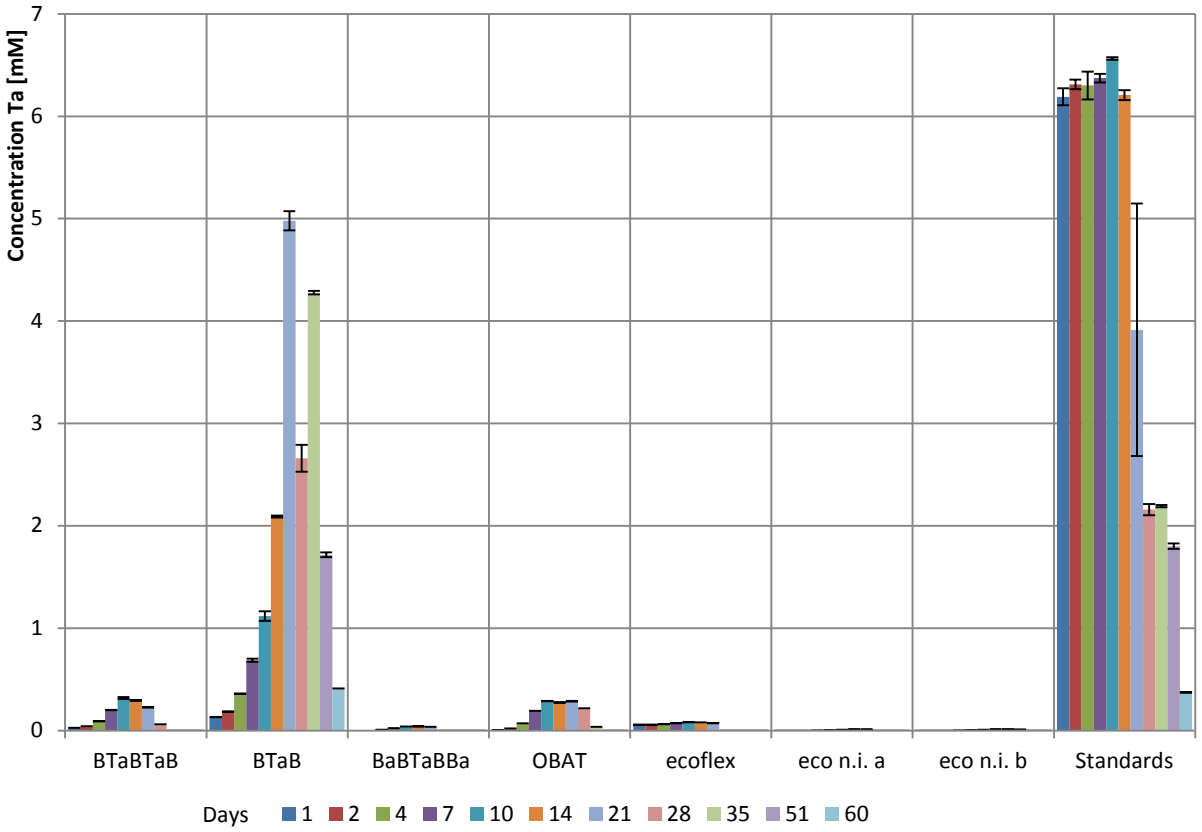


Figure 17: Concentrations of Ta in the anaerobic batches measured by HPLC. All batches showed a very distinctive metabolic lag phase of 14 to 28 days. After this metabolic lag phase the Ta concentration stopped to increase and began to decline. The Standards batch was initially incubatd with 6.6 mM Ada, 6.4 mM Ba and 6.7 mM Ta.

BTaB, which was used as model substrates in batch "BTaB", could only be found in this "BTaB" batch: Starting with a BTaB concentration of 6.4 mM at the beginning, the concentration of BTaB rapidly decreased to 1.9 mM after one day in the anaerobic sludge batch. Within one week, BTaB was completely hydrolysed to smaller intermediates.

Ba was only present in the Standards batch and the batch containing BaBTaBBa as model substrate. Starting with a Ba concentration of 6.4 mM in the Standards batch, Ba was mineralized within 35 days. In the "BaBTaBBa" batch Ba levels peaked at 0.16 mM within 10 days, indicating hydrolysis of model substrate BaBTaBBa. After 14 days no Ba could be detected. In soil samples, Ba was shown to be completely biodegraded after 20 days [50].

Ada was only found in the Standards batch, which had an initial Ada concentration of 6.6 mM as carbon source. Similar to Ba, Ada was completely mineralized within 28 days. Siotto *et.al.* reported an Ada mineralization rate of 84 % after 20 days [50].

BTa was found in low amounts in the batches “ecoflex” “eco n.i.a” and “eco n.i.b” that contained ecoflex as model substrate.

In abiotic control batches no hydrolysis products were detected. This leads to the assumption that the model substrates were not hydrolysed in the abiotic batches (data not shown).

Summing up, all batches showed a certain amount of degradation which can be attributed to microbial activity. However, after an adaption time of 21 days, the concentration of the hydrolysis products decreased again and were taken up into the bacterial cells and metabolized. As a consequence, the degree to which each polymer substrate was hydrolyzed cannot be determined definitively. While the model substrate BTaB was readily degraded to Ta and easily measurable, the other, bulkier substrates might just have formed larger intermediates in the beginning that could not be detected. When these intermediates where then broken down to Ta the lag phase had already been over and the monomers were hidden from detection inside the bacterial cells.

3.7 Discussion: Comparing the results of the activity assay with the HPLC data, and the influence of the microbial community composition

An important parameter when dealing with the mineralization of biodegradable polymers is the lag phase, which is the time a microbial community needs to adapt to certain substrates in the surrounding environment. For Ta degrading methanogenic bioreactors a start-up time from a few months to a year was reported until Ta was actually degraded [51]. This adaptation time was significantly shortened when using seeding sludge that had already been degrading phenolic compounds. *Kleerebezem et al*. [52] came to a similar conclusion when they used an upflow anaerobic sludge blanket reactor in two stages for Ta degradation in wastewater purification: The first stage, which was seeded with methanogenic granular sludge, showed a lag phase of 300 days before starting to degrade Ta. However, the second stage reactor was able to degrade Ta from the beginning, when using the suspended Ta degrading culture from the first stage as inoculum.

Looking at the data from the HPLC analysis, the concentration of Ta rises steadily until it starts to decline after 3 weeks. This trend can be seen in all batches. However, when comparing the different sludges used for ecoflex in detail, the metabolic lag phase is shorter for the sludge “ecoflex“ that had been in contact with synthetic and natural polyesters before – see Figure 18. The sludge “ecoflex” that was fed with PHA and that had previously been used in an experiment with synthetic polymer substrates started fully metabolizing Ta after 10 days, while the sludges “eco n.i.a” “eco n.i.b “ that were fed with maize silage began to metabolize Ta after 21 – 28 days.

The microbial community in the sludge batch "ecoflex" was in prolonged contact with PHA and cutina to induce enzyme expression while "eco n.i.a" and "eco n.i.b" were only fed with maize silage. This induction seems to reduce the metabolic lag phase and boost esterase expression compared to the not-induced sludges (see Table 13 for enzyme activity increases). The inducers PHA and cutina seem to facilitate the adaption of the microbial community to polymeric substrates.

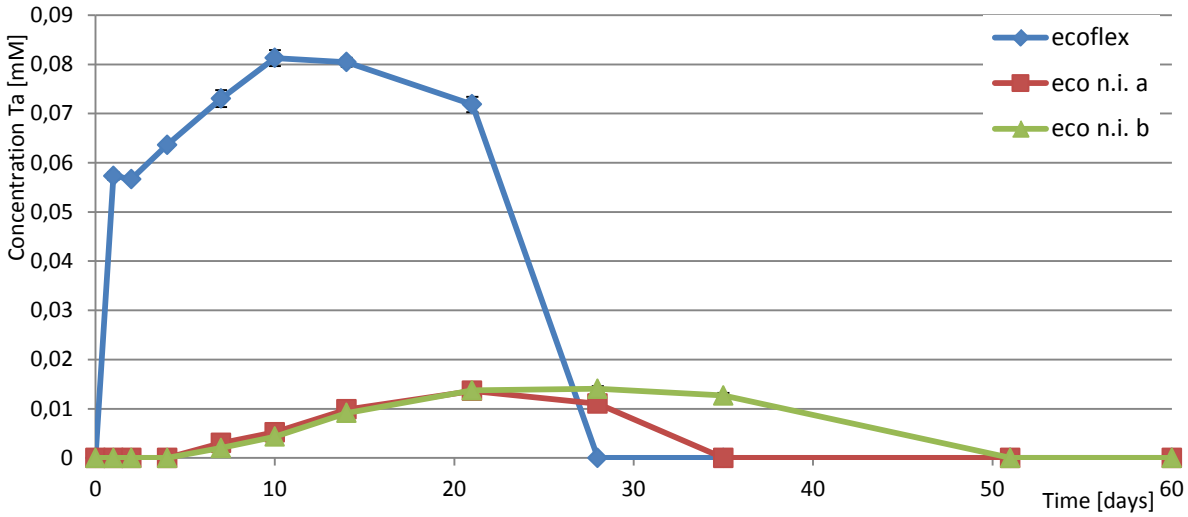


Figure 18: Comparing the metabolic lag phases of different sludges containing ecoflex. All three sludges used ecoflex as carbon source; however, their original inoculation sludges had different sources: "ecoflex" was previously used in experiments with other polymeric substrates and was fed with PHA; "eco n.i.a" and "eco n.i.b" were only in contact with maize silage prior to this experiment.

In general, the origin of the sludge is of utmost significance. It is common practice to give information about the C/N ratio, the CO_2 production and various ISO – standards when describing compost and sludge. However, this might just overlook the fact that there can be great differences in the overall microbial composition depending on their diet [53]. Microorganisms originating from similar thermophilic conditions can be classified according to the raw material. For example, fermenting bacteria are abundant in food-waste composting processes while actino-bacteria are found in sewage sludge [53]. When there is a low abundance of phthalate degrading organisms in the seeding sludge, the metabolic lag phase is increased tremendously. For that reason, selecting the right source of the seed sludge is a good way of speeding up the mineralization process.

The process of completely mineralizing PBAT anaerobically is very complex, and many different organisms are involved in a symbiotic manner. Each degradation step is performed by different species for their mutual benefit [54]. Regarding PBAT, three monomers can be released during the degradation: Ada, Ta and B. For an environmentally safe application of biodegradable plastics it is crucial that even these hydrolysis products do not have any ecotoxicological effects, because mainly these products are found in the environment [55]. A risk assessment using *Daphnia magna* and *Photobacterium phosphoreum* showed no indication of an environmental risk when PBAT like ecoflex or their intermediates are introduced into a composting environment [55]. However, when testing

the phytotoxicity on germination of young radish seeds and the cytotoxicity on HeLa cells, a human cell line derived from cervical cancer cells, the results are different: all monomers are potentially harmful, the aromatic compounds in general more than the aliphatic ones [56]. A concentration of 1 % Ta reduced germination to 70 % and notably retarded the growth of leaf, stem and root. 1 % Ada decreased the rate of germination even further to 47 %. 5 % of Ada or Ta totally prevented germination. Looking at HeLa cells viability, the system was more sensitive: 0.1 % Ta the cells showed 79 % viability with similar results for Ada. When increasing the concentration to 1 %, the cell viability plummets (Ada: 48 %, Ta: 16 %). In both tests, B turns out to be much more tolerable. Another study discovered that phthalates might have adverse effects on human health and reproduction [57].

When comparing the time it takes to mineralize each monomer aerobically in soil, respirometric tests based on the evolution of CO_2 indicated that Ta is much more stable than Ada and B [50]: While after 25 days 80 % of Ada and 100 % of B were mineralized, Ta only reached a degradation of 40 %.

Taking into account the toxicity and the stability of the monomers, it is evident that Ta can have the biggest impact on the environment and needs to be examined closely. A simplified schematic diagram for the degradation of PBAT to Ta and its mineralization is presented in Figure 19.

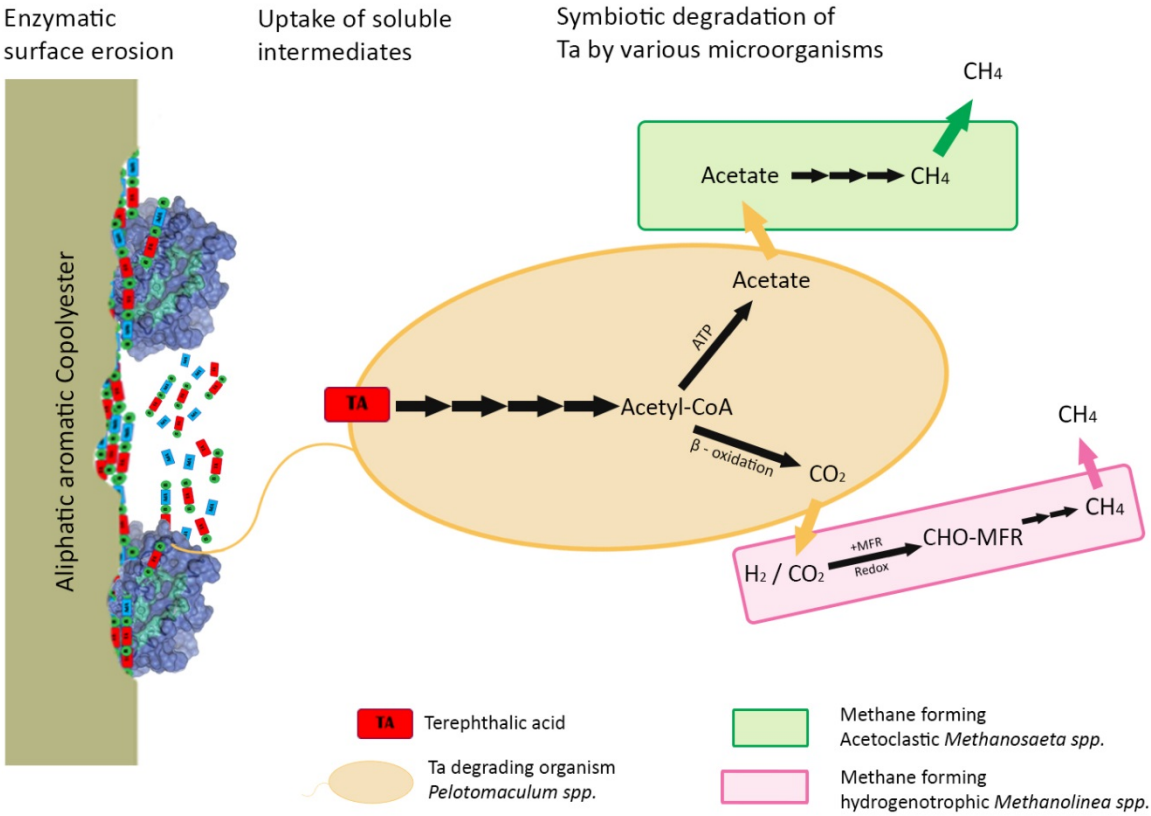


Figure 19: Schematic depiction of Ta degradation in a mixed microbial culture. Polymer chains are degraded in an enzymatic surface erosion process and soluble intermediates like Ta are released. Ta is taken up by Ta degrading organisms like *Pelotomaculum spp.* and through acetyl-CoA in different pathways acetate and CO_2 are formed, which are secreted and used by other organisms to form methane. [diagram modified after [59], [60]**]**

In the first step extracellular esterases are secreted that degrade the polymer chain. However, this could mainly be due to the fact that the microorganisms that secrete the esterases mistake the synthetic polymers for natural polymers like PHA, so they cannot process the unexpected released products of the polymer chain. That is where other organisms that are able to degrade phthalates take over.

Shotgun sequencing and metagenomics show a complex syntrophic cooperation between Ta degrading *Pelotomaculum spp*. and the two methane forming species, acetoclastic *Methanosaeta spp*. and hydrogenotrophic *Methanolinea spp.*[58]–[60]. Especially during exponential growth tight syntrophic coupling between fermenting organisms and methanogens was observed [61].

The delicate syntrophic cooperation could easily be disturbed by changes in the environment. As an example, changing the diet of a sludge can have an influence on the microbial community [47]: Using only xylan as carbon source caused hemocellolytic populations to grow rapidly. While this was the desired effect, a characterization of microbial communities by SSCP fingerprints showed that other species vanished. Enforcing certain catabolic pathways appears to cause breaches in syntrophic interactions, especially of methane producing microorganisms.

Going back to the results of the present study, the induction with model substrates did not seem to have a harmful effect on the microbial community. This factor should be further investigated by metagenomics or by monitoring the methane production.

Aerobic hydrolysis of PBAT was confirmed by several standard degradation tests; metabolizing PBAT under anaerobic conditions proved to be much more difficult [62]. The total microbial activity of the exposure environment and the composition of PBAT also play a great role when assessing the biodegradability [63]. Phthalate biodegradation is decreased by increasing polymer side chain length and high concentrations of phthalates or their metabolites can even inhibit their degradation [64].

In conclusion the esterase activity assay and the HPLC analysis of the degradation products can be useful tools to assess the biodegradability of PBAT and the enzymatic activity induced by model substrates. Yet comparing the results of these two methods did not provide new insight into the degradation process. While in theory the increase in enzymatic activity should result in larger amounts of detectable intermediates, these intermediates were mostly assimilated instantly by the microorganisms. The measurement of the hydrolysis products need to be improved to include the larger fragments that cannot be taken up into the cells so quickly.

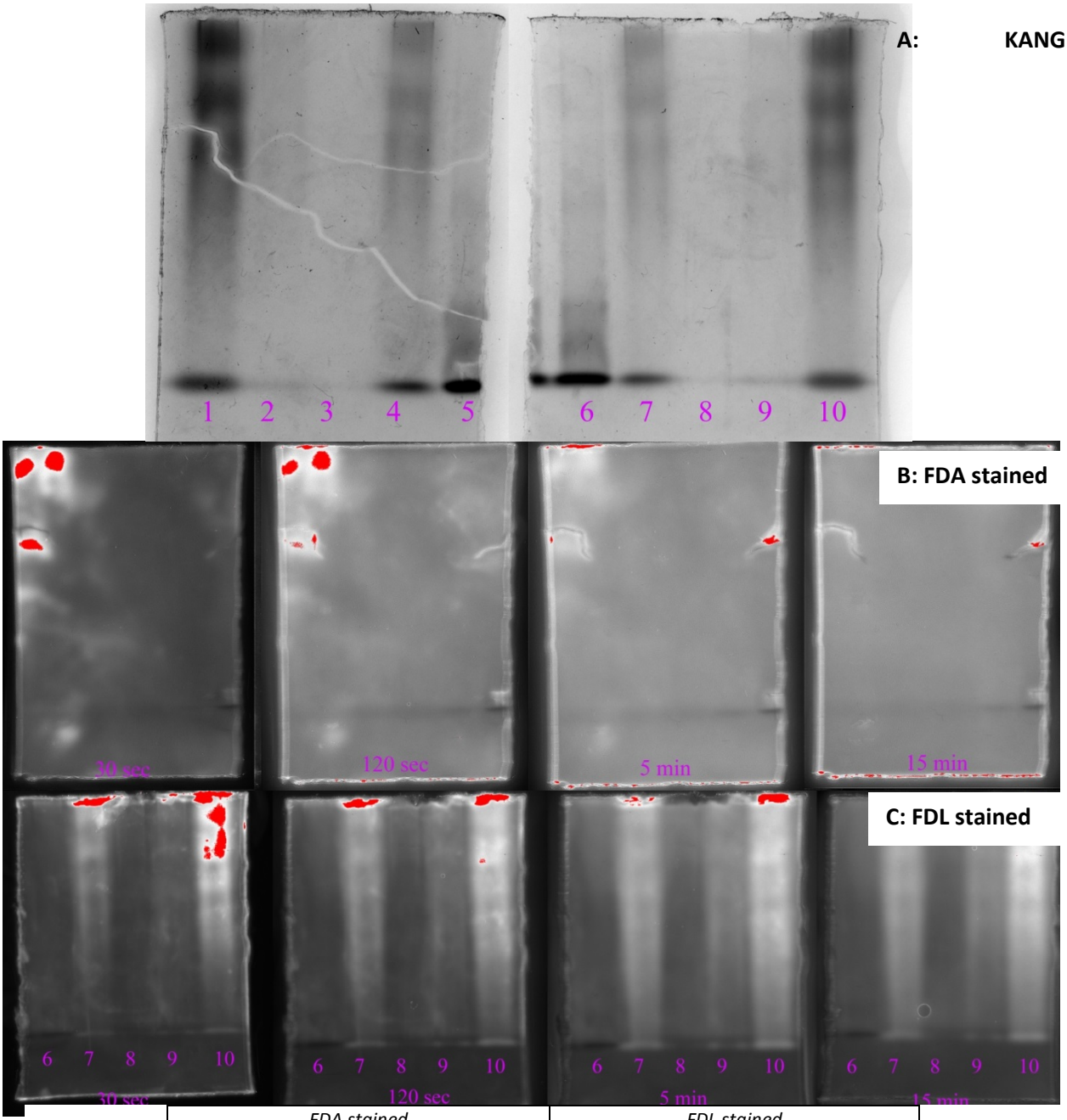
Nevertheless, we were able to show that the smaller model substrates containing only Ta and B did cause a larger increase in esterase activity and that different sludges adapt to the model substrates at varying speeds. On top of that the HPLC analysis revealed that ecoflex was degraded in the anaerobic sludge to a certain degree.

For future experiments changes in the microbial community should be closely monitored as they play a major role in the biodegradation of PBAT and could be useful for the search for sources of biopolymer degrading enzymes.

3.8 Separating extracellular proteins by PAGE and staining of hydrolases with FDL

The separation and isolation of extracellular proteins from the anaerobic batches proved to be a difficult task. An example of the results is given in Figure 20. In this gel HiC was used in different dilutions and to spike an anaerobic sludge (see 2.5 for details). By cutting the gel in halves and putting it into an FDA or FDL solution, bands containing enzymes with esterase activity were stained as they cut the fluorescein derivatives and produce the fluorescent fluorescein. A much better stability of FDL compared to FDA was observed; FDA showed a high amount of autocatalysis which made it impossible to distinguish between the particular lanes. The HiC dilutions 1:10 and 1:100 displayed enough activity to produce detectable lanes after 30 seconds of exposure to the FDL solution. While bands were visible, it was not possible to make a clear distinction between them, as the transitions between these bands were too blurry.

None of the changes made to the experimental setup could help to improve the resolution of the PAGE separation, the bands always showed a distinctive smudge. However, when looking at the KANG-solution stained gel, it appears that this was not a result of the fluorescence staining method itself, but rather of the complex sludge matrix. If the blurry lanes were a result of the FDL staining, the use of the KANG-solution should have produced sharper bands. This was not the case. If the resolution of the lanes was increased this method could be a very cost efficient alternative to labelling enzymes with commercially available products like the ActiveX Tamra FP probes, which were used in 2.14.



	FDA stained		*FDL stained*
1	HiC 1:10	6	Standard (PageRuler)
2	HiC 1:100	7	Sludge + HiC (1:10)
3	HiC 1:1000	8	HiC 1:1000
4	Sludge + HiC (1:10)	9	HiC 1:100
5	Standard (PageRuler)	10	HiC 1:10

Figure 20: 5 % PA gel after electrophoresis at 60 volt for 4 hours. "A" shows the KANG-stained gel after it was cut in half and stained with FDA / FDL. Parts B and C display the two halves of the gel incubated with FDA or FDL solution for the indicated time periods.

3.9 Biofilm formation on polymer foils - crystal violet staining

3.9.1 Biofilm formation assay with biofilm forming bacteria

Strains *E.coli* XL1 and the *S. aureus* "praktikum" both showed the same growth rate in the medium (OD_{600} after 8 hours: *E. coli* 2.1, *S. aureus* 2.2). On the other hand, the picture was quite different for their ability to form biofilms on polymer foils, as can be seen in Figure 21. In Table 14 the absorbance at 595 nm for the samples is shown.

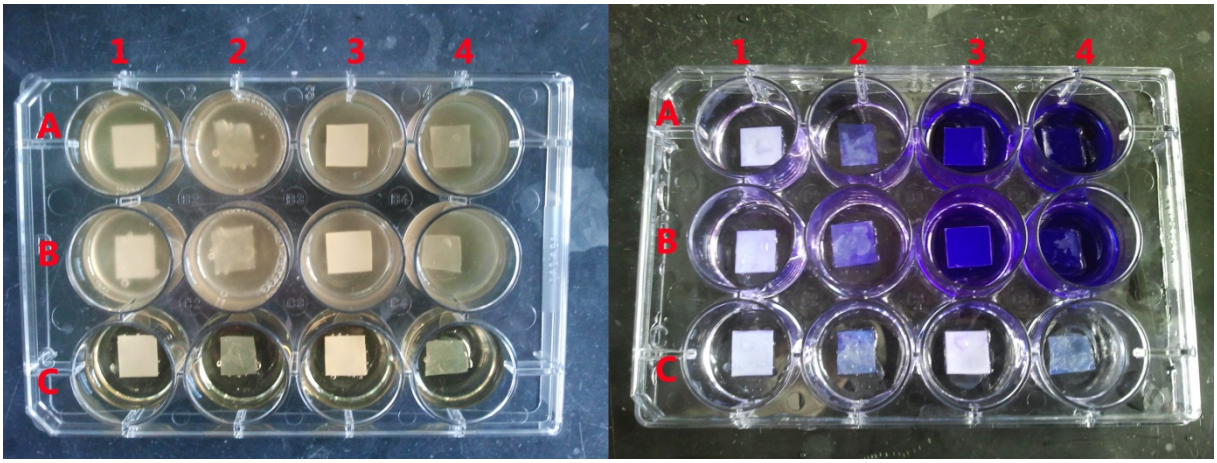


Figure 21: Left side: Incubation of polymer foils with *E. coli* and *S. aureus*. Right side: crystal violet remaining on the samples after washing with H_2O. Rows A and B are duplicates, C is the control with sterile medium. Column 1: *E. coli* 400 µm ecovio; Column 2: *E. coli* 50 µm ecoflex; Column 3: *S. aureus* 400 µm ecovio; Colum 4: *S. aureus* 50 µm ecoflex

While the *S. aureus* is readily building a biofilm on both ecovio and ecoflex, the amount of detected crystal violet for *E. coli* is only slightly higher than for the control group. This indicates that *E. coli* XL1 is not able to form biofilms on polymer foils of this type and in consequence is not efficiently able to biodegrade them. However, the *E.coli* XL1 used in this experiment is a laboratory strain and could have lost its ability to form biofilms all together.

Table 14: Absorbance at 595 nm of the re-dissolved crystal violet

polymer type	*E. coli*	*S. aureus*	Control
ecovio 400 µm	0.15 ± 0.001	6.1 ± 2.4	0.14 ± 0.11
ecoflex 50 µm	0.17 ± 0.02	6.7 ± 1.3	0.11 ± 0.02

3.9.2 Biofilm formation assay after incubation of plastic foils in sludge

In the next step, polymer foils were incubated in anaerobic sludge instead of defined cultures. The recovery of the plastic foils from the sludge proved to be quite difficult. As the pieces were sticking together, it was hardly possible to isolate a single piece without damaging a lot of the biofilm on its surface. Due to this experimental hindrance, the absolute values of the absorbance measurement

may be the subject of rather big variations. Nevertheless, our results indicated that with decreasing thickness of the foil a slight increase in biofilm formation was to be observed (see Figure 22 and Table 15).

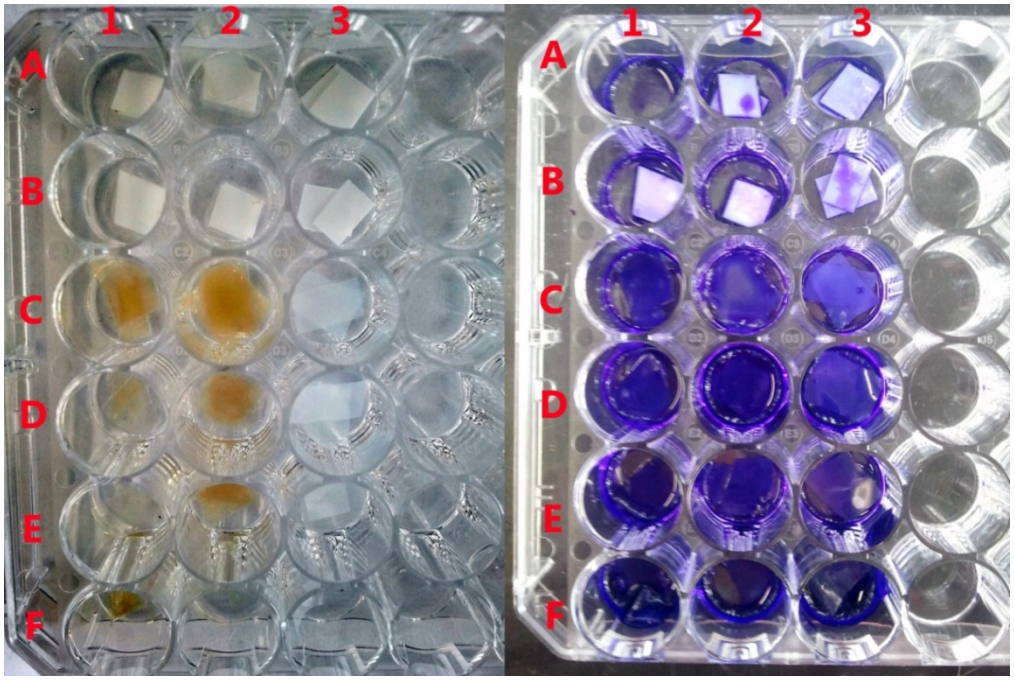


Figure 22: Left side shows the polymer foil pieces that were incubated in sludge, after washing in water; the right side shows the staining with crystal violet (before washing in water). Columns 1 and 2 are duplicates; Column 3 is the negative control with non-incubated plastic film pieces. Rows A to F contain decreasing strengths of foils: 520 µm ecovio, 400 µm ecovio, 50 µm ecoflex, 30 µm ecoflex, 25 µm bionolle, 15 µm bionolle

A visual inspection of the samples underlines this assumption. The thinner foil pieces all showed a darker colouring which originated from residual bacteria. The two samples with the highest thickness displayed no dark spots at all and were completely white. It is possible that not the thickness, but the material used for these two – ecovio – is responsible for this. On the other samples biofilm formation was shown with crystal violet staining.

Table 15: Absorbance at 595 nm of the re - dissolved crystal violet

polymer type	Abs.	Control
520 µm ecovio	0.11	0.21
400 µm ecovio	0.22	0.45
50 µm ecoflex	0.38 ± 0.09	0.16
30 µm ecoflex	0.37 ± 0.13	0.23
25 µm bionolle	0.21 ± 0.06	0.11
15 µm ecoflex	0.31 ± 0.08	0.22

3.10 LIVE/DEAD *Bac*Light Bacterial Viability Kit

The first analysis by CLSM in which only the sludge itself was stained clearly revealed a large number of live cells (data not shown).

In the subsequent experiment, foil samples were placed in the sludge for 3 weeks. Figure 23 shows the freshly prepared slides with Bionolle or ecoflex foils after staining with the LIVE/Dead Kit.

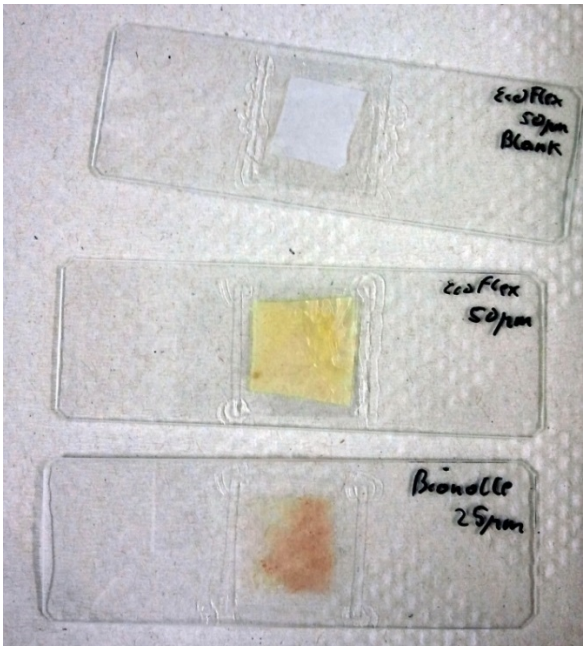


Figure 23: Comparison of ecoflex and Bionolle foil pieces that were incubated for 3 weeks in biogas sludge to a blank. From top to bottom: ecoflex 50 µm blank; ecoflex 50 µm; Bionolle 25 µm.

The following images (Figure 24) are the CLSM pictures of the samples, displayed at maximum projection of all acquired stacks. Bionolle and ecoflex both showed conglomerates of bacteria, with a large number of living individuals. However, the thinner Bionolle foil had a higher abundance of living microorganisms, as the green signal was far stronger. This could support the theory stated before that with decreasing thickness of the samples the biofilm formation is increased. The increased amount of living cells could also be explained by the difference in composition of Bionolle and ecoflex.

In addition to the channels for red and green a third spectrum for blue fluorescence was monitored. On all samples blue appearing cells were detected. This can be attributed to the presence of the co-factor F350: This protein is auto-fluorescent when excited at 350 nm and emits a blueish- white light [65]. It is mainly found in methanogenic organisms (archaea) and can therefore be used to monitor these species and differentiate between methanogenic and non-methanogenic bacteria.

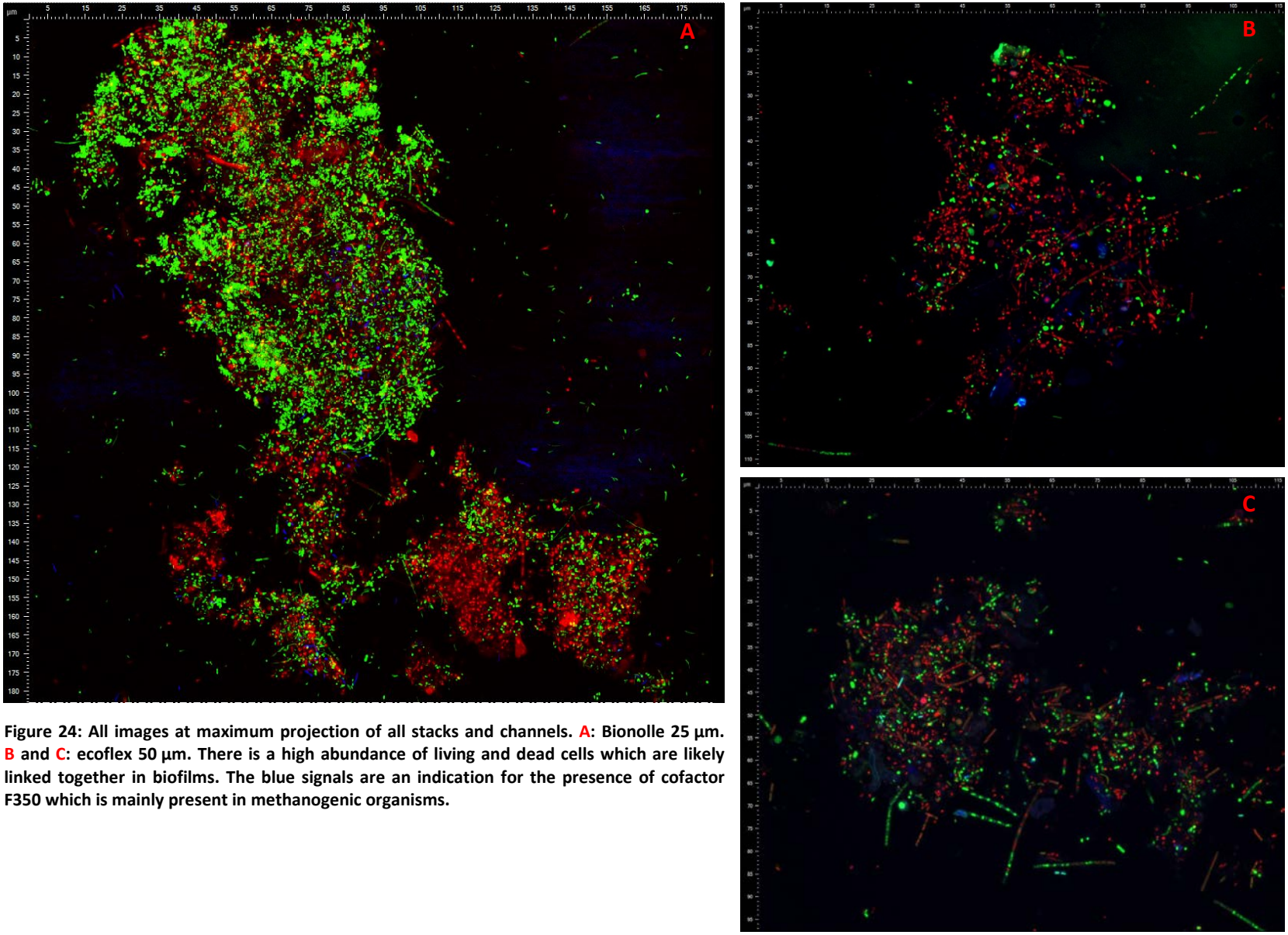


Figure 24: All images at maximum projection of all stacks and channels. A: Bionolle 25 µm. B and C: ecoflex 50 µm. There is a high abundance of living and dead cells which are likely linked together in biofilms. The blue signals are an indication for the presence of cofactor F350 which is mainly present in methanogenic organisms.

A second co-factor, F420, is used in the identification of methanogenic archaea[66]. It emits a green-yellow light when excited at 420 nm. In other archaea like halobacteria the co-factor F420 can be found as well, but in much smaller concentrations [67].

Additional experiments could be made with fluorescence in situ hybridization (FISH) probes that are more specific to certain organisms e.g. firmicutes. Using FISH, detailed information about the biofilm's composition and abundance of certain microorganisms can be gathered.

Part III: Enzyme isolation from *C. hathewayi*

3.11 PAGE and ActivX Serine hydrolases probes labelling

In an attempt to find novel polyesterases from a pure culture of *C. hathewayi*, the culture's active serine hydrolases were labelled with fluorescent probes that bind to the serine in the active center. After the labelling and protein separation by PAGE the gels were examined using fluorescent gel imaging: The bands containing labelled proteins emitted light. The gel was then stained with a KANG-solution, which colours all present proteins. The bands which emitted light before were cut out and their sequence analysed.

Figure 26 shows the result of the two labeling methods. Cultures C and D contained PBAT as inducer for enzyme expression during cultivation and are hence marked with a "*" in the depiction. Active esterases only appear in culture D. The bands that were cut out are marked in the figure in red: *d1* is from the supernatant of culture D, *d2* and *d3* appear in the pellet that was resuspended after the ultrasonification. *c1* was cut out due to its close proximity to *d1* and because it could contain inactive enzymes. To counter the low protein concentration in the samples, a methanol–chloroform precipitation was performed for the supernatants of all cultures and the procedure for the isolation of enzymes was repeated. The result of the protein separation is shown in Figure 25. The parts of the gel that were cut out are labeled in red in the figure (as *c2* and *d4*). They appeared to be in the same region as the bands *c1* and *d1*.

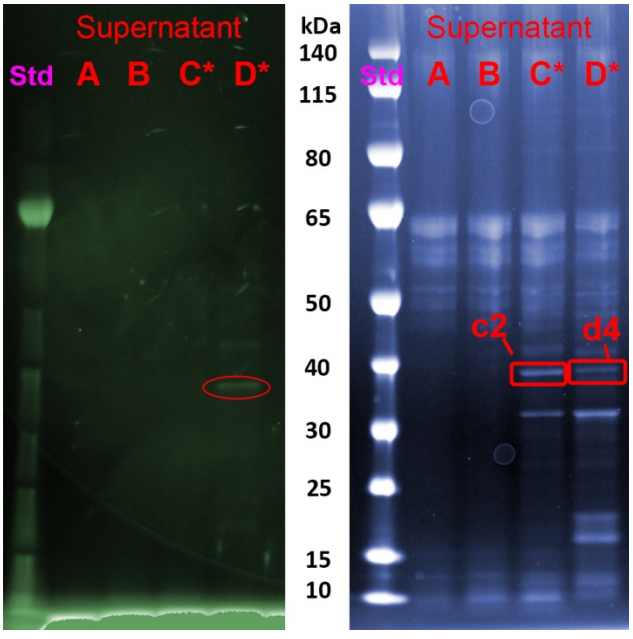


Figure 25: Protein separation with gel electrophoresis after a methanol-chloroform precipitation of the supernatants was performed. Cultures C* and D* were induced with PBAT during cultivation. The left gel image shows emitted light at 575 nm after excitation with a green laser, the band of interest is circled in red. On the right side is the gel after KANG-staining, the parts of the gel that were cut out and analyzed are marked with red squares. The standard is the Pageruler Prestained Protein Ladder (10 – 170 kDa, Fermentas).

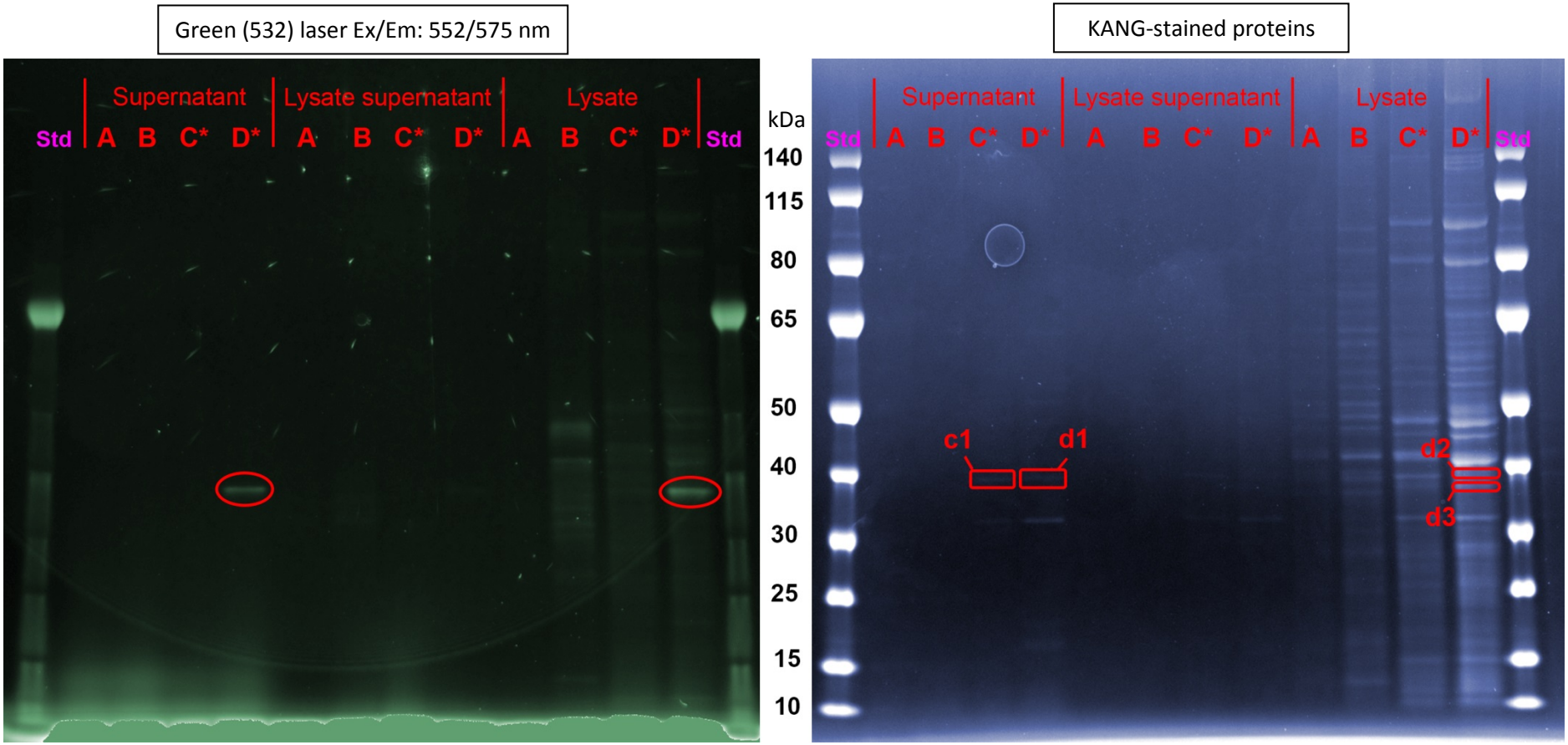


Figure 26: Gel images after protein separation with PAGE. *C.hathewayi* cultures C* and D* were induced with PBAT during cultivation. The left gel image shows emitted light at 575 nm after excitation with a green laser, the bands of interested are circled in red. On the right side is the gel after KANG-staining, the parts of the gel that were cut out and analyzed are marked with red squares. The standard is the Pageruler Prestained Protein Ladder (10 – 170 kDa, Fermentas).

The cut out gel bands were sent to an external lab for sequence analysis and the protein sequences were then compared to a databank using a BLAST search. In the following summary the samples which were situated next to each other on the gels are grouped:

The proteins in the bands c1 and d1 originated from the supernatant of the PBAT-induced cultures and contained no enzymes with potential hydrolytic activity. No proteins from *Clostridia* were found, the predominant organism is *Bacillus licheniformis*.

The samples d2 and d3 from the resuspended cell pellet after lysis showed many proteins from *Propionibacterium acnes* and again *B. licheniformis*, but no hydrolases were found.

The bands c2 and d4 were both from the methanol-chloroform precipitated supernatants. The prevalent organisms were once again *B. licheniformis* and *P. acnes*. However, in these samples two proteins with high homology to interesting enzymes could be identified: A serine protease from *B. licheniformis* and a possible cellulase from *P. acnes*.

Overall, the analysis of all bands showed that the cultures appear to contain a mix of *B. licheniformis* and *P. acnes*, rather than the expected *C. hathewayi* strain. The DNA sequencing of the cultures confirmed this assumption: None of the four received cultures were *C. hathewayi*. The amplified rRNA sequences revealed that cultures A and B contained *Staphylococcus epidermis*, culture C *B. licheniformis* and culture D *P. acnes*.

Although the cultures did not contain *C. hathewayi*, they still showed some hydrolytic activity on ecoflex (data not shown). This is an unexpected result which indicates that the diversity of organisms that might be able to degrade synthetic polymers is far larger than originally believed.

3.12 Evaluation of the results and method

The goal to isolate and identify enzymes from *C. hathewayi* could not be achieved due to a contamination and the experiment needs to be repeated. However, despite the contamination we were still able to establish a new method for the search of active serine hydrolases. The analysis of the gel bands by sequencing the containing proteins is a fast and reliable technique for gaining access to hydrolytic enzymes, which will be of great use in future experiments.

4 Conclusion and outlook

The difference between hydrolytic enzymes from aerobe and anaerobe sources was investigated with ThC_Cut1 and CB_EstA. As expected, ThC_Cut1 of aerobe origin displayed higher esterase activity on the tested PBAT model substrates. Regarding enzymatic hydrolysis of ecoflex, ThC_Cut1 released 35.2 % of total available Ta monomers in form of hydrolysis products, whereas CB_EstA released 0.4 %. However, on the model substrates of varying Ada : Ta ratio CB_EstA was able to catch up: When hydrolysing the model substrate Ada90_Ta10 over 40 % of available Ta was released with CB_EstA, compared to 90.9 % with ThC_Cut1. While there is still a significant gap in activity for the two enzymes, the change in the polymer composition clearly brings their performance closer together. It was also interesting to notice the different distribution of hydrolysis intermediates; ThC_Cut1 almost exclusively produced the monomer Ta, while CB_EstA favoured the production of larger intermediates like BTaB and BTa.

For the biogas producing sludge batches a novel esterase activity assay that can be performed directly in sludge samples was created. With fluorescein dilaurate a well-suited indicator was found as it proved to be stable in the sludge batches and enabled reliable measurements. The method was immediately put to the proof in the enzyme induction experiment and turned out to be a viable addition to other analytical techniques.

The induction of enzyme expression in sludges with the naturally occurring polyesters polyhydroxyalkanoate and cutina showed a slight improvement in esterase activity. Comparing the sludges that contained ecoflex as additional carbon source, the induced sludge showed an increase in esterase activity of 258 % within the first 35 days of the experiment, whereas the activity in sludges that were not fed with PHA upfront increased by 209 % on average. The data at hand suggests a successful induction. This assumption was supported by the HPLC analysis of hydrolysis intermediates: The characteristic metabolic lag phase that occurs for the mineralization of Ta was shorter for sludges that were induced with natural polymers. The reason for this could be that the adaption of the microorganisms to phthalate was accelerated, which could be useful for biotechnological applications. In general the monitoring of the microbial community is of great importance when investigating the biodegradation and mineralization of biodegradable polymers like ecoflex, as it is a delicate process with multiple different organisms involved.

For the biofilm formation on polymer foils a minor coherence between the thickness of the foils and the amount of biofilm formed was observed. After incubation in sludge, the polymer foils with the highest thickness had no biofilm attached that could be detected with crystal violet. However, the lack of biofilm could also be a result of the materials used. CLSM imaging revealed a high abundance

of living bacteria and possibly some methanogenic archaea attached to the foils. Additional experiments with specialized FISH probes could give more information about the involved microorganisms.

The sludge matrix proved to be too complex to be separated by PAGE, but the isolation and identification of proteins was continued with anaerobic cultures. A method for labelling and sequencing proteins directly out of the gel was established. Although the experiment could not be conducted with pure cultures of *C. hathewayi*, the technique itself proved to be a promising way of retrieving enzymes from anaerobic sources and is already in use in ongoing experiments.

This thesis investigated the difference between anaerobe and aerobe degradation mechanisms and established novel techniques for the search for polyester degrading esterases. The advances in the development of biodegradable polymers are exciting, and with increased insight in their degradation mechanisms and the discovery of new hydrolytic enzymes nothing will stand in the way of fully integrating biodegradable polymers in our daily lives.

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6 Appendix

6.1 Calculations for model substrates and results of enzymatic hydrolysis

App.Table 1: The calculations for the respective molar distribution of Ta and Ada for all tested model substrates in Part I of the experiments. The calculations are made for a hypothetical polymer consisting of 200 monomers as described in Table 1. During esterification the monomers condense and one H_2O molecule separates. To take the ester bonds into account, one H_2O molecule was subtracted from each monomer and linker B (1,4 butanediol). The monomer ratios and concentrations are presented for 10 mg substrate in 2 ml buffer.

	Ta [mol/L]	Ada [mol/L]	B [mol/L]	Total [mol/L]
Ada:Ta = 50:50	0.012	0.012	0.024	0.048
ecoflex (Ada:Ta = 53:47)	0.011	0.013	0.024	0.048
Ada:Ta = 60:40	0.010	0.014	0.024	0.048
Ada:Ta = 70:30	0.007	0.017	0.024	0.048
Ada:Ta = 80:20	0.005	0.019	0.024	0.048
Ada:Ta = 90:10	0.002	0.021	0.024	0.048
PBAda	0	0.024	0.024	0.048

App.Table 2: Summary of the results of the enzymatic degradation experiment. The amounts for Ta represent the sum of Ta, BTa and BTaB. T_M is the melting point of the substrate; ΔT_{MT} is the difference between the reaction temperature and the melting point. mol/mol E. is the amount of mol hydrolysis product released per mole enzyme.

				Sum of terephthalic acid*		adipic acid	
Substrate	**T_M [°C]**	**ΔT_{MT} [°C]**	**Enzyme**	**mol/mol E.**	**% of total**	**mol/mol E.**	**% of total**
ecoflex	125.3	75.3	ThC_Cut1	6577	35.2	4300	20.4
			CB_EstA	116	0.6	0	0
Ada50_Ta50	132.2	82.2	ThC_Cut1	8127	40.9	5219	26.3
			CB_EstA	303	1.5	0	0.0
Ada60_Ta40	101.4	51.4	ThC_Cut1	9322	58.6	8916	37.4
			CB_EstA	462	2.9	0	0.0
Ada70_Ta30	70.0	20.0	ThC_Cut1	8304	69.6	11101	39.9
			CB_EstA	857	7.2	510	1.8
Ada80_Ta20	47.8	-2.2	ThC_Cut1	1709	21.5	4636	14.6
			CB_EstA	967	12.2	694	2.2
Ada90_Ta10	52.8	2.8	ThC_Cut1	3614	90.9	18618	52.0
			CB_EstA	1581	39.8	1912	5.3
PBuAda	58.8	8.8	ThC_Cut1	/	/	24152	60.8
			CB_EstA	/	/	5911	14.9

6.2 Spectrometer and HPLC instrumental settings

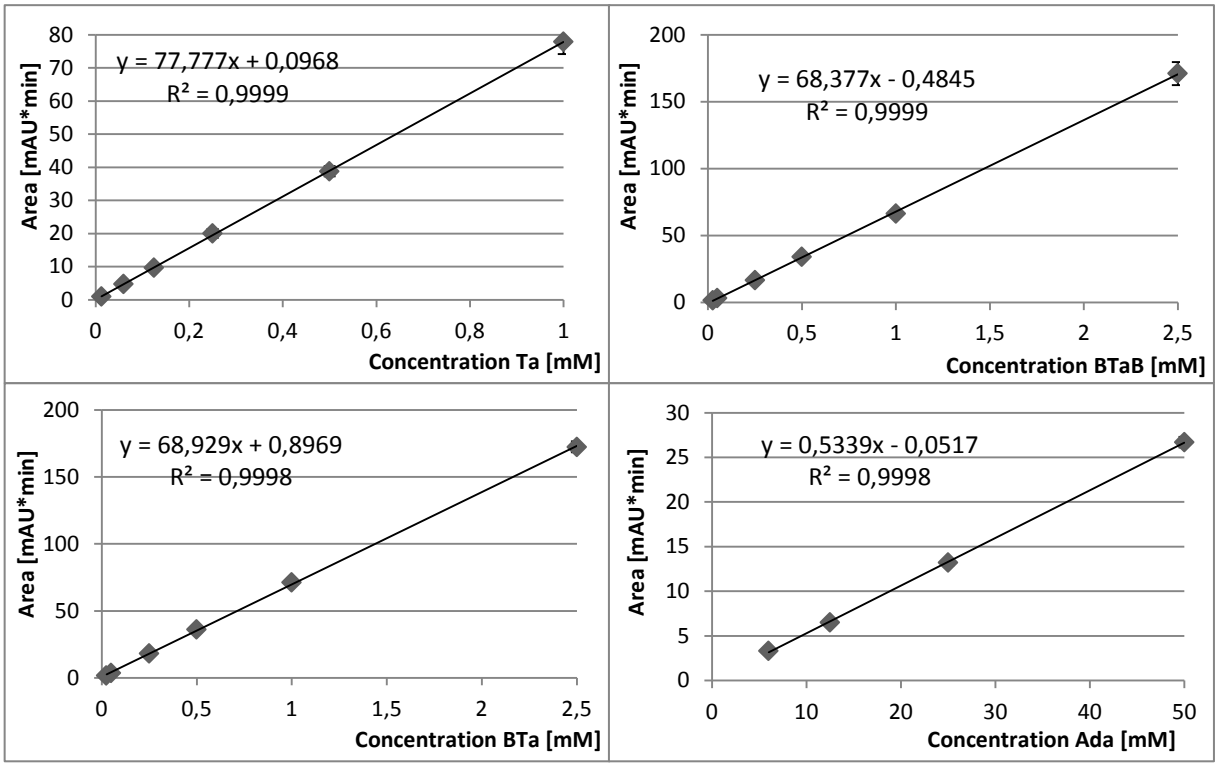
App.Table 3: Gradient settings for the HPLC analysis.

Parameter				
Temperature	Nominal	Lower limit	Upper limit	
	30.0 °C	20.0 °C	30.0	
Wavelengths	Channel 1	Channel 2	Channel 3	Channel 4
	241 nm	228 nm	254 nm	210 nm
Pump Pressure	Max Flow Ramp up	Lower limit	Upper limit	
	1.000 ml / min²	0 bar	300 bar	
Needle Wash	Wash volume	Wash speed		
	100.000 µl	20.000 µl / s		
Time	Pump % ddH2O	Pump % Acetonitrile	Pump % 0.1% formic acid	Pump Flow
0.000	72.0	8.0	20.0	0.500 ml/min
4.500	72.0	8.0	20.0	
5.000	60.0	20.0	20.0	
13.000	50.0	30.0	20.0	
17.000	30.0	50.0	20.0	
17.500	10.0	70.0	20.0	
18.000	10.0	70.0	20.0	
19.000	72.0	8.0	20.0	
25.000	72.0	8.0	20.0	

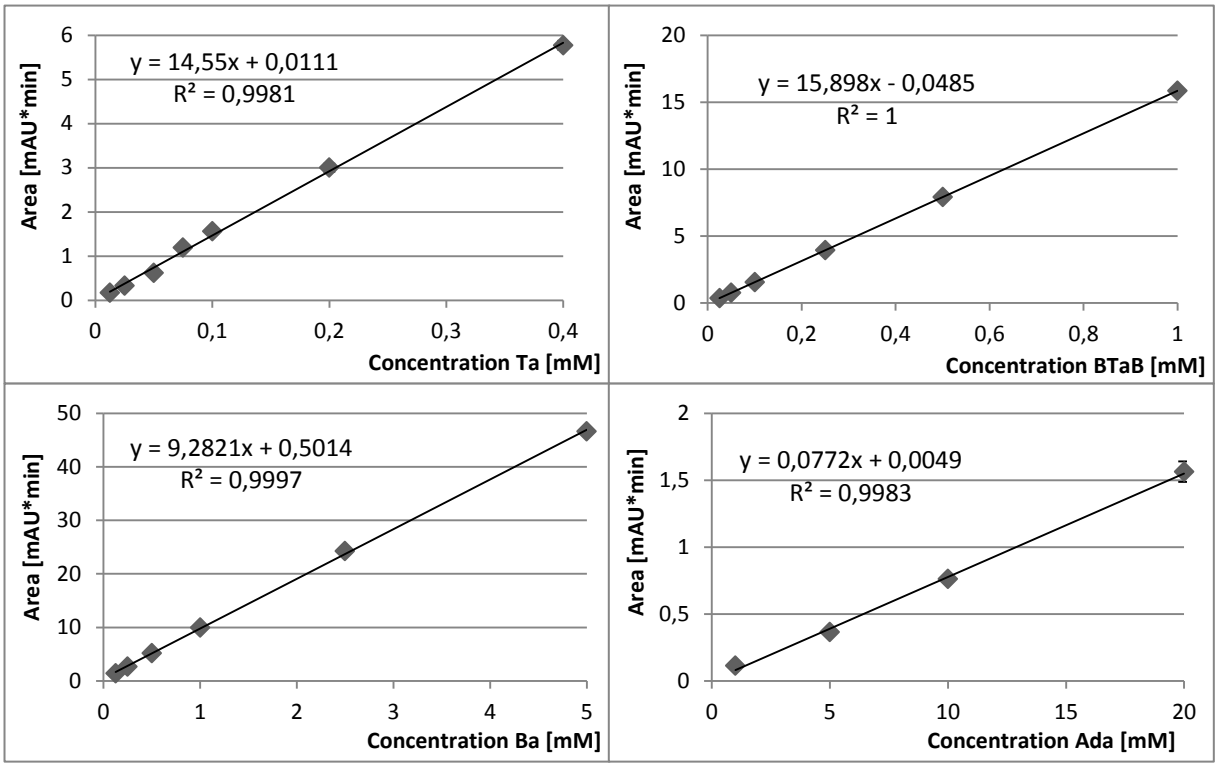
App.Table 4: Final settings for FDL activity assay in sludge for the spectrophotometer (Infinite 200, Tecan)

Plate	**Greiner 96 Flat bottom Transparent Polystyrol**	
Wait(Plate)	On	
Target Temperature	37	°C
Kinetic		
Shaking (Orbital) Duration:	20	s
Shaking (Orbital) Amplitude:	5	mm
Fluorescence		
Kinetic Measurement		
Kinetic Cycles	90	
Interval Time	00:01:00	
Mode	Fluorescence Top Reading	
Excitation Wavelength	485	nm
Emission Wavelength	520	nm
Excitation Bandwidth	9	nm
Emission Bandwidth	20	nm
Gain	80	Manual
Number of Reads	5	
Integration Time	20	µs
Lag Time	0	µs
Settle Time	0	ms

6.3 HPLC Standards

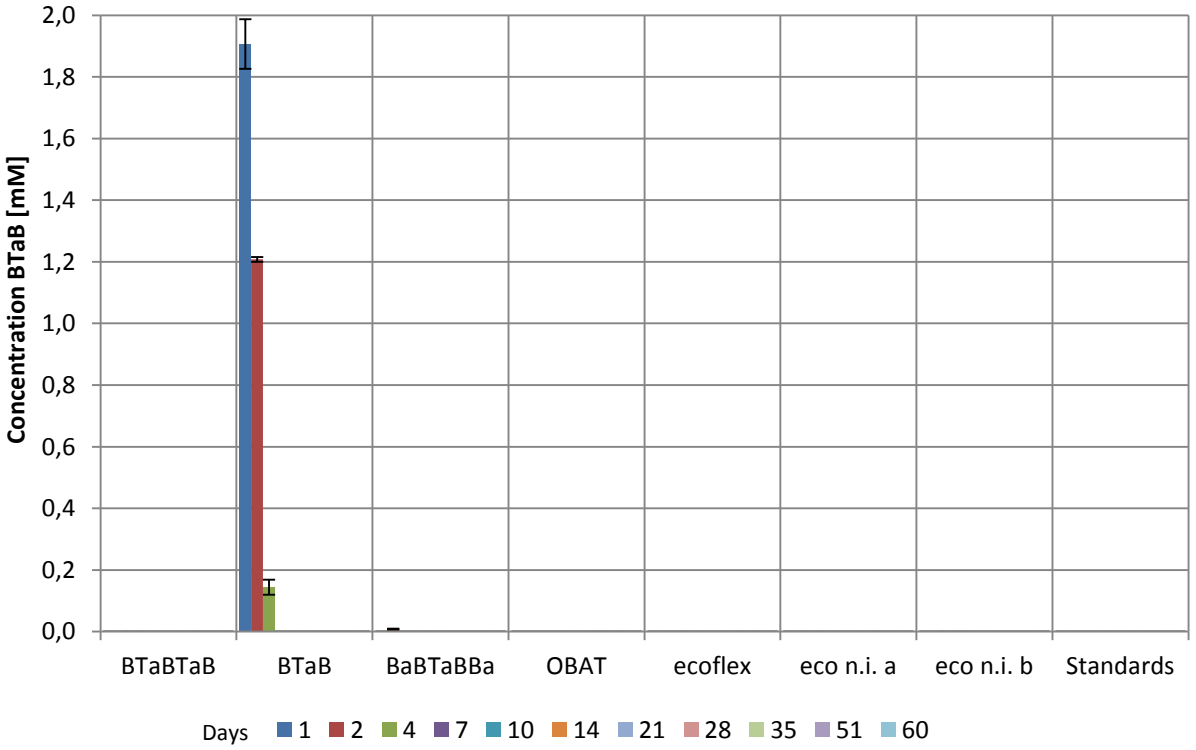


App.Figure 1: Ta, BTaB, BTa and Ada standards for quantification of degradation products created by enzymatic hydrolysis of AAC. The standards were dissolved in potassium phosphate buffer (0.1 M, pH 7.0) and a methanol precipitation was performed.

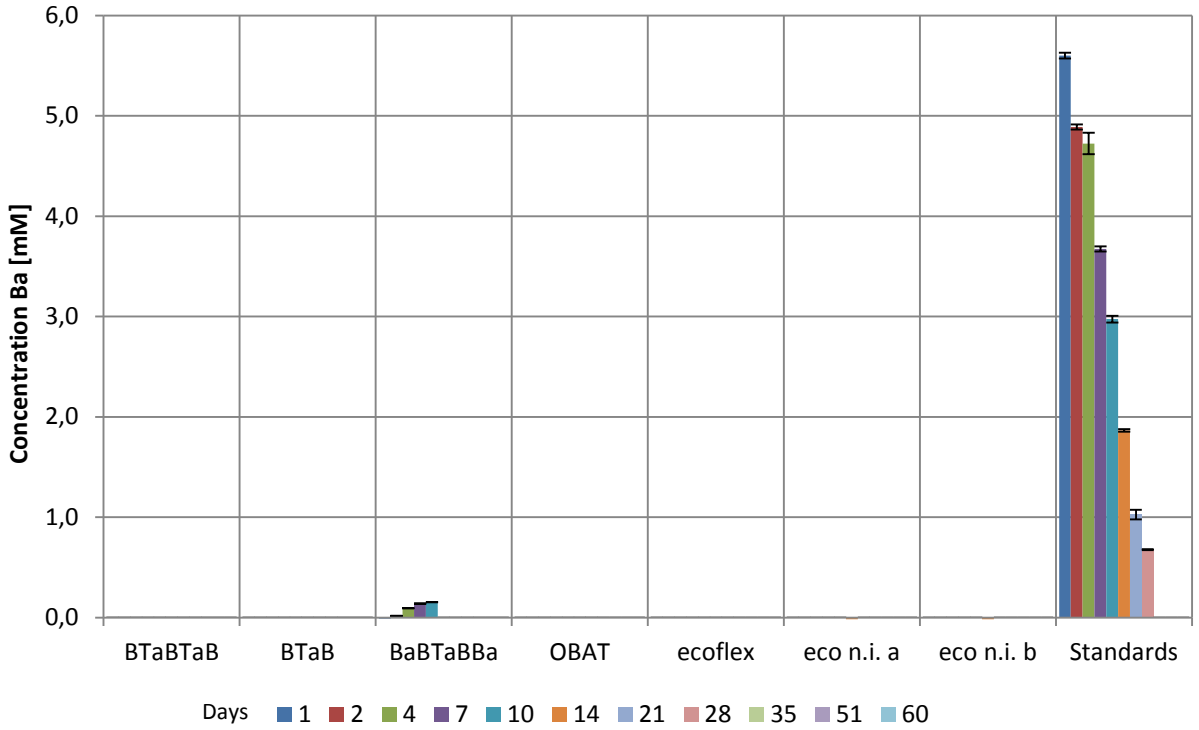


App.Figure 2: Ta, BTaB, Ba and Ada standards for quantification of degradation intermediates in the sludge experiment. The standards were dissolved in potassium phosphate buffer (0.1 M, pH 7.0) and a Carrez clarification was performed.

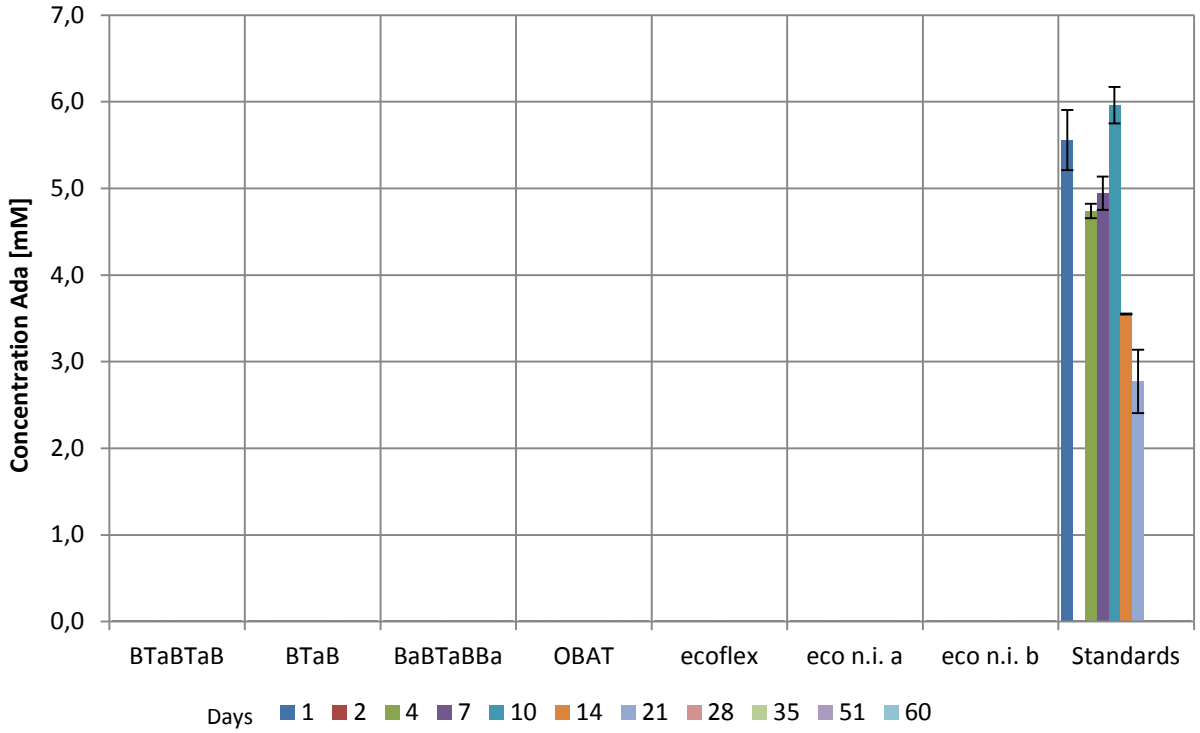
6.4 HPLC quantification of BTaB, Ba, Ada and BTa in the sludges



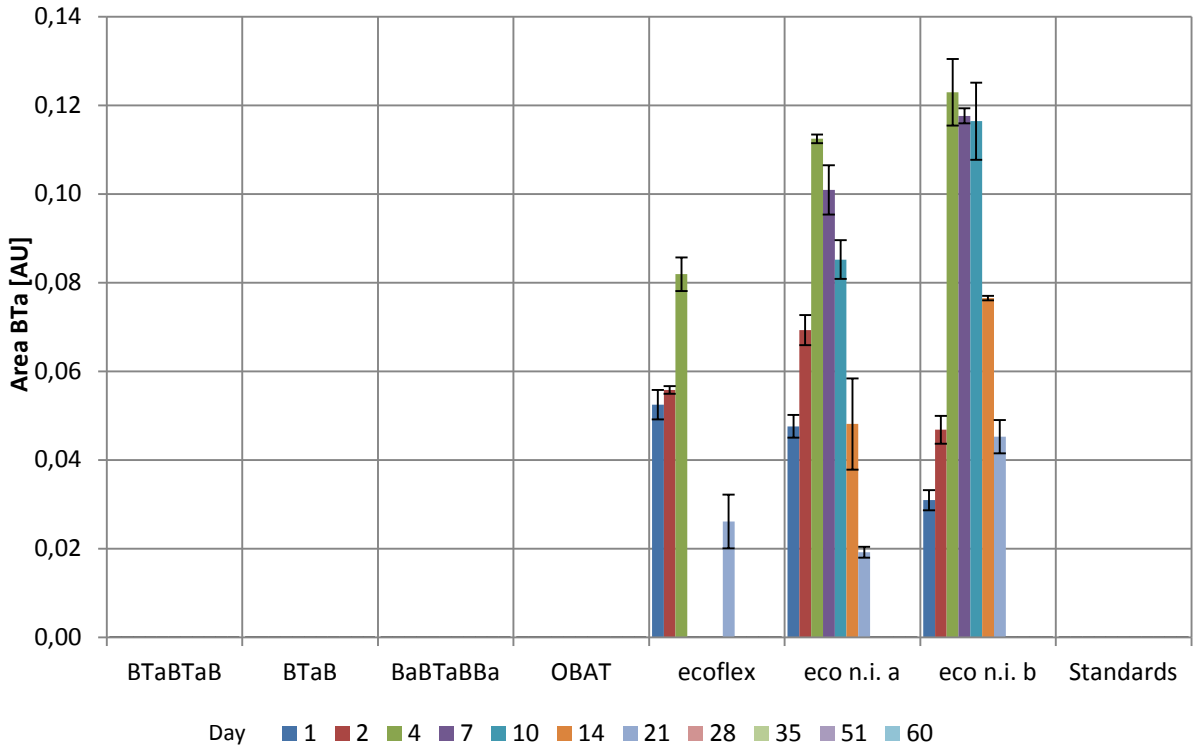
App.Figure 3: BTaB concentrations in the sludges measured by HPLC.



App.Figure 4: Ba concentrations in the sludges measured by HPLC. The Standards batch had an initial Ba concentration of 6.4 mM.



App.Figure 5: Ada concentrations in the sludges measured by HPLC. The Standards batch had an initial Ada concentration of 6.6 mM.



App.Figure 6: BTaB concentrations in the sludges measured by HPLC.

7 Equipment

Tool	Name	Manufacturer
Analytical Balance	2004 MP 6E	Sartorius,Göttingen / GER
Plate Reader	Infinite 200	Tecan Austria, Grödig / AUT
Spectrophotometer	Nanodrop 2002c	Thermo Scientific
Photometer	U – 2001	Hitachi Instruments
Gel Imaging	Universal Hood III	Bio Rad
Centrifuge	Biofuge Primo	Heraeus
Centrifuge	Minispin	Eppendorf, Hamburg / GER
Ultrasonic processor	Vibra Cell	*Sonics and Materials*
Purifier	AEKTA purifier	Amersham Pharmacia Biotech
Vortex	VF2	Janke & Kunkel IKA, Staufen / GER
Thermomixer	Thermomixer comfort	Eppendorf, Hamburg / GER
Centrifuge	Herme Z300K	MIDSCI, Missouri
Rotation Mixer	WiseMix RT 10	Wisd Laboratory Instruments Czech Republic
HPLC Components:		
Photodiode Array Detector	UVD 340 U	Dionex, Germering / GER
Solvent Rack	SR 3000	Dionex, Germering / GER
Quaternary Analytical Pump	LPG 3400 SD	Dionex, Germering / GER
Autosampler	ASI 100 T	Dionex, Germering / GER
Column Compartment	TCC 3000 SD	Dionex, Germering / GER
Column	XTerra RP 18 3.5µm	Waters

8 Abbreviations

°C	degree celsius
µm	micrometer
µM	micromole
AA	acrylamid
AAC	aliphatic – aromatic copolyesters
abs	absorbance
ACIB	Austrian Centre of Industrial Biotechnology
Ada	adipic acid
B	1,4 butanediol
Ba	benzoic acid
BCA	bicinchoninic acid
C	carbon
$CaCl_2*2H_2O$	calcium chloride dihydrate
CB_EstA	esterase A of *Clostridium botulinum*
CH_4	methane
CLSM	confocal laser scanning microscopy
CO_2	carbon dioxide
$CoCl_2*6H_2O$	cobalt(II) chloride hexahydrate
Cu	copper
$CuCl_2*2H_2O$	copper(II) chloride dihydrate
ddH_2O	bidest. water
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
FDA	fluorescein diacetate
FDL	fluorescein dilaurate
$FeCl_2*4H_2O$	iron(II) chloride tetrahydrate
g/mol	gram per mole
h	hour
HCl	hydrochloric acid
HeLa cells	cell line derived from cancer patient Henrietta Lacks
HiC	Cutinase 1 of *Humicola insolens*
HPLC	high pressure liquid chromatography
ISO	international organisation for standardization
K2HPO4	dipotassium phosphate
$K_4[Fe(CN)_6]$	potassium hexacyanoferrate(II) trihydrate
kcat	turnover number
KH_2PO_4	monopotassium phosphate
M	mol per liter
mg	milligram
$MgCl_2*6H_2O$	magnesium chloride hexahydrate
ml	milliliter
min	minute

Abbreviations

mM	millimole per liter
mmol	millimole
M_n	number-average
$MnCl_2*4H_2O$	manganese(II) chloride tetrahydrate
N	nitrogen
Na_2HPO_4	disodium phosphate
$Na_2HPO_4*12H_2O$	disodium hydrogen phosphate dodecahydrate
Na_2S*1H_2O	sodium sulfide hydrate
$Na_2SeO_3*5H_2O$	sodium selenite pentahydrate
NaH_2PO_4	monosodium phosphate
$NaMoO_4*2H_2O$	sodium molybdate dihydrate
NaN_3	sodium azide
NH_4Cl	ammonium chloride
$NiCl_2*6H_2O$	nickel(II) chloride hexahydrate
nm	nanometer
OBAT	oligobutyrate-adipate-terephthalate
PAGE	polyacrylamid gel electrophoresis
PBAda	poly(butylene adipate)
PBAT	poly(butylene adipate-co-terephthalate)
PBS	poly (butylene succinate)
PCR	polymerase chain reaction
PET	polyethylenterephthalate
PHA	polyhydroxyalkanoates
PHBH	type of medium chain length PHA
PLA	poly(lactic acid)
pNPB	para-nitrophenylbutyrate
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
sec	second
SOP	standard operating procedure
SSCP	single-strand conformation polymorphism
Ta	terephthalic acid
ThC_Cut1	cutinase 1 of *Thermobifida cellulosilytica*
T_M	melting point
tris	Tris(hydroxymethyl)aminomethane
TSB	tryptic soy broth
U	the conversion of 1 µmol substrate per minute
U/mg	specific activity
w/V	weight per volume
$ZnCl_2$	zinc chloride
$ZnSO_4$	zinc Sulfate
ΔE	change of absorbance per minute
ΔT_{MT}	difference of reaction temperature to melting point

9 Chemicals

40 % acrylamide, bis	Sigma Aldrich
acetone	Carl Roth GmbH
acetonitrile	Sigma Aldrich
aluminium sulfate 18 hydrate	Merck Millipore
ammonium chloride	Sigma Aldrich
ammonium persulfate	Sigma Aldrich
bicinchoninic acid	EMD Millipore chemicals
bromophenol blue	Sigma Aldrich
calcium chloride dihydrate	Sigma Aldrich
cobalt(II) chloride hexahydrate	Sigma Aldrich
Coomassie blue	Bio-Rad
copper(II) chloride dihydrate	Sigma Aldrich
dimethyl sulfoxide	Sigma Aldrich
dipotassium phosphate	Carl Roth GmbH
disodium phosphate	Carl Roth GmbH
ethanol (96%)	Carl Roth GmbH
fluorescein diacetate	Sigma Aldrich
fluorescein dilaurate	Sigma Aldrich
fluorescein sodium salt	Sigma Aldrich
formic acid	Sigma Aldrich
glycerol	Sigma Aldrich
glycine	Sigma Aldrich
hydrochloric acid	Carl Roth GmbH
iron(II) chloride tetrahydrate	Sigma Aldrich
manganese(II) chloride tetrahydrate	Sigma Aldrich
magnesium chloride hexahydrate	Sigma Aldrich
methanol	Carl Roth GmbH
monopotassium phosphate	Carl Roth GmbH
monosodium phosphate	Carl Roth GmbH
nickel(II) chloride hexahydrate	Sigma Aldrich
ortho-phosphoric acid (100 %)	Sigma Aldrich
potassium hexacyanoferrate(II) trihydrate	Sigma Aldrich
sodium azide	Sigma Aldrich
sodium dihydrogen phosphate dodecahydrate	Sigma Aldrich
sodium dodecyl sulfate	Sigma Aldrich
sodium molybdate dihydrate	Sigma Aldrich
sodium selenite pentahydrate	Sigma Aldrich
sodium sulfide hydrate	Sigma Aldrich
tetramethylethylendiamin	Sigma Aldrich
Tris(hydroxymethyl)aminomethane	Sigma Aldrich
Triton X	Sigma Aldrich
tryptic soy broth	Sigma Aldrich
zinc chloride	Sigma Aldrich
zinc sulfate	Sigma Aldrich

10 Acknowledgements

I want to dedicate this thesis to my parents Waltraud and Günter Bleymaier who have supported me all of my life in all ways imaginable and never lost patience or hope. I am eternally grateful to both of you for your love and the shining example you give for how to live a happy life.

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