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Assessing the effects of non-canonical amino acid incorporations on the polymer degradation capability of polyesterases

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ABSTRACT

It has been shown in previous studies that extracellular esterases derived from anaerobic microorganisms including *Clostridium species*, have the ability to act on complex synthetic polymers. In the natural environment, extracellular enzymes break down polymeric substrates to gather nutrients, which can then be taken up by the cell. Among others, anaerobes are commonly found in biogas plants, where they participate in the conversion of various biodegradable materials to methane. Combining polymer degradation on the one hand and anaerobic biogas production on the other hand, new possibilities for synthetic polymer recycling and disposal would open up. In contrast to the numerous studies about the aerobic biodegradation of plastics, only a few data are available for the anaerobic process. The present study describes the engineering of a potential polymer degrading enzyme from the anaerobic microorganism *Clostridium species* with the tools of synthetic biology and the subsequent assessment of the polymer degradation capability of the generated enzyme variants. Analysis of the crystal structure of the target protein identified methionine (Met) and tryptophan (Trp) as suitable targets for incorporation studies. Hereby, the natural substrate tolerance of the diverse processes involved in uptake, metabolism and translation of amino acids is exploited to introduce non-canonical amino acids (ncAAs) of various sizes, acidities, hydrophobicities and other (chemical) features into the target protein in a residue-specific manner. Expression protocols were optimized and adapted and an *in-vivo* synthesis and incorporation of Trp and Trp analogues with the tryptophan synthase (TrpS) from *Salmonella typhimurium* was performed. The expression of the esterases in the presence of the ncAAs was successfully confirmed by SDS-PAGE analysis; the target proteins were expressed in varying amounts depending on the ncAA. Kinetic characterization on the small, soluble substrate *para*-nitrophenyl butyrate (pNPB) was performed for the parent protein, the norleucine (Nle) and 1-methyltryptophan (1MeW) variants. In all cases it could be demonstrated that functional proteins were expressed under the chosen conditions. Subsequently the hydrolytic activity towards an aliphatic-aromatic copolyester was tested. All three enzymes exhibited degradation activity in varying amounts accompanied by a shift in the degradation patterns for the variant proteins. The degradation mechanism was subsequently studied in more detail on an aliphatic-aromatic model substrate. Exo-wise and endo-wise cleavage mechanisms were postulated and compared to the degradation patterns gained from the experiments. The results showed that rather a tendency towards exo- or endo-wise cleavage than a strict differentiation can be deduced from the degradation patterns. Analysis of analogue incorporation by MALDI-MS revealed that successful incorporation of Nle into the target protein can be assumed. For the proteins, which were expressed in the presence of the Trp analogue 1MeW, MALDI-MS revealed that the protein preparation may have been a mixture of non - and partially labelled protein.

ZUSAMMENFASSUNG

Mikroorganismen nutzen extrazelluläre Enzyme um polymere Substrate in ihrer Umgebung zu zerlegen, aufzunehmen und zu metabolisieren. Die Fähigkeit von extrazellulären Esterasen aus anaeroben Mikroorganismen wie *Clostridium species,* komplexe, synthetische Polymere abzubauen wurde bereits in vorangegangenen Studien gezeigt. Anaerobe Mikroorganismen sind unter anderem in Biogasanlagen zu finden, wo sie an der Umwandlung von biologisch abbaubaren Materialien zu Biogas teilnehmen. Durch die Kombination von Polymerabbau auf der einen- und anaerober Biogasproduktion auf der anderen Seite, eröffnen sich neue Möglichkeiten für die Verwertung von synthetischen Polymerabfällen. Im Gegensatz zu den zahlreichen Studien über die biologische Abbaubarkeit von Kunststoffen unter aeroben Bedingungen, sind nur wenige Daten zum anaeroben Prozess verfügbar. In der vorliegenden Studie wurden Methoden der Synthetischen Biologie eingesetzt um ein potentiell Polymer-abbauendes Enzym aus dem anaeroben Mikroorganismus *Clostridium sp.* zu verändern. Die generierten Varianten wurden anschließend auf ihre Aktivität gegenüber polymeren Substraten getestet. Im Zuge der Analyse der Kristallstruktur des Enzyms wurden die Aminosäuren (AS) Methionin (Met) und Tryptophan (Trp) als attraktive Angriffspunkte für Einbaustudien identifiziert. Hierbei wird die natürliche Substrattoleranz der verschiedenen Prozesse die an der Aufnahme und Translation von AS beteiligt sind, ausgenutzt um nicht-kanonische Aminosäuren (nkAS) verschiedenster chemischer Eigenschaften zielgerichtet in das Protein einzubringen. Expressionsprotokolle wurden optimiert und angepasst und eine *in-vivo* Synthese mit anschließendem Einbau von Trp und Trp Analoga mithilfe der Trp Synthase (TrpS) aus *Salmonella typhimurium* durchgeführt. Die Expression der Esterasen in Anwesenheit der nkAS wurde erfolgreich durch SDS-PAGE Analyse bestätigt. Die kinetische Charakterisierung auf dem kleinen, löslichen Substrat *para*-Nitrophenylbutyrat (*p*NPB) wurde für das kanonische Enzym als auch für die Norleucin (Nle) und die 1-Methyltryptophan (1MeW) Variante durchgeführt, wobei in allen Fällen Enzymaktivität nachgewiesen werden konnte. Anschließend wurde die hydrolytische Aktivität gegenüber einem aliphatisch-aromatischen Copolyester getestet. In allen Fällen konnte Abbauaktivität in unterschiedlichem Ausmaß nachgewiesen werden; weiters konnte eine Verschiebung der Abbaumuster bei den Varianten beobachtet werden. Der Abbaumechanismus wurde näher auf einem aliphatisch-aromatischen Modellsubstrat untersucht. Beim Vergleich mit den postulierten Exo-und Endo-Abbauwegen ergab sich, dass mehr eine Tendenz hinsichtlich Exo- oder Endoabbau als eine klare Trennung aus den Abbauprodukten erkennbar war. Die Analyse der Einbaueffizienz mittels MALDI-MS ergab, dass von einem erfolgreichen Einbau von Nle in das Zielprotein auszugehen ist. Für jene Proteine, die in Anwesenheit des Trp Analogons 1MeW exprimiert wurden, konnte kein erfolgreicher Einbau nachgewiesen werden.

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1. INTRODUCTION

1.1. Synthetic biodegradable polymers

Facing the diverse problems related with generation, handling, treatment and disposal of plastic waste, biopolymers became more and more important over the last decades. Going through the literature, the terms used in this context seem to be as diverse as the application of those materials. The term "biopolymers" can be seen as an umbrella term. It encompasses naturally occurring polymers like starch and bio-based polymers which are made from renewable resources. Furthermore it also includes biodegradable polymers that can be metabolized by microorganisms. Bio-based does not necessarily mean that the polymer is biodegradable, while biodegradable does not imply that the polymer is a naturally occurring polymer or derived from renewable resources. To sum up it can be stated that the biodegradability of a polymeric material is not defined by its origin but by its chemical structure (Siegenthaler et al., 2012, Witt et al., 1999).

Taking this into account it seems obvious that biodegradable polymers must share some common features which make them susceptible for metabolization by microorganisms like bacteria, fungi or yeast. So what are the principal prerequisites, which an organic molecule has to fulfil in order to be taken up and metabolized by microbial cells? First and foremost the molecules have to be water-soluble and small enough to be transported through the cell membranes. Usually polymers don't fulfil either one of those requirements. This is where extracellular enzymes come into play (Figure 1).

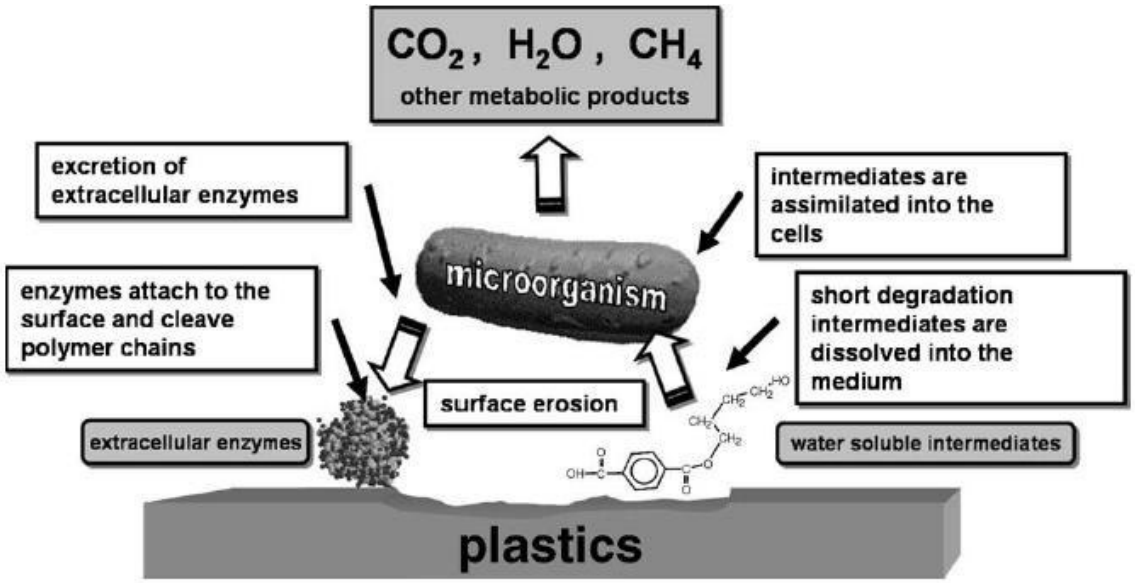


Figure 1: General mechanisms of enzyme catalysed polymer degradation.
Microorganisms excrete extracellular enzymes, which attach to the surface of the polymeric substrate and cleave the polymer chains. Water soluble degradation intermediates are released and dissolved in the medium. Afterwards the intermediates are assimilated into the cells where they are metabolized to CO_2, H_2O, CH_4 and other metabolic products. (Reproduced from Mueller, 2006: Figure 1)

Some microorganisms excrete extracellular enzymes to gather nutrients in their surroundings. The enzymes break down complex macromolecules into smaller fragments, which can then be taken up by the cells. Compared to the quite rapid mechanisms of enzyme excretion and uptake of degradation products into the cells, the enzymatic polymer degradation seems to be the rate-limiting step. First the enzymes must adsorb or somehow get access to the polymer. Secondly functional sites or chemical bonds which are susceptible for enzymatic attack must be available on the surface of the polymeric substrate, since enzymes are too large for penetrating deeper into the polymeric material (Mueller, 2006, Siegenthaler et al., 2012). In this context it early turned out that aliphatic polyesters like the natural polyester polyhydroxybutyrate (PHB) or the synthetic polyester poly(ε-caprolactone) (PCL) are quite suitable and their biodegradability was studied extensively (Tokiwa and Suzuki, 1978, Yakabe et al., 1992, Walter et al., 1995). However, the application areas of those polymers are quite restricted. For example PCL exhibits a very low melting point of about 60 °C. Besides, the relatively high price is another serious disadvantage (Müller, 2005). In contrast to aliphatic polyesters, aromatic polyesters like polyethylene terephthalate (PET) show excellent mechanical and chemical properties such as transparency, lightweight, low-cost, durability and resistance to carbon dioxide permeation (Andrady and Neal, 2009).

Combining the potential biodegradability of aliphatic polyesters on the one hand and the excellent material properties of aromatic polyesters on the other hand, aliphatic-aromatic copolyesters were developed (Deckwer et al., 1995). In this way structures susceptible to enzymatic attack are implanted into the resistant structures of aromatic polyesters. In 1995, Witt and co-workers described the synthesis of aliphatic-aromatic copolyesters by polycondensation of diols (1,2-ethanediol, 1,3-propanediol, 1,4-butanediol) and dicarboxylic acids (sebacic acid, adipic acid, terephthalic acid). Four types of copolyesters with varying monomer compositions were synthesized. In all cases it turned out that the degradation behaviour was primarily determined by the amount of terephthalic acid. The type of dicarboxylic acid seemed to be of minor importance. In subsequent studies, the potential of BTA-copolyesters consisting of 1,4-butanediol, terephthalic acid and adipic acid was identified. Besides their biodegradability and their promising material properties, the relatively easy and cheap synthesis from commodity chemicals represents a big advantage (Witt et al., 1997).

In 1998, BASF launched the biodegradable but completely fossil-based copolyester, ecoflex®. ecoflex® is a poly(butylene adipate co-terephthalate) (PBAT) synthesized from terephthalic acid, adipic acid and 1,4-butanediol (Figure 2). The statistical copolyester usually consists of 42-45 mol% terephthalic acid (Siegenthaler et al., 2012, Müller, 2005). For the present study a material containing

47 mol% terephthalic acid was used. Nevertheless, this lies within the range of 40 mol% to 50 mol%, which was found to be ideal for the terephthalic acid content (Witt et al., 1997).

ecoflex® is a thermoplastic. This type of plastic material can be processed above a specific temperature and solidifies upon cooling. The process can be repeated any number of times. ecoflex® exhibits thermal stability up to processing temperatures of 230 °C, compared to natural polymers like cellulose or starch which become instable above 170-180 °C. Another advantage is that the material can be melt-processed on standard polyolefin equipment, like the extruder. In this way, flexible, tear-resistant biodegradable polymeric material, with mechanical properties similar to PE can be produced. ecoflex® is primarily used in those applications, where biodegradability is a huge plus: biowaste bags, films for agriculture, coating for compostable paper cups. The films are breathable due to their moderate water vapour permeability, which is beneficial for packaging of food, especially vegetables.

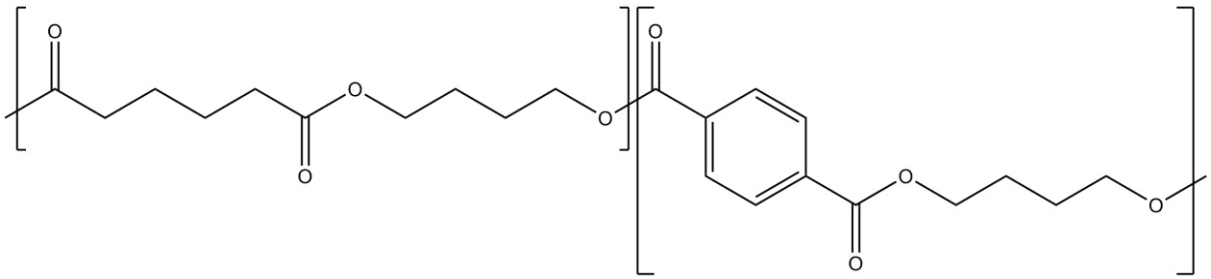


Figure 2: Chemical structure of ecoflex®.
(Reproduced from Siegenthaler et al., 2012: Figure 3)

The aerobic biodegradability of ecoflex®, was extensively studied and described in the literature. Not least because the material has to meet the requirements of various international standards and regulations (e.g. ISO 14855, ISO 17088, European standard EN 13432, American Standard ASTM 6400, Japanese standard GreenPla) before it can be launched on the market. In this context, one central test is the "controlled composting test". According to this test, 90% conversion to CO_2 must be achieved within 180 days. Therefore, the polymer to be tested is incubated with defined, mature compost in a composting vessel. To make sure that the process runs under optimum conditions, humidity, temperature and aeration with CO_2-free air are controlled. The biodegradation process is monitored by detecting the released CO_2 in the exhaust air. In parallel, the compost is incubated once without the polymer and once with cellulose instead of the polymer. In this way the amount of CO_2 related to the degradation of the polymer can be calculated and the degradation behaviour can be compared to the cellulose reference. The course of the degradation curve gained from the controlled composting test for ecoflex® is depicted in Figure 3. The criterion according to which 90% conversion to CO_2 must be achieved within 180 days, was already reached after 80 days. According to

that, ecoflex® is completely biodegradable under aerobic composting conditions (Siegenthaler et al., 2012, Müller, 2005).

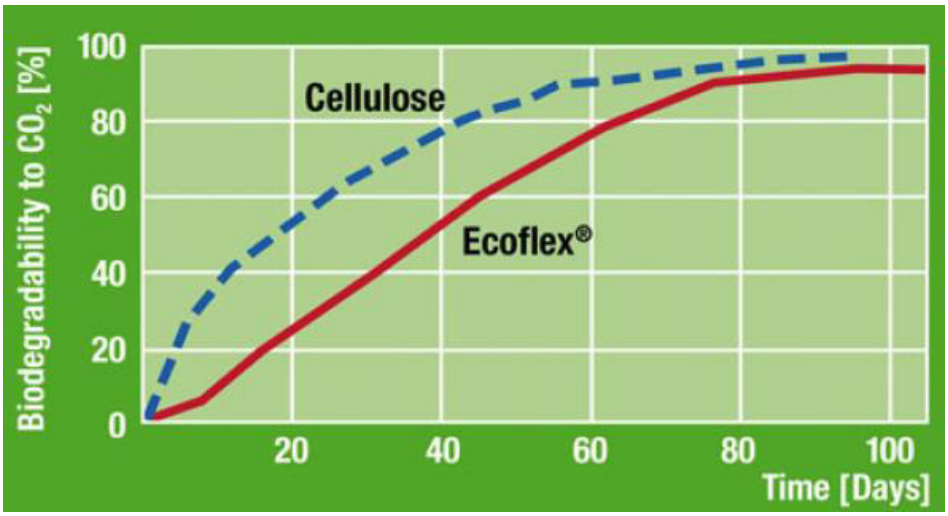


Figure 3: Controlled composting test of ecoflex®.
The biodegradability to CO_2 [%] is plotted over time [days] for ecoflex® and cellulose. The biodegradability to CO_2 [%] represents the actual CO_2 evolution attributed to the test polymer (ecoflex®/ cellulose) compared to the theoretical CO_2 evolution. In case of ecoflex® 90% of the theoretical CO_2 evolution is reached after 80 days. For cellulose, this value is already reached after 60 days (Reproduced from Siegenthaler et al., 2012: Figure 1).

In contrast to the numerous studies about the aerobic biodegradation of plastics, only a few data are available for the anaerobic process. Especially for biogas plants, biodegradability of plastics under anaerobic conditions would be beneficial. Combining polymer degradation on the one hand and anaerobic biogas production on the other hand, new possibilities for synthetic polymer recycling and disposal would open up. For the production of biogas, organic matter like manure, agricultural waste, municipal waste or sewage is incubated under anaerobic conditions. Specialized, anaerobic microorganisms assimilate and convert the organic matter to methane and carbon dioxide.

The biodegradability of aliphatic polyesters like the natural polyester polyhydroxybutyrate (PHB) or the synthetic polyester poly(ε-caprolactone) (PCL) under anaerobic conditions has been described in the literature (Abou-Zeid et al., 2001). Aliphatic-aromatic copolyesters turned out to be quite stable under anaerobic conditions. Within a test period of several months, no significant biodegradation of a BTA-copolyester with a terephthalic acid content of 40-45 mol% (like ecoflex®) could be observed. It seemed that the microorganisms were not able to degrade aliphatic-aromatic copolyesters in the absence of oxygen (Abou-Zeid, 2000).

Rather than optimizing the polymer in respect to its degradability under anaerobic conditions, recent efforts focussed on the second part which defines biodegradability: the microorganisms and especially their extracellular enzymes.

Using various approaches, the genomes of anaerobic microorganisms were analysed for extracellular or membrane bound proteins, belonging to the classes of lipases, cutinases, polymerases and esterases. Eight enzymes belonging to the esterase/lipase superfamily were identified in the organisms *Clostridium botulinum* and *Clostridium hathewayi*. The enzymes were successfully cloned, expressed and characterized. The two enzymes Est5 from *Clostridium hathewayi* and Est8 from *Clostridium botulinum* were identified as potential candidates for further research and optimization. Also promising tests were performed on various polymeric substrates (Baumschlager, 2013a & b). With those enzymes identified as promising targets, protein engineering seemed to be one of the next logical steps (Schöffmann, 2013).

1.2. Protein engineering using non-canonical amino acids

To meet the needs of industrial or medical processes, enzymes must fulfil a set of requirements such as stability under process conditions, high catalytic activity and binding specificity. Apparently, it is often necessary to improve wild-type enzymes using the tools of rational design or directed evolution (Bornscheuer and Pohl, 2001). Either way, those classical protein design and engineering approaches are limited to the 20 canonical amino acids (cAAs) as prescribed by the universal genetic code. The incorporation of non-canonical amino acids (ncAAs) offers the possibility to broaden the set of possible modifications by incorporating amino acid analogues of various sizes, acidities, nucleophilicities, hydrophobicities and other (chemical) features (Wang and Schultz, 2002).

NcAAs are as diverse as one could imagine, having in common that they are not encoded by the genetic code. Based on the similarity to their canonical counterparts, ncAAs are qualified for either one of the two basic *in vivo* approaches for genetic code engineering. The first approach exploits the natural substrate tolerance in the diverse processes involved in amino acid uptake, metabolism and translation – especially the substrate tolerance of the aminoacyl-tRNA synthetases (aaRSs). AaRSs play a crucial role in the translational process. During aminoacylation, the aaRS recognizes the tRNA and the corresponding cAA and joins them covalently (Figure 4a). Structurally and/or chemically similar ncAAs are recognized due to the substrate tolerance or “catalytic promiscuity” of the aaRS which results in incorrectly charged tRNAs (Figure 4b). Consequently, the ncAA is introduced into the protein (Hoesl and Budisa, 2012). This method only works properly, if the corresponding cAA is not available, which would obviously be incorporated more efficiently than the analogue. Therefore strains which are auxotrophic for a particular amino acid are used to prevent canonical aminoacylation in the presence of the non-canonical analogue.

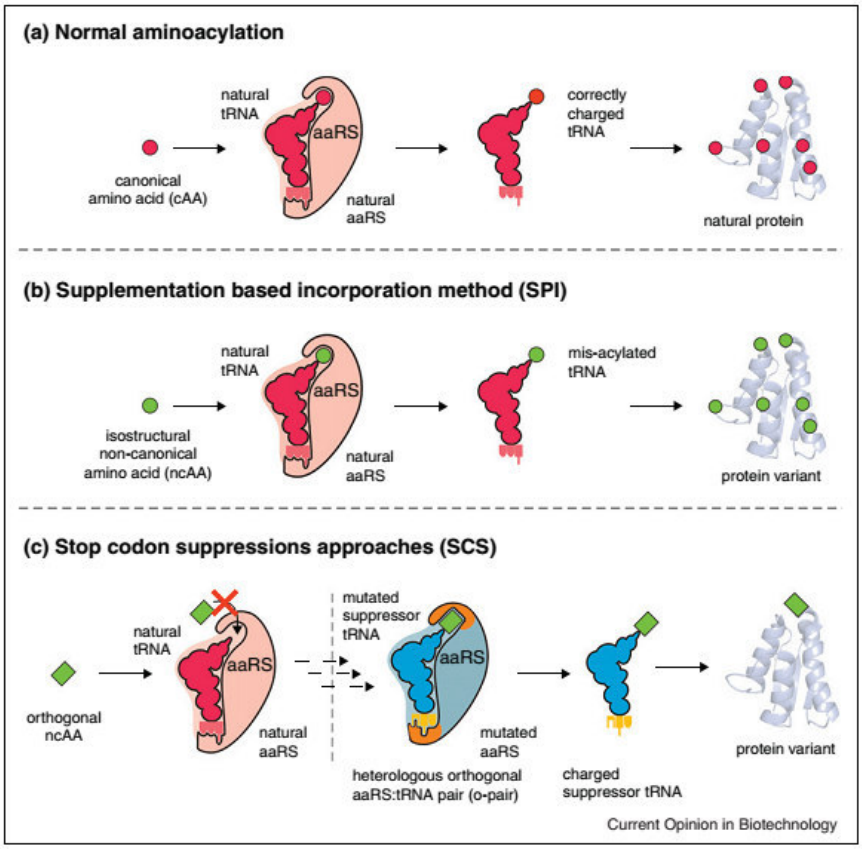


Figure 4: *In vivo* incorporation strategies for non-canonical amino acids.
(a) Normal aminoacylation. In case of canonical aminoacylation, the aminoacyl-tRNA synthetase (aaRS) recognizes the corresponding tRNA as well as the canonical amino acid (cAA) and joins them covalently, resulting in a correctly charged tRNA. (b) Supplementation based incorporation method (SPI). Structurally and/or chemically similar non-canonical amino acids (ncAAs) are recognized due to the intrinsic substrate tolerance of the aaRS and loaded onto the tRNA. A mis-acylated tRNA emerges, introducing the ncAA into the protein. (c) Stop codon suppression approaches (SCS). SCS use heterologous orthogonal aaRS/tRNA pairs (o-pairs) to incorporate an orthogonal ncAA in response to a stop or quadruplet codon. (Reproduced from Hoesl and Budisa, 2012: Figure 1).

Most of the amino acids outside the canonical pool are toxic to cells, or hamper cellular growth. To keep the toxic effects on the expression host at a minimum and incorporate the ncAA only into the target protein, the supplementation based incorporation approach (SPI) is applied. In the SPI approach the expression construct carrying the gene of interest under the control of an inducible promoter is introduced into an expression host that is auxotrophic for a certain amino acid. In the first phase of the incorporation, the host cells are grown to the desired density in minimal medium containing an appropriately limiting amount of the cAA. At the same time, the expression of the target gene is turned off in order to prevent its translation with the cAA. As soon as the cells are depleted of the cAA, which corresponds to a plateau in the growth curve, the ncAA is added and target protein expression is induced. In this stage, cellular growth almost comes to a complete stop. The host cells only serve as a factory, to express the desired modified protein. Commonly this expression phase is kept rather short, to circumvent the release of the corresponding cAA into the medium due to cell death and lysis (Wiltschi, 2012). The proteins, which originate from such expression experiments, are not mutants in a classical sense because they originate from the same

gene sequence, but contain a fraction of ncAA. Therefore they are called variants, alloproteins or congeners (Hoesl and Budisa 2011).

The second approach for genetic code engineering is suitable for non-cognate amino acids, which are chemically and/or structurally too different to be recognized by aaRSs. Suppressor tRNAs addressing one of the three termination codons UAG (amber), UGA (opal) and UAA (ochre) are chemically or enzymatically loaded with the ncAA and added to an *in-vitro* or *in-vivo* translation system. The stop codon acts no longer as stop codon but is suppressed by the misacylated tRNA, leading to the site-specific incorporation of the ncAA. Since only one stop codon is needed for efficient translation termination, the other two codons can be re-assigned to ncAAs (Wiltschi and Budisa, 2007). More efficient site specific incorporation methods make use of heterologous orthogonal aaRS/tRNA pairs, so called o-pairs. In this context, orthogonality describes the absence of cross-reactivity between the heterologous and the endogenous host aaRSs, amino acids and tRNAs. Heterologous aaRSs only aminoacylate heterologous tRNAs, which nonetheless function efficiently in host cell protein translation. Mostly the re-assigned coding units used for incorporation of such orthogonal amino acids are stop codons (stop codon suppression, SCS) or quadruplet codons (Figure 4c).

Both approaches for genetic code engineering the SPI, as well as the suppressor based methodology, have certain advantages and pitfalls. For the present study, the SPI approach was the method of choice, since residue-specific substitutions can be achieved by a relatively simple, reproducible methodology. Furthermore, high levels of protein expression, almost comparable to wild type proteins, are possible. This was especially of outstanding interest because sample-intensive activity measurements on diverse substrates were planned. Apart from that, the SPI approach is in general favourable, if labelled protein is needed for sample-intensive methods such as NMR-spectroscopy or X-ray crystallography. Biological functions of proteins, which are based on complex interactions of many amino acid residues, such as activity, stability or conformational orientation can be well studied by SPI. Contrarily, the SCS offers the possibility of site-specific incorporation of ncAAs into proteins. In this approach not one of the twenty cAAs is replaced, but a twenty-first one is added to the repertoire. Therefore it is a matter of expansion rather than supplementation (Hoesl and Budisa, 2012).

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial strains

Plasmid amplification was performed in *E. coli* TOP10F' (Life technologies, C665-11). The expression strain BWEC50 is a descendant of *E. coli* BL21-Gold(DE3) (Agilent technologies, Santa Clara, USA; #230132) carrying a genetic knockout of the methionine synthase, *metE*, which is responsible for the methionine auxotrophy of the strain. BWEC47 carries a genetic knockout of the N-(5-phosphoribosyl) anthranilate isomerase/ indole-3-glycerolphosphate synthase, *trpC*, which is responsible for the tryptophan auxotrophy of the strain. BWEC47 is also a descendant of *E. coli* BL21-Gold(DE3) (Agilent technologies, Santa Clara, USA; #230132) (Anderhuber, 2014). All bacterial strains used in this work, their genotype, application and the corresponding references are summarized in Table 1.

Table 1: Bacterial strains used in this work.

strain	genotype	description	reference
E. coli TOP10F'	F' {*lacI*^{q}*Tn*10(Tet^{R})} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	plasmid propagation	Life technologies, Carlsbad, USA
BWEC50	*E. coli* B F^{-} *omp*T *hsdS*(r_B^- m_B^-) dcm^{-} Tet^{r} gal λ(DE3) *endA* Hte *metE*	protein expression	(Anderhuber, 2014)
BWEC47	*E. coli* B F^{-} *omp*T *hsdS*(r_B^- m_B^-) dcm^{-} Tet^{r} gal λ(DE3) *endA* Hte *trpC*	protein expression	(Anderhuber, 2014)

2.1.2. Plasmids

The est8 gene from *Clostridium botulinum* was kindly provided by Armin Baumschlager (ACIB, Graz) cloned in the pET26b(+) vector (Novagen, Cat. No. 69862-3), carrying an N-terminal 6xHis-tag. The vector map of pET26b(+)_est8 is provided in the appendix in Figure 34. The pET26b(+) vector carries a T7*lac* promoter, which contains a 25 bp *lac* operator sequence immediately downstream from the 17 bp T7 promoter region. This provides a second *lacI*-based mechanism, besides the repression at the *lacUV5* in the *E. coli* genome. pET26b(+) vectors also carry a copy of *lacI,* ensuring that sufficient amounts of repressor are available. Furthermore, a high-copy-number ColE1 origin of replication and an aminoglycoside phosphotransferase gene (Kan^{R}), which confers resistance to kanamycin, are encoded on the plasmid. Finally, a f1 bacteriophage origin of replication is included in the pET26b(+) vector for mutagenesis and sequencing. For target protein expression in BWEC47 the est8 gene was subcloned into pQE-80L (Qiagen, Cat. No. 32943). pQE-80L_TT2_h6 was kindly provided by Patrik Fladischer (ACIB, Graz) and used as source of the pQE-80L vector backbone. pQE-80L carries a

bacteriophage T5 promoter for *E. coli* RNA polymerase, with an embedded *lac* operator element. A β-lactamase coding sequence confers resistance to ampicillin (Amp^R), while a ColE1 origin of replication confers a high copy number of the plasmid. The expression of the encoded *lacI* gene is controlled by the $lacI^q$ promoter, where a single base change in the promoter boosts expression of the *lacI* gene about 10-fold. Transcription terminator T1 from the *E. coli* rrnB gene is located downstream to the *lacI* gene, while the transcription terminator, t_0 from phage lambda is located downstream to the MCS. Furthermore, pQE-80L carries a promoterless CDS of the chloramphenicol acetyltransferase, which would confer resistance to chloramphenicol. Subcloning of the target gene est8 into pQE-80L was accomplished by Gibson assembly. The vector map of the assembled plasmid pQE-80L_est8 is provided in the appendix in Figure 35.

pSTWS1a was used for the inducible expression of the tryptophan synthase (TrpS) from *Salmonella typhimurium*. A vector map of pSTWS1a is provided in the appendix in Figure 36. The expression of the bicistronic gene *trpBA* is controlled by an IPTG inducible *trc* promoter. Transcription termination is accomplished by rrnB T1 and T2 transcriptional terminators. pSTWS1a also carries the lac-repressor gene, *lacI* and a Neo/Kan gene, which confers kanamycin resistance in bacteria and G418 resistance in mammalian cells. Replication in bacteria is facilitated by the p15A ori. For amplification and selection in yeast, the pSTWS1a also carries 2µ/URA3 sequences (Würstlin, 2010). pSTWS2 was used for the constitutive expression of the TrpS. In this construct, the trc promoter of pSTWS1a was exchanged by the high-level constitutive expression promoter, HCE. Furthermore the 2µ/URA3 sequences were removed, resulting in a significantly smaller plasmid (Hofmann, 2012). A vector map of the pSTWS2 construct is provided in the appendix in Figure 37. All plasmids used in this work, their most important features, application and the corresponding references are summarized in Table 2.

Table 2: Plasmids used and produced in this work.

plasmid	features	description	reference
pET26b(+)_est8	T7*lac* promoter, ColE1 ori, Kan^R, f1 ori, *lac*I	protein expression in BWEC50	(Baumschlager, 2013a, b)
pQE-80L_TT2_h6	PT5, *lac*O, ColE1 ori, Amp^R, $lacI^q$, T1 rrnB, t_0,	source of pQE-80L vector backbone	Patrik Fladischer
pQE-80L_est8	PT5, *lac*O, ColE1 ori, Amp^R, $lacI^q$, T1 rrnB, t_0,	protein expression in BWEC47	this work
pSTWS1a	*trpBA,* trc promoter, T1, T2 rrnB, *lac*I, Neo^R/ Kan^R, p15A ori, 2µ/URA3	inducible expression of TrpS	(Würstlin, 2010)
pSTWS2	*trpBA,* HCE promoter, T1, T2 rrnB, *lac*I, Neo^R/ Kan^R, p15A ori,	constitutive expression of TrpS	(Hofmann, 2012)

2.1.3. Primers

Table 3: Primers for subcloning of est8 into pQE-80L.
The colours highlight features like restriction sites (*Eco*RI (blue), *Nde*I (brown), *Hind*III (orange)), start codons (red), stop codons (red), ribosomal binding sites (RBS) (grey) and hexa-histidine-tags (His6) (green), introduced into the expression construct through the primers. The primers were designed with the software Oligo Explorer. The melting temperature, T_m of the priming sequences (underlined) was calculated with the nearest neighbor model (NN). 36- 39 nucleotides (nt) homology regions for the pQE-80L target vector were added to the priming sequences for the subsequent Gibson assembly.

primer	sequence (from 5´to 3´)	T_m [°C]	length [nt]
est8_fwd (BPp460)	36 nt homology region *EcoRI* **RBS** *NdeI* **start** tcaattgtgagcggataacaatttcacacagaattcAAGAAGGAGATATACATATGGCAG	58.2	60
his6_rev (BPp461)	39 nt homology region **stop** *HindIII* **|** His6 ttactggatctatcaacaggagtccaagctcagctaattAAGCTTCAATGATGGTGATG	59	59

2.1.4. Instruments and devices

Table 4: List of instruments and devices used in this work.

instrument	supplier
96 well microplate, PS, flat bottom	Greiner bio-one International AG, Kremsmünster, Austria
Äkta prime	GE Healthcare, Chalfont St Giles, UK
Avanti J-20 XP Centrifuge (JA 10 , JA 25.50 rotor)	Beckman Coulter, Brea, USA
BioPhotometer plus	Eppendorf, Hamburg, Germany
CELLSTAR® serological pipette (5 mL, 10 mL, 25 mL)	Greiner bio-one International AG
cellulose acetate filter (0.8 µm, 0.45 µm, 0.2 µm)	Sartorius AG, Göttingen, Germany
centrifuge 5810R with swing-bucket rotor A-4-62	Eppendorf AG, Hamburg, Germany
centrifuge buckets (400 mL, 50 mL)	Thermo Fisher Scientific, Massachusetts, USA
electrophoresis power supply EV261	Consort, Turnhout, Belgium
electroporation cuvettes, 0.1 cm	Cell Projects Ltd, Kent, UK
electroporator MicroPulser™	Bio-Rad Laboratories, Inc., Hercules, USA
Eppendorf safe-lock tubes™ (0.5 mL, 1.5 mL, 2 mL)	Eppendorf
Greiner centrifuge tubes (50 mL, 15 mL)	Greiner bio-one International AG
HisTrap 26/60 desalting column	GE Healthcare
HPLC vials (1.5 mL) and caps	Bruckner Analysentechnik, Linz, Austria
incubation shaker CERTOMAT BS-1	Sartorius AG
incubation shaker Multitron II	Infors AG, Bottmingen-Basel, Swiss
incubator	BINDER GmbH, Tuttlingen, Germany
Injekt® Luer Solo syringe (20 mL, 10 mL, 2 mL)	B. Braun Melsungen AG, Melsungen, Germany
laboratory glass bottles; various sizes	DURAN Group GmbH, Wertheim, Germany
LLG-electrical pipette pump	Lab Logistics Group GmbH, Meckenheim, Germany
microcentrifuge 5415R	Eppendorf
MiniTwist nutating mixer	Select BioProducts, Edison, USA
multichannel pipette Proline mechanical pipette, 12 channel	Sartorius AG
Nalgene™ Rapid-Flow™ filter unit	Thermo Fisher Scientific
NanoDrop 2000c spectrophotometer	Thermo Fisher Scientific
NuPAGE® Bis-Tris mini gels, 4-12%; 1.0 mm; 15 well	Life technologies, Carlsbad, USA
PCR tubes, thin- walled	Greiner bio-one International AG
PD-10 desalting columns	GE Healthcare
petri dishes	Greiner bio-one International AG
pipette tips	Greiner bio-one International AG
pipettes: P2 (0.2- 2 µL), P20 (2- 20 µL), P200 (20- 200 µL), P1000 (100- 1000 µL)	Gilson, Middleton, USA
platform shaker, Titramax 1000	Heidolph Instruments, Schwabach, Germany
Power Ease 500 power supply	Life technologies
PowerPac™ basic power supply	Bio-Rad Laboratories

instrument	supplier
PP columns (10 mL) with polyethylene filter discs and top and bottom press-on caps; for gravity flow	Thermo Fisher Scientific
Savant™ SPD131DDA SpeedVac™ concentrator	Thermo Fisher Scientific
scanjet 2200c	Hewlett-Packard Development Company, Palo Alto, USA
semi-micro-cuvettes, 1.6 mL, PS	Greiner bio-one International AG
shake flasks, baffled (250 mL, 1 L, 2 L)	DURAN Group GmbH
Sonifier® ultrasonic cell disruptor S-450A	Branson Ultrasonics, Danbury, USA
SpectraMax Plus384 absorbance microplate reader	Molecular Devices, Sunnyvale, USA
Sprout® mini-centrifuge	Heathrow Scientific LLC,Illinois, USA
sterile syringe filters, cellulose acetate (0.2 µm, 0.45 µm)	VWR, Radnor, USA
Sub-Cell GT electrophoresis cell	Bio-Rad Laboratories
thermal cycler 2720	Applied Biosystems, California, USA
thermomixer comfort with exchangeable thermoblock for: micro test tubes 1.5 mL, 2 mL 8 Falcon® tubes 15 mL 4 Falcon® tubes 50 mL	Eppendorf
UV/Vis spectrophotometer DU 800	Beckman Coulter
Vivaspin® 6 (10,000 MWCO, PES) centrifugal concentrator	Sartorius AG
Vortex 3	IKA, Staufen, Germany
Whatman® qualitative filter paper, Grade 1	GE Healthcare
XCell *SureLock™* mini-cell gel box	Life technologies

2.1.5. Chemicals and enzymes

Table 5: List of chemicals and enzymes used in this work.

reagent	Cat.Nr.	supplier
1,4-dithiothreitol (DTT)	6908.3	Carl Roth GmbH, Karlsruhe, Germany
1-methyl-L-tryptophan	447439-5G	Sigma-Aldrich, St.Louis, USA
4-aminoindole	25980595	Molekula, Dorset, UK
4-nitrophenyl butyrate (*p*NPB)	N9876	Sigma-Aldrich
7-fluoroindole	35427508	Molekula
acetic acid, glacial	6755.2	Carl Roth GmbH
acrylamide/bis solution, 30% (w/v)	10688.01	Serva, Heidelberg, Germany
aluminium chloride hexahydrate ($AlCl_3 \cdot 6\ H_2O$)	1010841000	Merck, Darmstadt, Germany
ammonium chloride (NH_4Cl)	5470.2	Carl Roth GmbH
ammonium persulfate (APS)	13375.01	Serva
ampicillin sodium salt	K029.1	Carl Roth GmbH
BaBTaBBa, GK2814/127-3 filled up: 15.11.2011	-	BASF, Ludwigshafen am Rhein, Germany
BaBTaBBa, GM0506-0110-3	-	BASF

reagent	Cat.Nr.	supplier
filled up: 26.09.2012		
BIO-RAD Protein Assay, dye reagent concentrate	500-0006	Bio-Rad Laboratories, Inc., Hercules, USA
Biozym LE agarose	840004	Biozym, Hessisch Oldendorf, Germany
boric acid (H_3BO_4)	1001625000	Merck
bovine serum albumin (BSA), standard ampule, 2 mg/mL	23209	Thermo Fisher Scientific, Massachusetts, USA
bromophenol blue	T116.1	Carl Roth GmbH
calcium chloride dihydrate ($CaCl_2 \cdot 2\ H_2O$)	T885.1	Carl Roth GmbH
CelLytic™ B cell lysis reagent	B7435	Sigma-Aldrich
citric acid monohydrate	3958.1	Carl Roth GmbH
cobalt chloride hexahydrate ($CoCl_2 \cdot 6\ H_2O$)	1025390100	Merck
Coomassie brilliant blue G-250	B8522	Fluka Analytical, St. Louis, USA
copper(II)chloride dihydrate ($CuCl_2 \cdot 2\ H_2O$)	1027330250	Merck
D(+)-biotin	3822.1	Carl Roth GmbH
dimethyl sulfoxide (DMSO)	4720.1	Carl Roth GmbH
disodium hydrogen phosphate (Na_2HPO_4)	T876.2	Carl Roth GmbH
DNA Loading Dye, 6 X	R0611	Thermo Fisher Scientific
DNase I	DN25-100mg	Sigma-Aldrich
dNTPs (2.5 mM each)	BH4401A	TaKaRa, Kyoto, Japan
dNTP-Set I (100 mM each)	K039.1	Carl Roth GmbH
double distilled water (ddH_2O) aqua bidest. „Fresenius“ water for injection purposes	-	Fresenius Kabi, Graz, Austria
*Dpn*I (2500 U, 10 U/µL)	ER1702	Thermo Fisher Scientific
ecoflex® F GK 2814/127-9 Code GKT/W filled up: 22.11.2011	-	BASF
ethanol, absolute, for analysis	20821.330	VWR International, Pennsylvania, USA
ethidium bromide	E1510	Sigma-Aldrich
Ex Taq® DNA polymerase (250 U, 5 U/µL)	RR001A	TaKaRa
Ex Taq® reaction buffer, 10 X	AB1101A	TaKaRa
FastDigest buffer, 10 X	B64	Thermo Fisher Scientific
FastDigest *Eco*RI	FD0274	Thermo Fisher Scientific
FastDigest green buffer, 10 X	B72	Thermo Fisher Scientific
FastDigest *Hind*III	FD0504	Thermo Fisher Scientific
formic acid	399388	Sigma-Aldrich
glycerol, anhydrous	7530.4	Carl Roth GmbH
glycine	3908.1	Carl Roth GmbH
glycine	3790.1	Carl Roth GmbH
hydrochloric acid 32% (HCl)	4625.2	Carl Roth GmbH

reagent	Cat.Nr.	supplier
hydrochloric acid, fuming (HCl)	4625.1	Carl Roth GmbH
imidazole	I202-2KG	Sigma-Aldrich
indole	I3408-25G	Sigma-Aldrich
iron(II) sulphate heptahydrate ($FeSO_4 \cdot 7\ H_2O$)	3965	Merck
isopropyl-β-D-thiogalactopyranosid (IPTG)	I-8000	BioSynth, Staad SG, Switzerland
kanamycin sulfate	T832.3	Carl Roth GmbH
L-alanine	3076.1	Carl Roth GmbH
L-arginine	3144.1	Carl Roth GmbH
L-asparagine monohydrate	HN23.2	Carl Roth GmbH
L-aspartic acid	1690.1	Carl Roth GmbH
LB agar (Lennox)	X965.3	Carl Roth GmbH
LB medium (Lennox)	X964.3	Carl Roth GmbH
L-cysteine	1693.2	Carl Roth GmbH
L-glutamic acid	1743.1	Carl Roth GmbH
L-glutamine	HN08.1	Carl Roth GmbH
L-histidine	3852.1	Carl Roth GmbH
L-isoleucine	3922.2	Carl Roth GmbH
L-leucine	1699.1	Carl Roth GmbH
L-lysine hydrochloride	9357.1	Carl Roth GmbH
L-methionine	1702.1	Carl Roth GmbH
L-methionine	64319	Fluka Analytical
L-norleucine	HAA1113-0025	Iris Biotech GmbH, Marktredwitz, Germany
L-phenylalanine	4491.3	Carl Roth GmbH
L-proline	1713.1	Carl Roth GmbH
L-serine	1714.1	Carl Roth GmbH
L-threonine	T206.1	Carl Roth GmbH
L-tryptophan	1739.2	Carl Roth GmbH
L-tyrosine	T207.1	Carl Roth GmbH
L-valine	4879.1	Carl Roth GmbH
lysozyme from egg white (20,000 U/mg)	8259.1	Carl Roth GmbH
magnesium chloride ($MgCl_2$)	5833	Merck
magnesium chloride hexahydrate ($MgCl_2 \cdot 6\ H_2O$)	2189.1	Carl Roth GmbH
magnesium sulphate heptahydrate ($MgSO_4 \cdot 7\ H_2O$)	T888.1	Carl Roth GmbH
manganese(II) sulphate monohydrate ($MnSO_4 \cdot H_2O$)	4487.1	Carl Roth GmbH
methanol, HPLC grade	87-56-1	Avantor Performance Materials, Center Valley, USA
N,N,N',N'-tetramethylethylendiamine (TEMED)	35930.01	Serva
Ni-NTA agarose, 50% slurry 25 mL nickel-charged resin	30210	Qiagen, Hilden, Germany
NuPAGE® LDS sample buffer, 4 X	NP0007	Life technologies
NuPAGE® MOPS SDS running	NP0001	Life technologies

reagent	Cat.Nr.	supplier
buffer, 20 X		
NuPAGE® reducing agent, 10 X	NP0004	Life technologies
PEG-8000	P5413-1KG	Sigma-Aldrich
Phusion® high-fidelity DNA polymerase (100 U, 2 U/µL)	M0530S	New England Biolabs, Frankfurt am Main, Germany
potassium chloride (KCl)	6781.1	Carl Roth GmbH
potassium dihydrogen phosphate (KH_2PO_4)	3904.1	Carl Roth GmbH
RNase A	R5503-100mg	Sigma-Aldrich
sodium carbonate (Na_2CO_3)	8563.1	Carl Roth GmbH
sodium chloride (NaCl)	3957.1	Carl Roth GmbH
sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$)	K300.2	Carl Roth GmbH
sodium dodecyl sulfate (SDS)	2326.1	Carl Roth GmbH
sodium hydroxide (NaOH)	6771.1	Carl Roth GmbH
sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2\ H_2O$)	1065241000	Merck
Surfact-Amps® detergent solution	PI-28314	Thermo Fisher Scientific
T5 exonuclease (1000 U, 10 U/µL)	162340	Biozym
Taq DNA ligase (10,000 U; 40 U/µL)	M0208L	New England Biolabs
thiamine hydrochloride	T911.1	Carl Roth GmbH
TRIS, PUFFERAN®	4855.3	Carl Roth GmbH
Tris-acetate-EDTA (TAE) buffer, 50 X	B49	Thermo Fisher Scientific
triton X 100	3051.3	Carl Roth GmbH
tryptone	211705	BD Biosciences, Franklin Lakes, USA
urea	2317.3	Carl Roth GmbH
yeast extract (Charge: 269107355)	2363.4	Carl Roth GmbH
zinc sulphate heptahydrate ($ZnSO_4 \cdot 7\ H_2O$)	1088830500	Merck
α-D(+)-glucose monohydrate ($C_6H_{12}O_6 \cdot\ H_2O$)	6780.2	Carl Roth GmbH
β-mercaptoethanol	4227.1	Carl Roth GmbH
β-nicotinamide adenine dinucleotide (NAD)	AE11.2	Carl Roth GmbH

2.1.6. Media and buffers

Unless otherwise specified all media and buffers were autoclaved or filter sterilized.

- **Luria Broth (LB)**

Medium: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl
Agar: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar-agar
1 mL of the appropriate antibiotic stock (1000 X) was added per litre of medium or agar.

- **SOC medium**

20 g/L tryptone, 5 g/L yeast extract, 0.58 g/L NaCl, 4.27 g/L $MgCl_2 \cdot 6\ H_2O$, 0.18 g/L KCl, 5.04 g/L $MgSO_4 \cdot 7\ H_2O$, 3.46 g/L α-D(+)-glucose monohydrate

- **5 X ISO buffer**

Table 6: Composition of 5 X ISO buffer.

component	c_{Stock}	volume/ amount
25% (w/v) PEG-8000	PEG-8000	1.5 g
500 mM Tris/Cl pH 7.5	1 M Tris/Cl pH 7.5	3000 µL
50 mM $MgCl_2$	2 M $MgCl_2$	150 µL
50 mM DTT	1 M DTT	300 µL
1 mM dATP	100 mM dATP	60 µL
1 mM dCTP	100 mM dCTP	60 µL
1 mM dGTP	100 mM dGTP	60 µL
1 mM dTTP	100 mM dTTP	60 µL
5 mM NAD	100 mM NAD	300 µL
sterile ddH_2O		up to 6000 µL

The PEG-8000 was weighed out first and then all other ingredients were added out of stock solutions. All ingredients were mixed and filter sterilized by using 0.45 µm sterile syringe filters. 100 µL aliquots were stored at -20 °C.

- **Assembly master mix**

Table 7: Composition of assembly master mix for Gibson assembly.

component	volume to add
5 X ISO buffer	320 µL
T5 exonuclease (10 U/µL)	0.64 µL
Phusion® High-Fidelity DNA Polymerase (2 U/µL)	20 µL
Taq DNA ligase (40 U/µL)	160 µL
sterile ddH_2O	fill up to 1200 µL

2.1.6.1. M9 based media and components

- **5 X M9 salts stock**

Table 8: Composition of 5 X M9 salts stock.

component	amount
Na_2HPO_4	33.9 g/L
KH_2PO_4	15 g/L
NaCl	2.5 g/L
NH_4Cl	5 g/L
ddH_2O	up to 1000 mL

- **Trace elements stock**

Table 9: Composition of trace elements stock.

component	amount
$FeSO_4 \cdot 7\ H_2O$	40.0 g/L
$MnSO_4 \cdot H_2O$	10.0 g/L
$AlCl_3 \cdot 6\ H_2O$	10.0 g/L
$CoCl_2 \cdot 6\ H_2O$	7.3 g/L
$ZnSO_4 \cdot 7\ H_2O$	2.0 g/L
$Na_2MoO_4 \cdot 2\ H_2O$	2.0 g/L
$CuCl_2 \cdot 2\ H_2O$	1.0 g/L
H_3BO_4	0.5 g/L
HCl conc. (37%, fuming)	414 mL/L
ddH_2O	up to 1000 mL

- **Simple M9 medium**

Table 10: Composition of simple M9 medium for high cell density SPI.

component	c_{stock}	c_{final}	1 L
M9 salts[3]	5 X	1 X	200 mL
glucose[3]	1 M	20 mM	20 mL
$MgSO_4$[3]	1 M	1 mM	1 mL
$CaCl_2$[3]	1 mg/mL	1 mg/L	1 mL
trace elements[3]	~18.7 X	1 X	60 µL
amino acid supplementation[3]	100 mM	1 mM	10 mL
appropriate antibiotics[3]	1000 X	1 X	1 mL
yeast extract[1]		x g[3]	x g[3]
ddH_2O[2]			780 mL

[1] Amounts of yeast extract were determined for the different strains in titration experiments: *E. coli* BWEC50: 3- 3.5 g/L (personal communication, P. Fladischer, ACIB, Graz)
[2] Amount of yeast extract was dissolved in 780 mL ddH_2O and autoclaved.
[3] All other ingredients were supplemented as sterile stocks.

- **M9 medium adapted for Est8 expression in BWEC50**

Table 11: Composition of M9 medium adapted for Est8 expression in Met auxotrophic *E. coli* BWEC50.

component	c_{stock}	c_{final}	1 L
M9 salts[2]	5 X	1 X	200 mL
glucose[2]	1 M	20 mM	20 mL
$MgSO_4$[2]	1 M	1 mM	1 mL
$CaCl_2$[2]	1 mg/mL	1 mg/L	1 mL
trace elements[2]	~18.7 X	1 X	60 µL
$ZnSO_4 \cdot 7\ H_2O$[3]	2 mg/mL	120 µg/L	60 µL
amino acid supplementation[2]	100 mM	1 mM	10 mL
appropriate antibiotics[2]	1000 X	1 X	1 mL
yeast extract[1]		3.5 g/L	3.5 g
ddH_2O[1]			780 mL

[1] 3.5 g YE were dissolved in 780 mL ddH_2O and autoclaved.
[2] All other ingredients were supplemented as sterile stocks.
[3] 120 µg $ZnSO_4 \cdot 7\ H_2O$/L were introduced with the trace elements – another 120 µg/L were introduced additionally.

- **1.1 X M9 medium for titration of the optimal YE concentration**

Table 12: Composition of 1.1 X M9 medium used in the YE titration experiments.

component	c_{stock}	c_{final}	600 mL
M9 salts[2]	5 X	1.1 X	132 mL
glucose[2]	1 M	22 mM	13.2 mL
$MgSO_4$[2]	1 M	1.1 mM	0.66 mL
$CaCl_2$[2]	1 mg/mL	1.1 µg/mL	0.66 mL
trace elements[2]	~18.7 X	1 X	40 µL
amino acid supplementation[2]	100 mM	1.1 mM	6.6 mL
kanamycin[2]	1000 X	1 X	600 µL
ddH_2O[1]			446.84 mL

[1] 446.84 mL ddH_2O were autoclaved in a 1 L glass bottle.
[2] All other ingredients were supplemented as sterile stocks.

- **M9 medium for the titration of the optimal Trp concentration**

Table 13: Composition of M9 medium used in the titration of the optimal Trp concentration.

component	c_{stock}	c_{final}	700 mL
M9 salts[2]	5 X	1 X	140 mL
glucose[2]	1 M	20 mM	14 mL
$MgSO_4$[2]	1 M	1 mM	0.7 mL
$CaCl_2$[2]	1 mg/mL	1 mg/L	1 mL
trace elements[2]	~18.7 X	1 X	42 µL
kanamycin	50 mg/mL	50 mg/L	0.7 mL
biotin[2]	1000 X	1 X	0.7 mL
thiamine[2]	1000 X	1 X	0.7 mL
ddH_2O[1]			542 mL

[1] 77 mL ddH_2O (67 mL ddH_2O for the positive control) were added per flask and autoclaved.
[2] All other ingredients were supplemented as sterile stocks.
Amounts of Trp added are indicated in Table 20.

- **M9 medium used in the growth test of *E. coli* BWEC47**

Table 14: Composition of M9 medium used in the growth test of *E. coli* BWEC47 under different conditions and with different plasmids.

component	c_{stock}	c_{final}	amounts per flask
M9 salts	5 X	1 X	20 mL
glucose	1 M	20 mM	2 mL
$MgSO_4$	1 M	1 mM	0.1 mL
$CaCl_2$	1 mg/mL	1 mg/L	0.1 mL
trace elements	~18.7 X	1 X	0.006 mL
appropriate antibiotics	50 mg/mL	50 mg/L	0.1 mL
biotin	1000 X	1 X	0.1 mL
thiamine	1000 X	1 X	0.1 mL

- **M9 medium adapted for high cell density SPI of indole/ Trp analogues in *E. coli* BWEC47**

Table 15: Composition of M9 medium adapted for high cell density SPI of indole/Trp analogues in Trp auxotrophic *E. coli* BWEC47.

component	c_{stock}	c_{final}	1 L
M9 salts	5 X	1 X	200 mL
19 cAA stock (-Trp)	10 X (0.5 g/L each)	1 X	100 mL
glucose	1 M	20 mM	20 mL
$MgSO_4$	1 M	1 mM	1 mL
$CaCl_2$	1 mg/mL	1 mg/L	1 mL
trace elements	~18.7 X	1 X	60 µL
$ZnSO_4 \cdot 7\ H_2O$[1]	2 mg/mL	120 µg/L	60 µL
tryptophan	10 mM	18 µM	1.8 mL
thiamine	10 mg/mL	10 mg/L	1 mL
biotin	10 mg/mL	10 mg/L	1 mL
appropriate antibiotics	1000 X	1 X	1 mL
ddH_2O			up to 1000 mL

[1] An additional amount of 120 µg/L zinc was added to the medium only for the expression of Est8[1MeW].

2.1.6.2. SDS-PAGE (Laemmli system)

- **10% separating gel (amounts for 2 gels, 0.75 mm thickness)**

3.35 mL	30% (w/v) acrylamide/bis
2.50 mL	1.5 M Tris-HCl pH 8.8 + 0.4% (w/v) SDS
4.10 mL	ddH_2O
50 µL	10% (w/v) ammonium persulfate
5 µL	N,N,N',N'-tetramethylethylendiamine (TEMED)

- **5% stacking gel (amounts for 2 gels, 0.75 mm thickness)**

650 µL	30% (w/v) acrylamide/bis
1 mL	0.5 M Tris-HCl pH 6.8 + 0.4% (w/v) SDS
2.37 mL	ddH_2O
20 µL	10% (w/v) ammonium persulfate
2 µL	N,N,N',N'-tetramethylethylendiamine (TEMED)

- **5 X SDS sample buffer**

10% (w/v) SDS, 0.08 M Tris-HCl (pH 6.8), 12.5% (v/v) glycerol, 0.2% (w/v) bromophenol blue, 4% (v/v) β-mercaptoethanol (added fresh before use)

- **10 X SDS-PAGE running buffer**

1% (w/v) SDS, 1.92 M glycine, 0.25 M Tris

- **1 X SDS-PAGE running buffer**

0.1% (w/v) SDS, 192 mM glycine, 25 mM Tris
100 mL 10 X SDS-PAGE running buffer up to 1000 mL with ddH_2O.

- **Coomassie stain**

0.1% (w/v) Coomassie blue G-250, 40% (v/v) ethanol, 10% (v/v) acetic acid, up to 1000 mL with ddH_2O.

- **Destaining solution**

40% (v/v) ethanol, 10% (v/v) acetic acid, up to 1000 mL with ddH_2O.

2.1.6.3. Protein purification

- **Ni-NTA lysis buffer**

50 mM $NaH_2PO_4 \cdot H_2O$, 300 mM NaCl, 10 mM imidazole, pH 8.0 in ddH_2O

- **Ni-NTA wash buffer**

50 mM $NaH_2PO_4 \cdot H_2O$, 300 mM NaCl, 20 mM imidazole, pH 8.0 in ddH_2O

- **Ni-NTA elution buffer**

50 mM $NaH_2PO_4 \cdot H_2O$, 300 mM NaCl, 250 mM imidazole, pH 8.0 in ddH_2O

2.1.6.4. *p*-Nitrophenyl ester substrate solutions

- ***p*NPB assay**

Solution A: 3 mL DMSO, 258 µL *p*NPB

Solution B: 2 mL solution A, 50 mL Tris-HCl, pH 7

Table 16: Composition of different substrate dilutions used in the *p*NPB activity assays and the corresponding concentrations of *p*NPB per well.

dilution	DMSO [mL]	solution B [mL]	buffer [mL]	*p*NPB per well [mM *p*NPB]
1	0	10.00	0	15.750
2	0.132	6.70	3.168	10.553
3	0.200	5.00	4.800	7.875
4	0.240	4.00	5.760	6.300
5	0.268	3.30	6.432	5.198
6	0.284	2.90	6.816	4.568
8	0.320	2.00	7.680	3.150
9	0.332	1.70	7.968	2.678
11	0.350	1.25	8.400	1.969
12	0.356	1.10	8.544	1.733
14	0.373	0.67	8.957	1.055
15	0.380	0.50	9.120	0.788
16	0.387	0.33	9.283	0.520
17	0.390	0.25	9.360	0.394
19	0.396	0.10	9.504	0.158
20	0.398	0.05	9.552	0.079

2.1.6.5. Buffers for polymer degradation assays

Buffers were prepared according to Stoll and Blanchard (1990).

- **Citrate- phosphate buffer, pH 6**

17.9 mL 0.1 M citric acid solution, 32.1 mL 0.2 M Na_2HPO_4 solution, 50 mL ddH_2O; adjusted to pH 6.

- **Phosphate buffer, pH 8**

5.3 mL 0.2 M NaH_2PO_4 solution, 94.7 mL 0.2 M Na_2HPO_4 solution, 100 mL ddH_2O; adjusted to pH 8.

2.1.7. Commercial kits, DNA and protein ladders

2.1.7.1. Commercial kits

- **GeneJET™ Plasmid Miniprep Kit**

(Cat.Nr. K0503, Lot. 00127914), Fermentas /Thermo Fisher Scientific, Massachusetts, USA

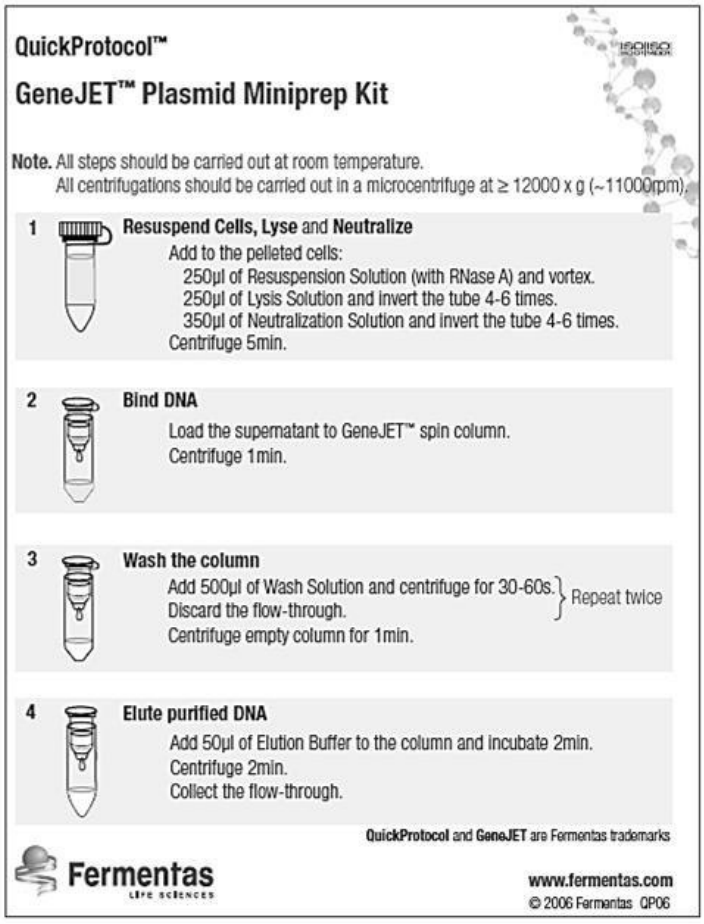
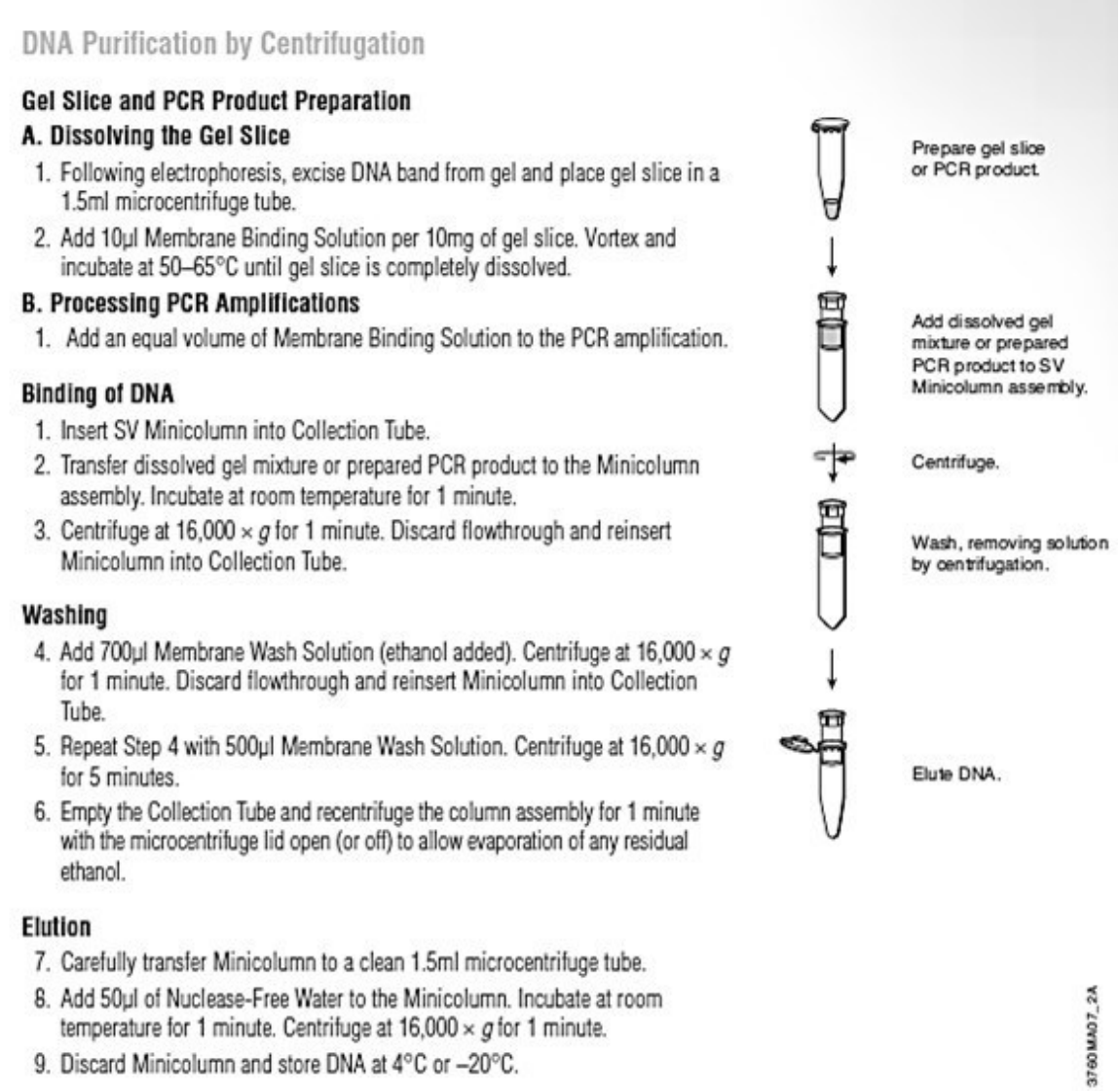


Figure 5: Overview of the purification procedure performed with the GeneJET™ Plasmid Miniprep Kit (Fermentas).

- **Wizard SV Gel and PCR Clean-Up System, Promega**

(Cat.Nr. A9282), Promega, Fitchburg, USA



DNA Purification by Centrifugation

Gel Slice and PCR Product Preparation

A. Dissolving the Gel Slice

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5ml microcentrifuge tube.
2. Add 10µl Membrane Binding Solution per 10mg of gel slice. Vortex and incubate at 50–65°C until gel slice is completely dissolved.

B. Processing PCR Amplifications

1. Add an equal volume of Membrane Binding Solution to the PCR amplification.

Binding of DNA

1. Insert SV Minicolumn into Collection Tube.
2. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.
3. Centrifuge at 16,000 × *g* for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

Washing

4. Add 700µl Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × *g* for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
5. Repeat Step 4 with 500µl Membrane Wash Solution. Centrifuge at 16,000 × *g* for 5 minutes.
6. Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution

7. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.
8. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × *g* for 1 minute.
9. Discard Minicolumn and store DNA at 4°C or –20°C.

Figure 6: Overview of the DNA and PCR product extraction and purification out of the gel, using the Wizard SV Gel and PCR Clean-Up System (Promega).

2.1.7.2. DNA and protein ladders

- **O' GeneRuler DNA Ladder Mix, ready-to-use**

(Cat.Nr. SM1173, Lot. 00038194), Fermentas /Thermo Fisher Scientific, Massachusetts, USA

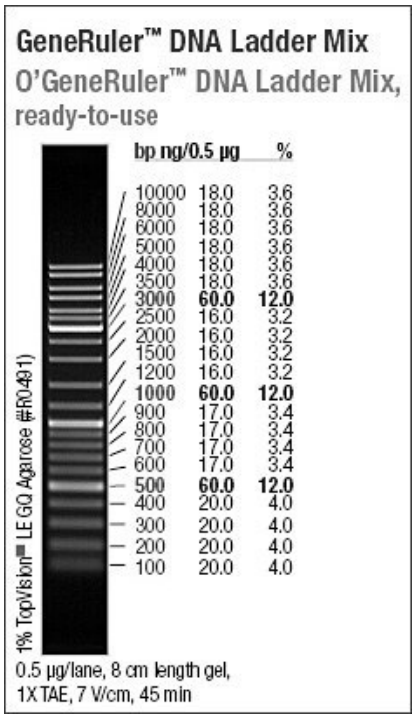


Figure 7: Migration pattern of O'GeneRuler™ DNA Ladder Mix, ready-to-use in 1% agarose gel.

- **Page Ruler™ Prestained Protein Ladder**

(Cat.Nr. 26616, Lot. 00107239) Thermo Fisher Scientific, Massachusetts, USA

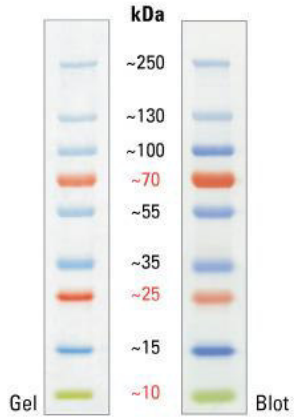


Figure 8: Band profile of the Page Ruler™ Prestained Protein Ladder, apparent MW, kDa.

2.2. Methods

2.2.1. Preparation of electrocompetent *E. coli* cells

For the preparation of electrocompetent *E. coli* cells, 50 mL LB medium in a 250 mL baffled shake flask were inoculated with a glycerol stock of the desired *E. coli* strain (for the strains used in this work see section 2.1.1). The culture was incubated at 37 °C and 110 rpm shaking over night. After incubation the OD_{600} of the overnight culture (ONC) was determined. A main culture of 50 mL LB in a 250 mL baffled shake flask was inoculated with the ONC to an initial OD_{600} of 0.1 and incubated at 37 °C and 110 rpm shaking until an OD_{600} between 0.6 and 0.8 was reached. Then the cells were transferred into pre-chilled 50 mL tubes and harvested for 15 minutes at 4000 rpm and 4 °C. The supernatant was discarded and the cells were gently resuspended in 1 mL of sterile, ice-cold 10% (v/v) glycerol. The cell suspension was filled up to a volume of approximately 50 mL with sterile, ice-cold 10% (v/v) glycerol and harvested again for 15 minutes at 4000 rpm and 4 °C. This washing procedure was performed twice, before the remaining cell pellet was resuspended in 500 µL sterile, ice-cold 10% (v/v) glycerol. The cells were dispensed into 40 µL aliquots in 0.5 mL Eppendorf tubes, flash frozen in liquid nitrogen and stored at -80 °C.

2.2.2. Electroporation of *E. coli*

Plasmid DNA was introduced into electrocompetent *E. coli* cells by electroporation. Therefore a 40 µL aliquot of the desired electrocompetent cells was thawed on ice and supplemented with 10-100 ng plasmid DNA to be transformed. As sterile control, 1 µL ddH_2O was added per 40 µL electrocompetent cells. The mixtures were transferred into pre-chilled 0.1 cm electroporation cuvettes. The electroporation was performed using the fixed program Ec.2 on the Bio-Rad micropulser with 2.50 kV for 4-6 ms. Afterwards 800 µL pre-warmed SOC medium were added immediately and the cells were regenerated for 1 hour at 37 °C and 600 rpm shaking. After regeneration, the cells were either platted on selective LB plates or directly transferred into fresh LB medium supplemented with the appropriate antibiotic for an ONC. 100 µL of the sterile control were platted on a selective LB plate and incubated at 37 °C over night.

2.2.3. Preparation of glycerol stocks

Glycerol stocks of the transformed strains were prepared by inoculating 10- 50 mL LB medium supplemented with the appropriate antibiotic, either with a single colony from the corresponding LB plate or with the regenerated cells after transformation. The culture was incubated at 37 °C and vigorous shaking over night. After incubation, the OD_{600} of the culture was determined and an

amount of cells corresponding to an OD_{600} of 20 was transferred into 15 mL tubes. The cells were harvested for 15 minutes at 3000 rpm and 10 °C and the supernatant was discarded. The pellet was resuspended in 1 mL LB medium and 1 mL 70% (v/v) glycerol was added. The cells were dispensed into 80 µL aliquots and stored at -80 °C.

2.2.4. Expression test with BWEC50

In order to assess the influences of various parameters like medium composition, expression temperature and duration of expression on the protein yield, the parent protein Est8[Met] was expressed in Met auxotrophic *E. coli* BWEC50 {pET26b(+)_est8} cells under different conditions.

Initially an ONC of Met auxotrophic *E. coli* BWEC50 {pET26b(+)_est8} cells in 100 mL LB+Kan (50 mg/L) medium in a 250 mL baffled shake flask was inoculated with 80 µL of the corresponding glycerol stock. The culture was incubated at 37 °C and 110 rpm shaking over night. After determination of the OD_{600}, amounts of cells corresponding to an OD_{600} of 0.1 in 250 mL were harvested per flask. The centrifugation was performed for 15 minutes at 3000 rpm and 10 °C. The pellets were resuspended in 500 µL LB and 500 µL of 70% (v/v) glycerol were added respectively. M9 medium was prepared in 1 L baffled shake flasks as described in section 2.1.6.1, Table 10, containing 3.5 g/L YE as the limiting source of Met and 50 mg/L kanamycin for selection. Additionally, varying amounts of zinc sulphate were added to the cultures, as outlined in Table 17. Starting from 120 µg $ZnS0_4 \cdot 7\ H_20$/L which was introduced into the M9 medium through the trace elements stock (for composition of trace elements stock see Table 9), one culture was supplemented with the double, one with the five-fold amount of zinc sulphate. Two flasks containing LB+Kan (50 mg/L) medium, one with and one without baffles were also prepared, to evaluate the differences between the expression in complete (LB) and minimal (M9) medium and to assess the effect of the baffles on the cell density and protein yield. For the cultivation and expression of Est8[Met] in LB medium, the protocol “Fermentation in shake flasks” from Armin Baumschlager (2013a) was applied.

The six 1 L flasks, containing 250 mL culture medium were inoculated to an initial OD_{600} of 0.1 and incubated at 37 °C and 140 rpm shaking. Samples were taken regularly and the OD_{600} was measured in duplicate or triplicate determination. As soon as the LB cultures 1 and 2 (Table 17), reached an OD_{600} of 0.6, the temperature was adjusted to 20 °C. The two cultures were further incubated until an OD_{600} of 0.8 was reached to make sure that the culture medium had the appropriate temperature upon induction. Right before induction with 0.5 mM IPTG, 2x 1 OD_{600} of each of the two cultures were transferred into 1.5 mL Eppendorf tubes and harvested for 2 minutes at 13200 rpm and RT (non-induced probe). The supernatants were discarded and the pellets were stored at -20 °C until the

preparation of the SDS-PAGE. For the LB cultures, protein expression was performed for 20 hours at 20 °C and 140 rpm. 4 hours after induction and at the end of expression, 2x 1 OD_{600} samples were taken for SDS-PAGE analysis (induced probe).

The M9 cultures, 3, 4, 5 and 6 (Table 17) were cultivated at 37 °C until Met depletion, which was expected to be reached 5- 6 hours after inoculation. As soon as no change in the OD_{600} of the cultures was detectable anymore, 2x 1 OD_{600} samples were taken for SDS-PAGE and treated as described above (non-induced probe). The cultures were supplemented with 1 mM Met and target protein expression was induced by the addition of 0.5 mM IPTG. Protein expression was performed at 20 °C for 20 hours for the cultures 3, 4 and 5. The control culture 6 was treated according to the standard protocol for high cell density SPI in *E. coli* (Fladischer, 2013) and was incubated at 28 °C for 4 hours. For the M9 cultures, also 2x 1 OD_{600} samples for SDS-PAGE were taken 4 hours after induction and at the end of expression (induced probe). For cell harvest, the cultures were transferred into tared centrifuge buckets and centrifuged for 20 minutes at 4000 x g and 4 °C. The supernatants were discarded and the pellets were resuspended in 30 mL 0.9% NaCl. The pellet solutions were transferred into 50 mL falcon tubes and harvested again for 15 minutes at 4000 rpm and 4 °C. After discarding the supernatant, the pellets were stored at -20 °C until protein purification.

Table 17: Medium compositions, zinc contents and expression conditions of the cultures used in the expression test of Met auxotrophic *E. coli* BWEC50 {pET26b(+)_est8}.

culture	medium	total $ZnSO_4 \cdot 7\ H_20$ [µg/L]	expression temperature [°C]	duration of expression [h]
1	LB	-	20	20
2	LB	-	20	20
3	M9[1]	120	20	20
4	M9[1]	240[2]	20	20
5	M9[1]	600[2]	20	20
6	M9[1]	120	28	4

[1] M9 medium containing 3.5 g/L YE and 50 mg/L kanamycin was prepared according to Table 10.
[2] Additional amounts of zinc were introduced with 15 µL or 60 µL from a 2 mg/mL $ZnSO_4 \cdot 7\ H_2O$ stock, respectively.

2.2.5. Subcloning of est8 into pQE-80L

To avoid plasmid incompatibility with the pSTWS plasmids carrying the tryptophan synthase, the gene of interest est8, was subcloned from the pET26b(+) construct into the pQE-80L vector system using the Gibson cloning strategy (Gibson et al., 2009). For this strategy, the used DNA fragments must be linear carrying a 40- 60 bp overlapping homology region for efficient recombination. Therefore, homology hooks for the desired vector were attached to the 5´-ends of the primers for target gene amplification. Figure 9 shows a schematic representation of the cloning strategy. The primers for amplification are listed in Table 3. The vector pQE-80L was double digested with the appropriate restriction enzymes *Eco*RI/*Hind*III to generate linear fragments, as described in section 2.2.5.2.

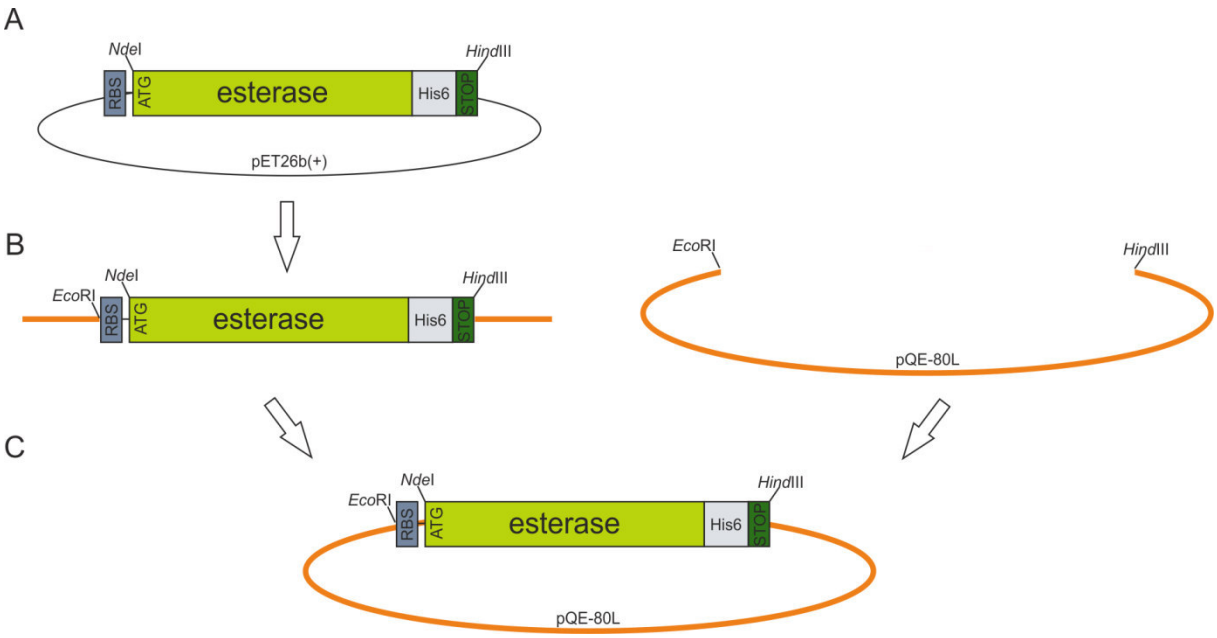


Figure 9: Schematic representation of the cloning strategy for the subcloning of the esterase gene est8, from pET26b(+) into the pQE-80L vector system by Gibson assembly.
The esterase gene was amplified out of the pET26b(+) construct (A) by PCR, using the primers depicted in Table 3. (B) The primers contained 36- 39 nucleotides (nt) homology regions for the pQE-80L target vector, which were added to the target gene in the course of PCR amplification (indicated with orange lines on both sides of the gene). The PCR fragments were assembled with *Eco*RI/*Hind*III cut pQE-80L by *in-vitro* homologous recombination, yielding the expression construct pQE-80L_est8 (C). A vector map of the assembled pQE-80L_est8 construct is provided in Figure 35. RBS, ribosomal binding site; His6, hexa-histidine-tag.

2.2.5.1. Polymerase chain reaction

The inserts for Gibson cloning were prepared by PCR. The primers listed in Table 3, were designed using the software Oligo Explorer and ordered from Integrated DNA Technologies (IDT, Coralville, USA). Appropriate amounts of ddH_2O were added to the delivered primers, to reach a concentration of 100 pM. The tubes were incubated for 10 minutes at 40 °C and subsequently centrifuged for

1 minute at 13200 rpm and room temperature (RT) to spin down possible primers attached to the walls of the tubes. The 100 pM stocks were further diluted to a concentration of 25 pM with ddH_2O and used in the PCR for amplification and attachment of the homologous regions to the esterase gene. Four PCR mixes were prepared in thin-walled PCR tubes as indicated in Table 18.

Table 18: PCR mix for amplification and attachment of homologous regions to the esterase gene.

component	final concentration	volume to add
dsDNA template[1]	~ 40 ng	1 µL
forward primer (25 pmol/µL)	0.2 – 1.0 µM	0.5 µL
reverse primer (25 pmol/µL)	0.2 – 1.0 µM	0.5 µL
10 X reaction buffer	1 X	2.5 µL
dNTPs (2.5 mM each)	0.2 mM each	2.0 µL
DNA polymerase (5 U/µL)	1.0 – 2.5 U	0.5 µL
ddH_2O	-	18 µL
total volume		25 µL

[1] Plasmid preparation (pET26b(+)_est8 (10.10.2012): 39 ng/µL) kindly provided by Armin Baumschlager (ACIB, Graz), was used as PCR template.

The components were thawed on ice. 18 µL ddH_2O were inserted first; the polymerase was thoroughly kept on ice and added the last. The tubes were inverted, the liquid was shortly spun down and a standard three-step PCR was run with the temperature programme shown in Table 19.

Table 19: Temperature programme of the standard three-step PCR, performed to amplify and attach homologous regions to the esterase gene.

step	temperature	time	cycles
initial denaturation	95 °C	5 min	1 x
denaturation	95 °C	30 sec	25 x
annealing	56 °C[1]	1 min	
extension	72 °C	1 min 40 sec (1 min/kb of amplicon length)	
end-elongation	72 °C	10 min	1 x

[1] Annealing temperature (T_{anneal}) was set 2-3 °C lower than the melting temperature (T_m) of the primers calculated with Oligo Explorer.

After PCR, a *Dpn*I digest was performed, to eliminate the methylated template DNA. 1 µL *Dpn*I was added to each 25 µL PCR mix and incubated at 37 °C for 1 hour. Subsequently, *Dpn*I was heat inactivated at 80 °C for 20 minutes. Afterwards the PCR products were stored at -20 °C until further analysis and purification by agarose gel electrophoresis.

2.2.5.2. Preparation of the target vector pQE-80L

The target vector pQE-80L was amplified in *E. coli* TOP10F' cells. Therefore 1 µL plasmid preparation (pQE80L_TT2_h6 (+seq 05.06.12), kindly provided by Patrik Fladischer (ACIB, Graz)) was transformed per 40 µL electrocompetent cells, as described in section 2.2.2. 50 mL LB medium supplemented with 100 mg/L ampicillin in a 250 mL baffled shake flask were inoculated with the transformed cells and incubated at 37 °C and 110 rpm over night. The next day, four plasmid preparations were made from the ONC. Per preparation, 2x 2 mL ONC were harvested for 1 minute at 13200 rpm and RT. The supernatants were discarded and the pelleted cells were treated according to the instruction manual of the GeneJET™ Plasmid Miniprep Kit (see section 2.1.7.1, Figure 5); the elution volume was 50 µL. The concentration of the plasmid preparations was determined with the NanoDrop 2000c Spectrophotometer. 2 µL of the samples were applied, while 2 µL ddH_2O were used as blank.

To open the vector backbone and generate linear fragments for Gibson assembly, the pQE-80L target vector was cut with *Eco*RI and *Hind*III. Per plasmid preparation (~48 µL), 6 µL 10 X FastDigest buffer, 2.5 µL FastDigest *Eco*RI and 2.5 µL FastDigest *Hind*III were added. The restriction digest was performed at 37 °C for 3 hours and 30 minutes. Afterwards, the restriction enzymes were heat inactivated at 80 °C and 600 rpm for 10 minutes. The cut vector was stored at -20 °C until further analysis and purification by agarose gel electrophoresis.

2.2.5.3. Agarose gel electrophoresis

The analysis and purification of DNA fragments by agarose gel electrophoresis was performed according to a standard protocol (Armstrong and Schulz, 2008). A 1% agarose gel solution was prepared by mixing 2 g agarose with 200 mL TAE buffer and adding one drop of ethidium bromide. During polymerization of the gel, the samples were prepared for gel electrophoresis by adding appropriate amounts of 6 X DNA Loading Dye. The samples and 10 µL of a DNA Ladder were loaded onto the gel. The preparative gel was run at 90 V for 2.5 hours. Afterwards, the size of the DNA fragments was estimated by comparing with the standard (Figure 7), the bands of the desired sizes (4658 bp pQE-80L$_{Eco\text{RI}/Hind\text{III}}$; 1483 bp est8_hom$_{\text{pQE-80L}}$) were cut out with a scalpel and transferred into 1.5 mL Eppendorf tubes. The extraction of the DNA and PCR products out of the gel was performed according to the Quick Protocol of the Wizard SV Gel and PCR Clean-Up System (see section 2.1.7.1, Figure 6). Afterwards, constructs of the same type were pooled and the nucleic acid concentrations were determined with the NanoDrop 2000c Spectrophotometer. 2 µL of the samples were applied, while 2 µL ddH_2O were used as blank. To verify the correct size and purity of the cut vector and PCR construct which should be used for Gibson assembly, an analytical gel was run. 200 ng of each

construct were supplemented with appropriate amounts of 6 X DNA Loading Dye and loaded together with a DNA Ladder (6 µL) onto a 1% agarose gel. The gel electrophoresis was performed at 120 V for 45 minutes. Afterwards, the size of the DNA fragments was verified by comparing with the standard (Figure 7).

2.2.5.4. Gibson assembly

For Gibson assembly at least 100 ng of each DNA fragment should be used. Furthermore, the inserts and vectors used for the assembly should be applied in equimolar amounts. For the inserts a fixed value of 100 ng was chosen. Based on that, equimolar amounts needed of the vector were calculated using the following formula:

$$\text{pmols} = \frac{\text{weight in ng} \cdot 1000}{\text{bp} \cdot 650 \text{ dalton}}$$

Formula 1: Formula used for the calculation of the number of pmols needed of each fragment for optimal assembly, based on fragment length and weight.
650 dalton is the average weight of a DNA basepair. The formula is taken from the Instruction Manual of the Gibson Assembly™ Cloning Kit (NEB, Ipswich, USA).

The calculated amounts of insert and vector were mixed in 1.5 mL Eppendorf tubes. Since only a maximum volume of 5 µL of DNA to be assembled can be added to a 15 µL assembly master mix, excess liquid was removed with the Speedvac device for 40 minutes. The DNA was re-dissolved in 5 µL ddH_2O and incubated at 40 °C and 600 rpm for 20 minutes. Meanwhile, 15 µL assembly master mix aliquots were thawed (for composition see section 2.1.6, Table 7). Then the DNA to be assembled was added to the assembly master mix and the reactions were incubated at 50 °C for 60 minutes. Afterwards, the tubes were cooled down on ice for 10- 15 minutes.

The assembled DNA fragments were transformed into electrocompetent *E. coli* TOP10F' cells as described in section 2.2.2. Positive clones were selected on LB-Amp (100 mg/L) plates, upon incubation at 37 °C over night. Four positive clones of each construct were streaked out on ½ LB-Amp (100 mg/L) plate and incubated over weekend at RT. After incubation, the cells were harvested from the plates with a sterile tooth pick and resuspended in 250 µL resuspension solution. Plasmid preparations were prepared according to the instruction manual of the GeneJET™ Plasmid Miniprep Kit (see section 2.1.7, Figure 5); the elution volume was 50 µL. Afterwards, the nucleic acid concentration of the plasmid preparations was determined with the NanoDrop 2000c Spectrophotometer. 2 µL of the samples were applied, while 2 µL ddH_2O were used as blank.

The correct assembly of the DNA molecules was verified by restriction enzyme digestion as well as sequencing. For the restriction digest, 100- 200 ng plasmid DNA were supplemented with 0.5 µL

FastDigest *Eco*RI, 0.5 µL FastDigest *Hind*III and 1 µL 10 X FastDigest green buffer. Appropriate amounts of ddH_2O were added to reach a final volume of 10 µL. The restrictions digests were incubated at 37 °C for 30 minutes. Afterwards, the restriction enzymes were heat inactivated at 80 °C for 10 minutes. 10 µL of the cut fragments were loaded onto a 1% agarose gel, together with a standard. Gel electrophoresis was performed at 120 V for 45 minutes. The size of the DNA fragments was verified by comparing with the standard (Figure 7). Furthermore, two clones were sent for sequencing to LGC Genomics GmbH (Berlin, Germany). 20 µL of plasmid preparation with a concentration of at least 50 ng/µL were sent for analysis with standard forward and reverse primers for the vectors by LGC Genomics.

2.2.6. Titration of the optimal YE concentration as limiting source of cAA

In order to assess the yeast extract (YE) concentration that is optimal to reach late log phase, titration experiments were performed as described by Fladischer (2013). Therefore the cultures were grown in minimal M9 medium containing different YE dilutions and the OD_{600} was recorded over time. The titration experiments were performed for the tryptophan (Trp) auxotrophic strain, *E. coli* BWEC47 once carrying the constitutive (pSTWS2), once carrying the inducible (pSTWS1a) expression plasmid for the tryptophan synthase (TrpS).

Initially, an ONC of the desired strain was inoculated in 50 mL LB medium in a 250 mL baffled shake flask with appropriate antibiotic supplementation and incubated at 37 °C and 110 rpm overnight. After incubation, the OD_{600} of the culture was determined. An amount of cells corresponding to an OD_{600} of 0.05 in 600 mL, equalling 30 OD_{600} units, was transferred into a 15 mL tube. The cells were harvested for 15 minutes at 3000 rpm and 10 °C and the supernatant was discarded. The pellet was resuspended in 500 µL LB medium and 500 µL of 70% (v/v) glycerol were added. The rest of the culture was either used to prepare glycerol stocks, as described in section 2.2.3 or discarded. In sterile 250 mL baffled shake flasks, 10 mL of different YE solutions were prepared by diluting a sterile 60 g/L YE stock with sterile ddH_2O. As the positive control, a 250 mL baffled shake flask with 10 mL of 1 g/L YE and 10 mM of the corresponding canonical amino acid (cAA) was prepared. 600 mL 1.1 X M9 medium, supplemented with the appropriate antibiotic were prepared as described in section 2.1.6.1, Table 12 and inoculated with the cell suspension to an OD_{600} of 0.05. 90 mL thereof were added to each 250 mL flask containing 10 mL of the YE dilution. The cultures were incubated at 37 °C and 140 rpm until stationary phase was reached. The OD_{600} was determined in triplicates regularly and an OD_{600} vs. time growth curve was recorded. The reading intervals were shortened in the log phase in order to get better resolution.

2.2.7. Titration of the optimal Trp concentration as limiting growth source

Since it was assumed that the constitutively expressed tryptophan synthase from the pSTWS2 plasmid may exploit unknown sources in the YE to synthesize Trp, in the next step a medium without YE as the source of Trp was tested.

An ONC in 50 mL LB medium in a 250 mL baffled shake flask supplemented with 50 mg/L kanamycin was inoculated with 80 µL glyercol stock of *E. coli* BWEC50 {pSTWS2} and incubated at 37 °C and 110 rpm shaking. After incubation, the OD_{600} of the culture was determined. An amount of cells corresponding to an OD_{600} of 0.05 in 700 mL, equalling ~41 OD_{600} units, was transferred into a 15 mL tube. The cells were harvested at for 15 minutes at 3000 rpm and 10 °C and the supernatant was discarded. The pellet was resuspended in 500 µL LB medium and 500 µL of 70% (v/v) glycerol were added. Minimal M9 medium was prepared in six sterile 250 mL baffled shake flasks as described in section 2.1.6.1, Table 13. Therefore 77 mL of ddH_2O (67 mL for the positive control) were autoclaved in the flasks and different amounts of 100 mM Trp stock were added, as depicted in Table 20. 158 mL M9 medium were inoculated to an initial OD_{600} of 0.05 and 23 mL thereof were added per flask.

Table 20: Initial Trp concentrations in the cultures of the titration experiment and the corresponding amounts of Trp/indole which were added after Trp depletion.

culture	initial Trp concentration	addition after Trp depletion
1 (positive control)	10 mM	-
2	30 µM	1 mM Trp
3	30 µM	1 mM indole
4	15 µM	1 mM Trp
5	15 µM	1 mM indole
6 (negative control)	-	-

The cultures were incubated at 37 °C and 140 rpm shaking. Samples were taken regularly and the OD_{600} was measured in triplicates. After ~3 hours of Trp depletion, 1 mM Trp or 1 mM indole were added respectively to flasks number 2- 5 (Table 20). Subsequent to that, samples were again taken regularly and the OD_{600} was measured in triplicates. After a total 52 hours of incubation, the cultures were discarded and an OD_{600} vs. time growth curve was compiled.

2.2.8. Testing the growth behaviour of BWEC47 under different conditions and plasmids

The plasmids of interest, pSTWS2, pSTWS1a and pQE-80L_est8 were retransformed into electrocompetent Trp auxotrophic *E. coli* BWEC47 cells as described in section 2.2.2 and used to

inoculate ONCs in 50 mL LB medium in 250 mL baffled shake flasks with appropriate antibiotic supplementation. After incubation at 37 °C and 110 rpm overnight, the OD_{600} of the cultures was determined. For each flask, amounts of cells corresponding to an OD_{600} of 0.1 in 100 mL were transferred into 15 mL tubes. The cells were harvested for 15 minutes at 3000 rpm and 10 °C and the supernatant was discarded. The pellets were resuspended in 1 mL M9 medium, taken from the corresponding flasks to be inoculated.

For the preparation of the M9 medium, up to 78 mL of ddH_2O were autoclaved in 250 mL baffled shake flasks, depending on the subsequently added amounts of 19 cAA stock and 100 mM tryptophan stock, which are depicted in Table 21. The other ingredients were combined in a sterile glass bottle as described in section 2.1.6.1, Table 14. 23 mL thereof were added to each flask, resulting in a total volume of 100 mL in each flask.

Table 21: Initial concentrations of Trp and 19 cAA stock in the cultures of the growth experiment and the corresponding amounts of Trp/indole which were added after Trp depletion.

culture	initial Trp concentration	initial concentration of 19 cAA	addition after Trp depletion
1 BWEC47 {pSTWS2} (negative control)	-	0.05 g/L each	-
2 BWEC47 {pSTWS2} (positive control)	0.5 mM	0.05 g/L each	-
3 BWEC47 {pSTWS2}	18 µM	0.05 g/L each	1 mM indole
4 BWEC47 {pSTWS1a}	18 µM	0.05 g/L each	1 mM Trp
5 BWEC47 {pSTWS1a}	18 µM	-	-
6 BWEC47 {pQE-80L_est8}	18 µM	0.05 g/L each	1 mM Trp
7 BWEC47 {pQE-80L_est8}	18 µM	-	-

The cultures were incubated at 37 °C and 140 rpm shaking. Samples were taken regularly and the OD_{600} was determined in duplicates. Upon depletion of Trp, corresponding to a plateau in the growth curve, 1 mM Trp or 1 mM indole were added to flasks number 3, 4 and 6 (Table 21). Subsequent to that, samples were again taken regularly and the OD_{600} was measured in duplicates. After a total 22.8 hours of incubation, the cultures were discarded and an OD_{600} vs. time growth curve was compiled.

2.2.9. High cell density SPI in *E. coli*

High cell density supplementation incorporation (SPI) was conducted based on the protocol developed by Fladischer (2013). The high cell density SPI of non-canonical amino acids (ncAAs) into target proteins was performed in *E. coli* strains, which were auxotrophic for the canonical-amino acid

(cAA) to be substituted. Every SPI experiment can be separated in two phases. In phase I (growth phase) the auxotrophic strain is grown with a limiting concentration of the cAA to be substituted. In phase II, after depletion of the cAA, the ncAA to be incorporated (analogue culture) or the cAA (canonical culture) is added to the medium and protein expression is induced by the addition of IPTG.

Phase I: Cell growth

The main cultures were always inoculated out of freshly prepared ONCs. For the ONCs 50 mL LB medium in 250 mL baffled shake flasks were supplemented with appropriate antibiotics and inoculated with single colonies from the corresponding agar plates or with 80 µL of the corresponding glycerol stocks. After incubation at 37 °C and 110 rpm the OD_{600} of the ONCs was determined. Amounts of cells corresponding to an OD_{600} of 0.1 in the volume of the main culture were harvested for 15 minutes at 3000 rpm and 10 °C. The pellets were resuspended in 500 µL LB and 500 µL of 70% (v/v) glycerol were added. For the main cultures, 100- 1000 mL M9 medium were prepared in 250- 2000 mL baffled shake flasks depending on the enzyme amount needed. The composition of the M9 medium is outlined in section 2.1.6.1, Table 10. For the expression of Est8 in Met auxotrophic *E. coli* BWEC50 cells and Trp auxotrophic *E. coli* BWEC47 cells, the medium was adapted as outlined in Table 11 and Table 15, respectively. Before inoculation 1 mL medium samples were taken which were later used as blanks in the OD_{600} measurements. The main cultures were inoculated to an initial OD_{600} of 0.1 and incubated at 37 °C and 140 rpm. Upon depletion of the cAA corresponding to a plateau in the growth curve, SDS samples were taken (non-induced probe). Therefore volumes of cell suspension equalling 1 OD_{600} were transferred into 1.5 mL Eppendorf tubes. The cells were pelleted for 2 minutes at 13,200 rpm and RT. The supernatants were discarded, and the cells were stored at -20 °C until the preparation of the SDS-PAGE.

Phase II: Expression and supplementation with the ncAA/ cAA

After the depletion phase, the cultures were supplemented with 1 mM ncAA to be incorporated (analogue culture) or 1 mM cAA (canonical culture). ncAA and cAA stocks were usually prepared in ddH_2O and solution was facilitated by adding a few drops of HCl conc. In case of the Trp analogue 1MeW, a 20 mM stock was prepared in 0.1 N NaOH as described by Opitz et. al. (2011). Target protein expression was induced with the addition of 0.5 mM IPTG. Protein expression was performed for 4 hours at 20 C or 28 °C as indicated and vigorous shaking. At the end of the expression phase the OD_{600} was determined in triplicates. For SDS-PAGE 2x 1 OD_{600} of each culture were transferred into 1.5 mL Eppendorf tubes and harvested for 2 minutes at 13,200 rpm and RT. The supernatants were discarded, and the cells were prepared for SDS-PAGE as described in section 2.2.11. For the incorporation of tryptophan analogues, which were *in-vivo* synthesized from the corresponding

indole analogues by the tryptophan synthase, the protein expression was performed for 21 hours. In case of these cultures, the OD_{600} was determined in triplicates after 4 h, 16 h and at the end of expression. At these time points, volumes of cell suspension equalling 1 OD_{600} were transferred into 1.5 mL Eppendorf tubes. The cells were pelleted for 2 minutes at 13,200 rpm and RT. The supernatants were discarded, and the cells were prepared for SDS-PAGE as described in section 2.2.11. For cell harvest, the cultures were transferred into tared centrifuge buckets and centrifuged for 20 minutes at 4000 x g and 4 °C. The supernatants were discarded and the pellets were resuspended in 30 mL 0.9% NaCl. The pellet solutions were transferred into 50 mL falcon tubes and harvested again for 15 minutes at 4000 rpm and 4 °C. After discarding the supernatant, the pellets were stored at -20 °C in case the protein purification was performed in a timely manner. For long-time storage, the pellets were stored at -80 °C until protein purification.

2.2.10. Protein purification

All proteins used in this work carried a 6xHis-tag and were purified by Ni-chelate affinity chromatography according to already established protocols (Baumschlager, 2013a, Wiltschi, 2010). Desalting/ buffer exchange was usually performed using PD10 columns. In case of the Trp parent and variant proteins, Est8[Trp], Est8[4NW] and Est8[7FW], buffer exchange was performed using the ÄKTA prime system.

2.2.10.1. Cell disruption by sonication

The deep-frozen cell pellets were thawed at RT and chilled on ice. 100 mL Ni-NTA lysis buffer, pH 8 were supplemented with 100 mg lysozyme, 100 µL of RNase A (10 mg/mL) and 50 µL of DNase (2 mg/mL) stock. 30 mL thereof were added to the thawed cell pellets, respectively. The cells were resuspended and incubated for 30 minutes on ice. Afterwards, the cells were disrupted by sonication. Therefore the cell suspensions were transferred into metal tubes bathed in ice cooled water to keep the temperature of the suspension low during sonication. The sonication was performed for 6 minutes with the following settings: duty cycle: 70%, output: 7-8, sonication tip Φ~1 cm. After sonication, the soluble parts were separated from the insoluble cell parts by centrifugation for 42 minutes at 40,000 x g and 4 °C. The lysates were separated from the pellets; the volumes were estimated and the lysates were filtered through 0.45 µm syringe filters. The sonication pellets were resuspended in 5 mL 2% (w/v) SDS. For SDS-PAGE, samples were taken from the lysates and pellet solutions and prepared as described in section 2.2.11. The SDS-samples were stored in the freezer until the preparation of the SDS-PAGE. The lysates were stored on ice until 6xHis-tag purification while the pellet solutions were discarded.

2.2.10.2. Ni-chelate chromatography by gravity flow

Affinity chromatography was performed using self-packed gravity flow columns with Ni-NTA agarose resin. One column was prepared for each enzyme variant as follows. In a first step, two filter discs were soaked in Surfact-Amps® detergent solution. Then one filter disc was inserted into an empty 10 mL PP column and moved to the bottom. 2 mL of properly mixed nickel-charged resin were loaded onto the filter disc and the liquid was drained which resulted in 1 mL column volume (CV). The column was filled with water; the second filter disc was inserted and moved down below the water surface until it was about 2 mm above the resin. The column was rinsed several times with ddH_2O, to even out the surface of the resin. The set-up of the chromatography equipment was conducted at RT, while the purification procedure was performed at 4 °C in the fridge. After the assembly of the gravity flow column, the resin was equilibrated with 20 CVs Ni-NTA lysis buffer and the flow through was discarded. Subsequently the lysate was loaded onto the column and the flow through was collected. The resin was washed with 50 CVs Ni-NTA wash buffer to remove unbound protein and other components. The wash fraction was collected. The bound protein was eluted with 4 CVs Ni-NTA elution buffer, the eluate fraction was collected and stored at 4 °C. For further use, the resin was regenerated with 30 CVs of 0.5 M NaOH to remove remaining protein from the resin. If a second sample of the same protein was purified with the column, the column was equilibrated with 20 CVs Ni-NTA lysis buffer and the procedure was performed as described above. For storage, the resin was rinsed with 10 CVs 30% (v/v) ethanol subsequent to regeneration, filled with 30% (v/v) ethanol, sealed and stored refrigerated. For SDS-PAGE, 80 µL samples of the flow through, wash and eluate fractions were taken and supplemented with 20 µL of 5 X SDS sample buffer. The SDS samples were stored at -20 °C until the preparation of the SDS-PAGE.

2.2.10.3. Desalting/ Buffer exchange

For better protein stability, the eluates were desalted immediately after purification using PD-10 desalting columns. At first the PD-10 columns were equilibrated with 25 mL of the desired buffer, in this case 50 mM Tris-HCl, pH 7. Then 3 mL of the purified protein were applied onto the columns and the flow through was discarded. Finally the proteins were eluted from the columns with 3.5 mL buffer. For SDS-PAGE, 80 µL eluate were supplemented with 20 µL of 5 X SDS sample buffer and stored at -20 °C until the preparation of the SDS-PAGE. The protein solutions were aliquoted into 1.5 mL Eppendorf tubes and stored at -20 °C. For further use, the columns were rinsed with ddH_2O, 20% (v/v) ethanol and regenerated with the desired buffer. In this way, 3-4 samples of the same protein were desalted using one column. For storage, the columns were filled with 20% (v/v) ethanol and stored at RT. In case of the Trp parent and variant proteins, Est8[Trp], Est8[4NW] and Est8[7FW],

buffer exchange was performed on an ÄKTA prime system. A HisTrap 26/60 Desalting column was used at a flow of 7 mL/min. The separation column was always equilibrated with 20 CV storage buffer, in this case 50 mM Tris-HCl, pH 7. At maximum, 15 mL of sample could be loaded via a 20 mL sample loop. 5 mL fractions were collected. Fractions with a UV_{280} signal were pooled and analysed with SDS-PAGE.

2.2.11. SDS-PAGE

All proteins were analysed by SDS-PAGE as described by Laemmli (1970). The polyacrylamide gels were composed of 5% stacking gel on top and 10% separating gel at the bottom (see section 2.1.6.2). To follow protein expression, 1 OD_{600} samples were taken in the course of the expression experiments, as described in section 2.2.9. The cells were pelleted for 2 minutes at 13,200 rpm and RT, the supernatant was discarded and the cells were resuspended in 80 µL ddH_2O and 20 µL of 5 X SDS sample buffer were added. Additionally, another 1 OD_{600} sample of each culture was harvested at the end of the expression phase, to analyse the soluble- insoluble distribution of the target proteins. Therefore the pelleted cells were resuspended in 200 µL CelLytic™ B. The mixture was incubated for 10 minutes at RT and 900 rpm shaking. Afterwards the mixture was centrifuged for 10 minutes at 13200 rpm and RT. 80 µL of the lysate were mixed with 20 µL of 5 X SDS sample buffer (soluble probe). The rest of the lysate was discarded. 200 µL 6 M urea were added to the pellet and the mixture was incubated for 10 minutes at RT and 900 rpm shaking. 80 µL thereof were mixed with 20 µL of 5 X SDS sample buffer (insoluble probe).

Table 22: Amounts of protein samples applied onto SDS gels.

sample	V_{load} [µL]
non-induced main culture	10
induced main culture	10
soluble (CelLytic™ B)	10
insoluble (CelLytic™ B)	10
lysate	2- 5
pellet	10
flow through	2.5- 10
wash	10
eluate	2- 5
eluate (after buffer exchange)	2- 5

To analyse the protein purification, 80 µL samples of the lysate, pellet, flow through, wash and eluate fractions were taken and mixed with 20 µL of 5 X SDS sample buffer. Prior to loading on the gel, all SDS samples were heated at 98 °C for 15 minutes and centrifuged shortly at 13,200 rpm. Afterwards

amounts of protein samples were loaded onto the gel as outlined in Table 22. In order to be able to estimate the size of the protein bands, 5 µL of Page Ruler™ Prestained Protein Ladder were loaded onto the gel (Figure 8). The stacking gel was run for 15 minutes at 100 V, the separating gel for 55 minutes at 180 V in 1 X SDS-PAGE running buffer.

NuPAGE® precast gels were used according to the manufacturer's instructions. The 1 OD_{600} samples which were taken in the course of the expressions, were pelleted as described above. Instead of dissolving the pellets in 80 µL ddH_2O, they were dissolved in 75 µL ddH_2O and 25 µL of 4 X LDS sample buffer were added. The soluble- insoluble distribution was analyzed with CelLytic™ B. Therefore the pelleted cells, equalling 1 OD_{600} were dissolved in 200 µL CelLytic™ B and treated as described above. 65 µL of the lysate and pellet thereof were mixed with 25 µL of 4 X LDS sample buffer and 10 µL of ddH_2O. The protein samples were loaded onto the SDS gels as indicated in Table 23 and gel electrophoresis was performed in 1 X MOPS SDS running buffer at 200 V for 50 minutes, according to the manufacturer's instructions.

Table 23: Amounts of protein samples applied onto precast NuPAGE® Bis-Tris mini gels.

sample	V_{sample} [µL]	4 X LDS sample buffer [µL]	10 X reducing agent [µL]	ddH_2O [µL]	V_{load} [µL]
non-induced main culture	≙ 1 OD_{600}	25	-	75	10
induced main culture	≙ 1 OD_{600}	25	-	75	10
soluble (CelLytic™ B)	≙ 1 OD_{600}	25	-	10	10
insoluble (CelLytic™ B)	≙ 1 OD_{600}	25	-	10	10
lysate	6	2.5	1	0.5	10
pellet	1- 1.2	2.5	1	5.3- 5.5	10
flow through	6	2.5	1	0.5	10
wash	10	4	1.6	0.4	16
eluate	1	2.5	1	5.5	10
eluate (after buffer exchange)	1	2.5	1	5.5	10

After gel electrophoresis all SDS gels were soaked in Coomassie staining solution for 15 minutes at RT and gentle agitation on a platform shaker. Subsequent to pouring off the Coomassie stain, the gels were rinsed with ddH_2O and soaked in destaining solution. To speed up destaining, the gels were heated up in a microwave oven. Finally the gels were scanned and analysed.

2.2.12. Determination of protein concentration

Before further analysis the protein concentrations were determined with the Bio-Rad protein assay. 4 mL Bio-Rad reagent were mixed with 16 mL of ddH_2O and filtered. The samples were diluted 1:5,

1:10 or 1:20 with the storage buffer, 50 mM Tris-HCl, pH 7. For the preparation of a calibration curve BSA dilutions with the following concentrations were prepared: 0.0625/ 0.125/ 0.25/ 0.5/ 1 mg/mL. 50 mM Tris-HCl, pH 7 was used as blank. 10 µL of the BSA dilutions, the samples and the blank were added to the wells of a 96 well microplate in triplicates. Following the addition of 200 µL Bio-Rad reagent solution per well, the plate was incubated for 5 minutes at RT and 400 rpm on a platform shaker. The absorption was determined at 595 nm. With the measured absorbance of the BSA dilutions and the corresponding BSA concentrations, a calibration curve was compiled. Out of the linear equation, the protein concentrations of the unknown samples were calculated.

In case of the Trp parent and variant proteins, Est8[Trp], Est8[4NW] and Est8[7FW], the eluates were concentrated using Vivaspin® centrifugal concentrators. One column was used exclusively for one enzyme variant. Initially, the columns were filled with ddH_2O up to the 5 mL mark and centrifuged for ~20 min at 3000 rpm and 4 °C. The water in the lower part of the concentrators was discarded and the washing procedure was repeated eight to ten times, to get rid of detergents in the columns originating from the production process. Subsequently, the eluates were filled into the concentrators and centrifuged for ~20 min at 3000 rpm and 4 °C. The flow through in the lower part of the columns was discarded. Afterwards, the protein concentrates were transferred into fresh 1.5 mL Eppendorf tubes and filled up to a volume of 1 mL with 50 mM Tris-HCl, pH 7.0. These protein solutions were stored at 4 °C.

2.2.13. *p*-Nitrophenyl ester assay

The specific enzyme activity was determined on the substrate *para*-nitrophenyl butyrate (*p*NPB) as described previously (Dellacher, 2012, Baumschlager, 2013b). Therefore, different dilutions of the substrate were prepared, using DMSO and 50 mM Tris-HCl, pH 7, as described in section 2.1.6.4. All the solutions containing the substrate were freshly prepared and carefully wrapped in cephalofoil to prevent excess exposure to UV light, since the substrate is very light sensitive. Then the substrate dilutions were aliquoted to 200 µL into 96 well microplates. The protein samples were thawed on ice and diluted to an uniform initial concentration. Starting from that, the protein solutions were subsequently diluted using 50 mM Tris-HCl, pH 7. 20 µL of enzyme dilution were quickly added to three rows of substrate dilutions in the 96 well microplates. As blank, 20 µL buffer were added to one row of substrate dilutions. The change in the absorbance at 405 nm was recorded for 5 minutes at RT (intervall: 14 seconds, reads: 22). For each substrate concentration, the measurements were performed in six repetitions.

2.2.14. Polymer degradation assay

The polymer degradation capability of the enzymes was tested on the polymeric substrate ecoflex® and the model substrate BaBTaBBa. Initially, amounts of substrates (ecoflex®: 10 mg; BaBTaBBa: 7.09 mg) were weighed into 1.5 mL Eppendorf tubes. The buffers, citrate-phosphate buffer, pH 6 and phosphate buffer, pH 8 were prepared as described in section 2.1.6.5. The enzymes were thawed on ice and the protein concentration was determined with the Bio-Rad protein assay as described in section 2.2.11. Enzyme- buffer solutions containing 0.6 µM enzyme (≙ 0.03102 mg/mL) were prepared and aliquoted to 1 mL into the 1.5 mL Eppendorf tubes containing the substrates. As blank, 1 mL buffer without enzyme was added to the substrate. The samples were incubated at 50 °C and 100 rpm shaking for at least 24 hours up to 120 hours depending on the substrate. For each pH and time point, the reaction mixtures were prepared in triplicates; the corresponding blanks were prepared in duplicates. The reactions were stopped after the given time periods by mixing 500 µL reaction mixture with 500 µL ice-cold methanol. For precipitation, the samples were incubated on ice for 15 minutes and subsequently centrifuged for 15 minutes at 13200 rpm and 0 °C. The supernatants (800 µL) were transferred into fresh 1.5 mL Eppendorf tubes and adjusted to pH 4 by adding 2 µL formic acid. Then the probes were stored at 4 °C for 1 hour. In case, precipitates were visible after this time period, the samples were centrifuged for 15 minutes at 13200 rpm and 0 °C. 600 µL of the reaction mixtures were transferred into HPLC vials and provided to Veronika Perz (ACIB, AG Gübitz) for HPLC analysis of the release products.

2.2.15. MALDI-MS analysis

To roughly determine the incorporation efficiency of the amino acid analogues into the proteins, samples were provided to the ACIB Core Facility Proteomics (Medical University of Graz) for analysis. Therefore 3 µg of the proteins were applied onto 4-12% precast NuPAGE® Bis-Tris gels and electrophoresis was performed according to the manufacturer's instructions. The bands of the appropriate size were excised and stored in 250 µL 10% (v/v) ethanol at -20 °C until MS analysis.

3. RESULTS AND DISCUSSION

3.1. Structural analysis identifies Met and Trp as suitable targets for SPI

It has been shown in previous studies that extracellular esterases derived from anaerobic microorganisms including *Clostridium botulinum*, have the ability to act on complex synthetic polymers. The esterase Est8, which is subject of this study, was identified in the course of NCBI genome and protein database searches. In preceding work it was successfully cloned, expressed and characterized. Also promising tests were performed on various polymeric substrates (Baumschlager, 2013a, b). In the next step it was of great interest to assess the possible effects of amino acid replacements on the polymer degradation capability of that enzyme. Therefore, the supplementation incorporation of non-canonical amino acids (ncAAs), which allows the global substitution of particular amino acid residues with synthetic analogues, was chosen.

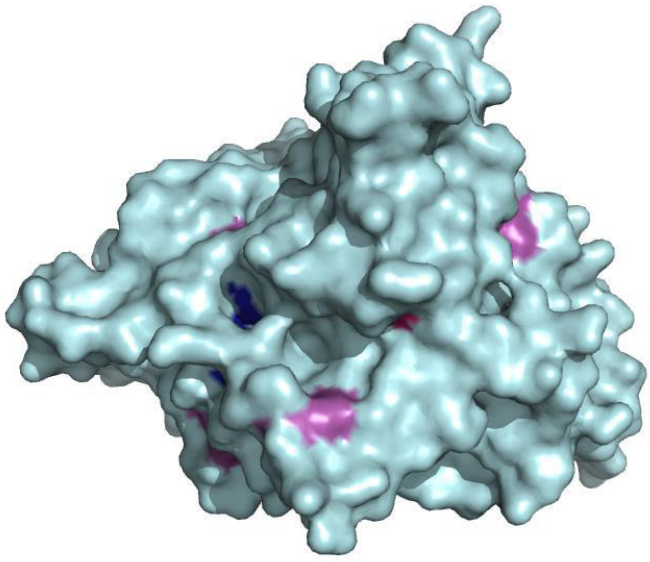


Figure 10: Surface representation of Est8, showing the methionine residues located on the surface.
The surface representation of Est8 was created in PyMol, based on the available crystal structure of the protein. The 5 methionine residues (Met54, Met240, Met352, Met418 and Met441) located on the surface are shown in pink. One tryptophan residue (Trp439) is also visible in this view and is shown in blue.

The first target which was selected for replacement studies was the sulphur containing amino acid methionine (Met, M). Met appears relatively rarely in proteins. It accounts for only 1.5% of the residues in all proteins of known structure (Rose et al., 1985). Furthermore, the Met residues are usually located in the protein interior, at positions inaccessible to the bulk solvent (Budisa, 2004). The analysis of the amino acid sequence of Est8 revealed a relatively high Met content. The amino acid sequence of Est8 is provided in the appendix in Figure 39. Of the total 461 amino acids of Est8, 10 amino acids are Met residues, equalling 2.2%. On the top of that, analysis of the available crystal structure of the protein showed that 5 out of 9 Met (the start codon is not included in the structure) are located on the surface of the protein (Figure 10).

When seen in the context of polymer hydrolysis, this surface localisation of the Met residues qualifies Met as an excellent target for incorporation studies. A major drawback of most polymeric compounds derived from fossil carbon sources is their resistance to biological degradation and their hydrophobicity. To be able to hydrolyse synthetic polymers, enzymes must adsorb to the surface or somehow get access to the polymer. Besides the active site, the overall surface of the enzyme might be crucial for polymer hydrolysis (Guebitz and Cavaco-Paulo, 2008).

When looking for a suitable Met analogue, norleucine (Nle) was the analogue of choice – not least because the successful expression of Est8 in the presence of Nle has already been shown in the preceding project lab (Schöffmann, 2013). In the structure of Nle a CH_2 group substitutes the sulphur, making it strongly hydrophobic and highly resistant to oxidation (Figure 11). It was expected that the incorporation of Nle, especially at Met positions on the surface, might change and even improve the interaction of the enzyme with the polymeric substrates.

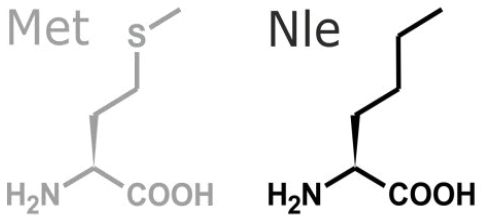


Figure 11: Structure of methionine (Met) and its analogue norleucine (Nle).
(Modified from Wiltschi and Budisa, 2007: Figure 4)

The second target which was selected for incorporation studies was the amino acid tryptophan (Trp, W). Similar to Met, Trp also occurs quite rarely, accounting for about 1% of all residues of globular proteins (Budisa, 2004). The analysis of the amino acid sequence of Est8, revealed a Trp content of 2.2% - 10 out of 461 amino acids of Est8 are Trp residues (Figure 39).From the 10 Trp residues, 6 are located on the surface of the protein. They are all centred on one side of the protein as shown in Figure 12a. Moreover, Trp129 and Trp274 are located on both sides of the zinc molecule – but too far away for direct interaction (Figure 12b).

In case of tryptophan, only a few tryptophan analogues are commercially available. Most of them are fairly expensive, making a routine application very costly. Exploiting the ability of the tryptophan synthase (TrpS) from *Salmonella typhimurium,* to convert indole analogues to the corresponding Trp analogues in a non-reversible condensation of indole and serine, this drawback can be circumvented (Würstlin, 2010, Hofmann, 2012).

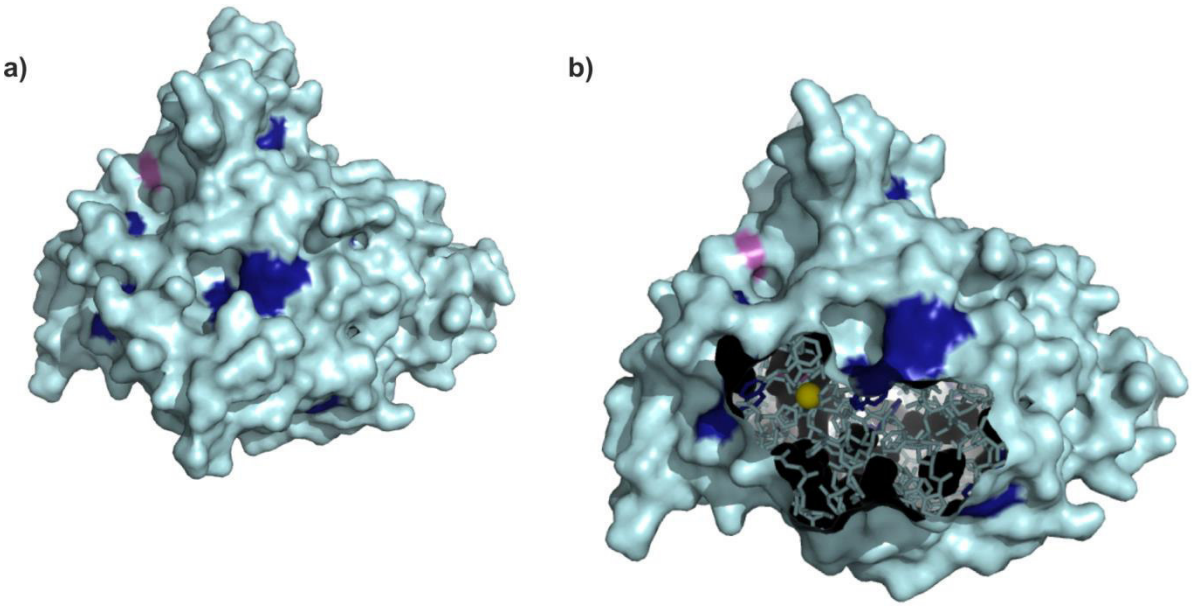


Figure 12: Surface representations of Est8, showing the tryptophan residues located on the surface and the zinc ion in the protein interior.
The surface representation of Est8 was created in PyMol, based on the available crystal structure of the protein. The 6 tryptophan residues (Trp129, Trp220, Trp274, Trp297, Trp319 and Trp380) located on the surface are shown in blue. One methionine residue (Met240) is also visible in this view and is shown in pink. In b) the surface representation was partially removed, to show the localization of the zinc in the protein interior.

Based on costs and availability the indole analogues 4-aminoindole (4NInd) and 7-fluoroindole (7FInd) were selected for the *in-vivo* synthesis of Trp analogues by TrpS (Figure 13). Compared to tryptophan, 4-aminotryptophan contains an additional NH_2 group which acts as an electron donating group. In contrast, 7-fluorotryptophan contains an additional fluorine atom, which acts as an electron withdrawing group. Since it is difficult to predict the global effects of amino acid replacements on the protein and its functions, frequently analogues of opposite properties, e.g. containing electron donating/ electron withdrawing groups, are tested. Additionally, the Trp analogue 1-methyltryptophan (1MeW) was chosen for a classical incorporation experiment, where the analogue is added directly. Due to the CH_3 group which is attached to the nitrogen, this analogue exhibits hydrophobic behaviour (personal communication P. Fladischer, ACIB Graz).

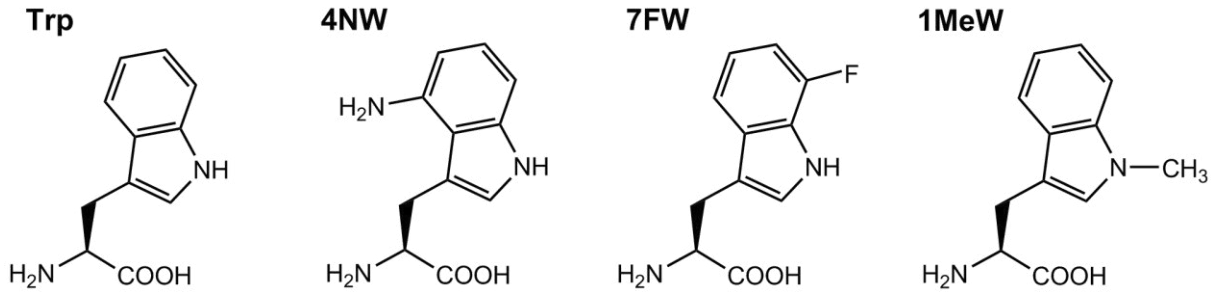


Figure 13: Structure of tryptophan (Trp) and its analogues 4-aminotryptophan (4NW), 7-fluorotryptophan (7FW) and 1-methyltryptophan (1MeW).

3.2. Methionine – a sulphur containing amino acid

3.2.1. Refinement of the expression protocol

As first target for replacement studies, the sulphur containing amino acid methionine was selected. In the course of the preceding project lab, the successful expression of Est8 in the presence of the Met analogues norleucine (Nle) and methoxinine (Mox) was already shown (Schöffmann, 2013). For further refinement of the protocol and to assess the influences of various parameters like medium composition, expression temperature and duration of expression on the expression level, the parent protein Est8[Met] was expressed in Met auxotrophic *E. coli* BWEC50 cells under different conditions. The experiments were conducted as described in section 2.2.4. The growth of the cultures was monitored by regular determination of the OD_{600}, which was then plotted over time (Figure 14). In the cultures 1 and 2, Est8[Met] was expressed in LB medium, following the protocol "Fermentation in shake flasks" from Armin Baumschlager (2013a). This was done to evaluate the differences between the expression in complete (LB) and minimal (M9) medium and to assess the effect of the baffles on the cell density and protein yield. The LB cultures showed quite similar growth behaviour with culture 2 always exhibiting lower OD_{600} values. This may be due to the fact that culture 1 was inoculated to an initial OD_{600} of 0.107, while the initial OD_{600} of culture 2 was 0.094. Subsequent to 20 hours of protein expression both LB cultures reached the same final OD_{600} of ~4. The determined cell wet weight (CWW) was 0.3 g higher for culture 1, with 2.3 g CWW compared to 2 g CWW for culture 2 (Table 24). The protein yield was also significantly higher for culture 1 with 4 mg protein/g CWW compared to 3.7 mg protein/g CWW for culture 2. Although the differences between the two cultures were not that drastic, these results upheld the decision to use baffled shake flasks for the cultivations. Especially in larger culture volumes oxygen supply can be a limiting factor. The baffles break the laminar flow in the flask, thus creating a turbulent flow which provides a larger surface for gas exchange. In this way the oxygen supply can be increased.

Besides the evaluation of the flasks, the medium composition was the main point to analyse in this expression test. Cultures 3, 4 and 5 were cultivated in minimal M9 medium under the same conditions, with the only difference being the amount of zinc sulphate added to the medium (Table 17). Regarding the progression of the OD_{600} over time, no difference was observable between culture 3, 4 and 5 (Figure 14). After Met depletion at an OD_{600} of ~3.6, Met was added, protein expression was induced and the cultures resumed growth. All three cultures reached the same final OD_{600} of ~6.7. When looking at the CWW (Table 24) culture 4, with a double amount of zinc sulphate in the medium yielded the highest CWW of 2.4 g, while the cultures 3 and 5 both yielded 2 g CWW.

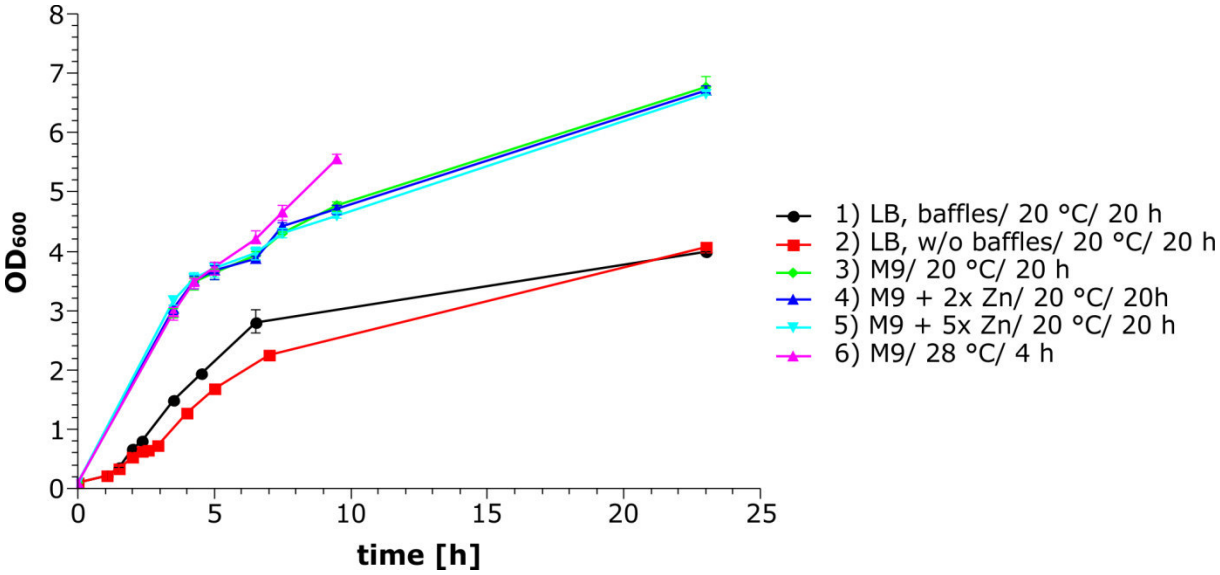


Figure 14: Progression of OD_{600} over time for Met auxotrophic *E. coli* BWEC50 {pET26b(+)_est8} cultivated in different growth media under different conditions.
Media were prepared as outlined in Table 10, Table 17 and described in section 2.2.4. Cultures were inoculated out of the same pre-culture of *E. coli* BWEC50 {pET26b(+)_est8} to an initial OD_{600} of 0.1 and incubated at 37 °C and 140 rpm shaking. OD_{600} was determined regularly in at least duplicate determination. Protein expression was induced in cultures 1 and 2 when reaching OD_{600} 0.8 and conducted as indicated. M9 cultures were cultivated until Met depletion, followed by the induction of protein expression after 5.5 h. Protein expression was performed in the M9 cultures at the temperatures and times given in the legend. Finally the average OD_{600} values were plotted over time. Error bars indicate the difference from the mean value to the highest and the lowest measured value of a duplicate or triplicate determination.

Comparing the amounts of purified protein gained from the expressions, the differences between the cultures 3, 4 and 5 became more obvious. Culture 3 yielded 7 mg, culture 4 yielded 12.5 mg and culture 5 yielded 9.9 mg purified Est8[Met]. The significant difference between the protein yields of the three cultures supports the conclusion, that the expression of the zinc containing enzyme Est8[Met] may somehow be improved in the presence of zinc sulphate. It seemed that the double amount of zinc sulphate was preferred to the five-fold amount. Despite the fact that, protein purification depends on so many different factors like the quality of the cell disruption, the course of the Ni-chelate chromatography itself and the desalting step, the difference due to the addition of zinc sulphate seemed to be provable.

Table 24: Cell wet weight, amount of purified protein and protein yields of Met auxotrophic *E. coli* BWEC50 {pET26b(+)_est8} cultures.

culture	cell wet weight [g]	purified Est8[Met] [mg]	protein yield [mg protein/g CWW]
1) LB, baffles/ 20 °C/ 20 h	2.3	9.2	4
2) LB, w/o baffles/ 20 °C/ 20 h	2	7.4	3.7
3) M9/ 20 °C/ 20 h	2.2	7.0	3.2
4) M9 + 2x Zn/ 20 °C/ 20 h	2.4	12.5	5.2
5) M9 + 5x Zn/ 20 °C/ 20 h	2.2	9.9	4.5
6) M9/ 28 °C/ 4 h	2.55	6.6	2.6

Additionally, a control or reference culture, culture 6 was also included in the expression test. This culture was cultivated under the conditions which were used in the preceding project lab (Schöffmann, 2013). The initial growth phase at 37 °C until Met depletion was followed by an expression phase at 28 °C for 4 hours. This culture reached a final OD_{600} of ~5.6, which can be explained by the higher expression temperature compared to the other cultures. The CWW was also quite high with 2.55 g but the amount of purified protein was with 6.6 mg expectably lower than for the other cultures. The resulting protein amounted to 2.6 mg protein/g CWW. Finally, when comparing the protein yields of the LB cultures to the M9 cultures, no considerable difference could be detected.

Based on these results it was decided to continue to perform the expressions in baffled shake flasks, as they ensure proper aeration of the *E. coli* cultures. Regarding the expression temperature it was decided to reduce it from 28 °C to 20 °C. Target protein expression at 28 °C leads to increased amounts of CWW, but not to substantially increased protein amounts. Furthermore, a higher expression temperature puts more stress on the expression strain. Since the culture with the double amount of zinc sulphate showed the highest protein yield, it was concluded to add an additional amount of 120 µg/L zinc sulphate to the medium, besides the 120 µg/L zinc sulphate which are already introduced through the trace elements. Despite the fact that longer expression times resulted in higher protein yields, the duration of expression should not be prolonged. Especially when expressing synthetic variants, it is absolutely necessary that the corresponding cAA is not available. To circumvent the release of the corresponding cAA into the medium due to cell death and lysis, the expression phase should be kept rather short.

3.2.2. Protein expression in the presence of Nle

In the course of the preceding project lab, protein expression in the presence of norleucine (Nle) was successful and yielded suitable protein amounts (Schöffmann, 2013). To further study the activity and behaviour of the enzyme on various substrates, this is a prerequisite. With Nle as a feasible analogue and the optimized protocol described in section 3.2.1 and Table 11, the high cell density supplementation incorporation was performed. The cultivation was conducted in semi-minimal M9 medium adapted for Est8 expression in Met auxotrophic *E. coli* BWEC50 (Table 11). The cultures showed exponential growth behaviour until Met depletion at OD_{600} ~4.5, followed by a stagnation of the OD_{600} for 30 minutes. The analog cultures were supplemented with Nle, while Met was added to the parent culture. After 4 hours of protein expression, no significant increase of the OD_{600} was detected for the analogue cultures. In contrast, the parent culture resumed growth after Met addition and reached a final OD_{600} of ~5.4. After cultivation, the cells were harvested and the

proteins were purified by Ni-chelate affinity chromatography as described in section 2.2.10. Target protein expression, solubility as well as the quality of the 6xHis-tag purification was analysed by SDS-PAGE. In Figure 15 the SDS-gel of the purification of the parent as well as the Nle variant is depicted. For both proteins, a prominent band at the expected size of ~52 kDa was found in the lysate (LY) (Figure 15, lanes 1, 5). The insoluble, pellet fractions (P) contained bands at this size as well (Figure 15, lanes 2, 6). The desalted eluates (E) (Figure 15, lanes 3, 7) contained in both cases only one band at the expected size of ~52 kDa, which indicates a high purity of the protein.

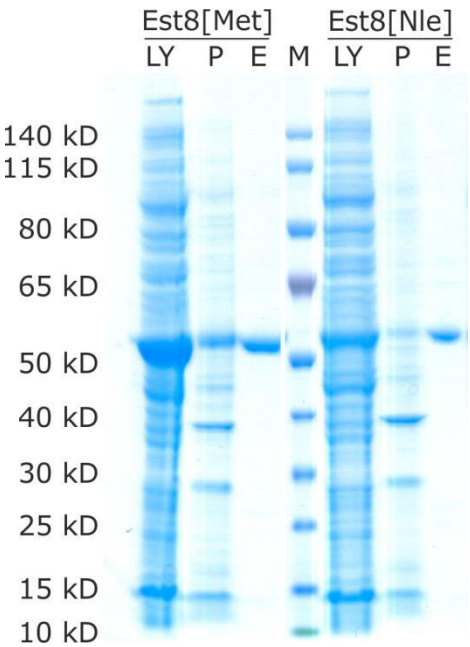


Figure 15: SDS-PAGE analysis of cell disruption and 6xHis-tag purification of Est8[Met] and Est8[Nle].
6 µL lysate (LY), 1 µL pellet solution (P) and 1 µL eluate (after buffer exchange) (E) were applied. Marker: Page Ruler™ Prestained Protein Ladder, 3 µL. 4- 12% NuPAGE® precast SDS gels, Coomassie stain. Abbreviations: norleucine (Nle). Gel image was edited, indicated by a gap between the assembled parts.

Finally the total protein concentration in the eluates was determined with the Bio-Rad protein assay. In the Est8[Met] eluate pool, which was gained from the purification of a total of 10.86 g CWW, a total protein amount of 11.3 mg was determined. The protein yield is therefore 1.04 mg protein/g CWW, which is rather low, compared to the values of the expression test in Table 24. The eluate pool of Est8[Nle] contained a total of 11.7 mg protein, which was obtained from the purification of 19.22 g CWW. The therefor calculated protein yield amounts to 0.609 mg protein/g CWW. The reason for these relatively low protein yields may be the high amounts of pellet (up to 5 g) which were applied for one cell disruption run. Most probably the cells were not completely disrupted. Furthermore, due to the high amounts of total protein which were in further consequence applied onto the gravity flow columns, it is likely that also here significant amounts of target protein were lost. Nevertheless, enough target protein was gained from the expressions for the following kinetic characterization, activity assays and MS analysis.

3.3. Tryptophan – the rarest amino acid

3.3.1. Subcloning of the esterase gene into pQE-80L

For the incorporation of Trp analogues, it was necessary to subclone the gene of interest from the pET26b(+) construct into the pQE-80L vector system first. Since most of the Trp analogues are not commercially available or very expensive it was decided to perform an *in-vivo* synthesis of Trp analogues using the tryptophan synthase (TrpS) from *Salmonella typhimurium.* Plasmids carrying the TrpS under the control of an inducible promoter (pSTWS1a) as well as for constitutive expression (pSTWS2) were available (Würstlin, 2010, Hofmann, 2012). Regarding the selection marker both plasmids were not compatible with the pET26b(+) construct carrying the est8 gene, which made a subcloning experiment necessary. As target vector the pQE-80L vector system (Qiagen, Cat. No. 32943), carrying an ampicillin resistance cassette was chosen.

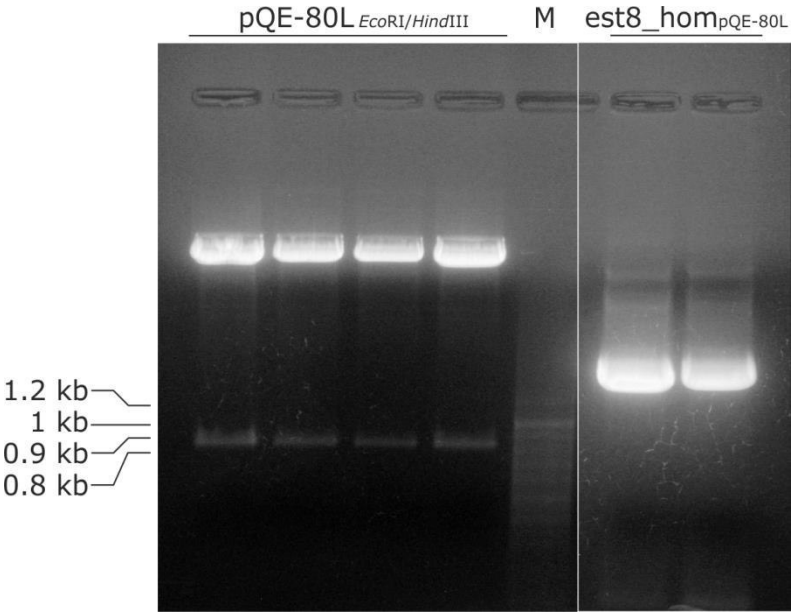


Figure 16: Preparative gel of target vector and insert for Gibson assembly.
The esterase gene was amplified out of the pET26b(+)_est8 construct by a standard three-step PCR, using the primers depicted in Table 3. The primers contained 36- 39 nucleotides homology regions for the pQE-80L target vector, which were added to the target gene in the course of the PCR. The target vector pQE-80L was amplified in *E. coli* TOP10F' and digested with *Eco*RI and *Hind*III. PCR products and cut vector preparations were supplemented with 6 X DNA Loading Dye and loaded onto the gel. Marker: O' GeneRuler DNA Ladder Mix, 10 µL. 1% agarose gel. The gel image was edited, indicated by a gap between the assembled parts.

Subcloning was performed as described in section 2.2.4 using the Gibson cloning strategy (Gibson et al., 2009). The esterase gene was amplified out of the pET26b(+)_est8 construct and simultaneously equipped with homology regions for the pQE-80L target vector in a standard three-step PCR. The hereby generated fragment was purified by agarose gel electrophoresis. The corresponding gel image is depicted in Figure 16. In lanes 6 and 7 the PCR products of est8 were applied. Identification of the size of the fragments based on the marker (lane 5), was hardly possible due to the high order

of degradation of the same. Nevertheless it was assumed, that the distinct bands located above the 1.2 kb lane of the marker, can be assigned to the PCR products of the esterase gene (1483 bp). The target vector for Gibson assembly pQE-80L was amplified in *E. coli* TOP10F' cells, digested with *Eco*RI and *Hind*III and purified by agarose gel electrophoresis (Figure 16). Four vector preparations were applied onto the agarose gel (lanes 1- 4). In all four lanes distinct bands were visible, being notably bigger in size than the PCR products of the esterase gene. It was assumed, that also these fragments had the correct size of 4658 bp. Furthermore, bands of smaller size of ~850 bp were also visible in all four lanes, accounting for the part of the vector (TT2_h6) which was removed by the linearization with *Eco*RI and *Hind*III. After gel analysis, the bands of the desired sizes (4658 bp pQE-80L$_{EcoRI/HindIII}$; 1483 bp est8_hom$_{pQE-80L}$) were cut out of the gel and the DNA was extracted using standard procedures. For verification of the correct size and purity of the cut vector and PCR products, an analytical gel was run (Figure 17).

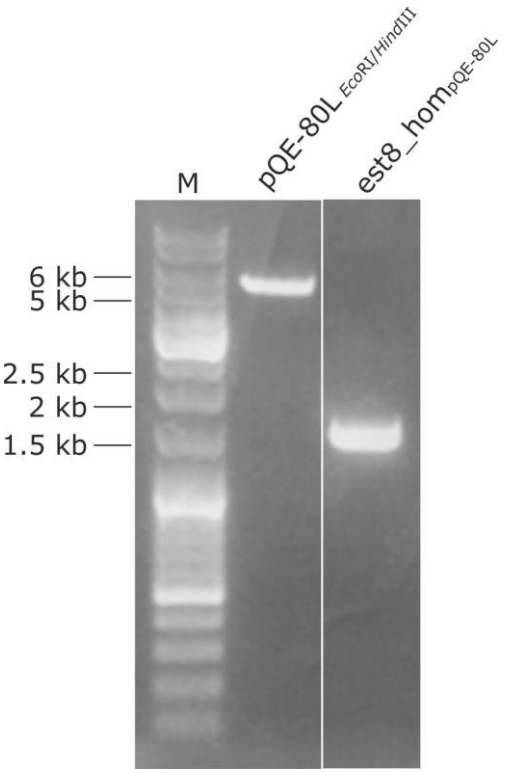


Figure 17: Analytical gel of target vector and inserts for Gibson assembly, after gel extraction.
*Eco*RI/ *Hind*III cut target vector pQE-80L and the gene of interest were purified by agarose gel electrophoresis (Figure 16) and extracted from the gel using the Wizard SV Gel and PCR Clean-Up System. Constructs of the same type were pooled and the nucleic acid concentrations were determined with the NanoDrop 2000c Spectrophotometer. 200 ng of each construct were supplemented with 6 X DNA Loading Dye and loaded onto the gel. Marker: O' GeneRuler DNA Ladder Mix, 10 µL. 1% agarose gel.

In lane 2, where the *Eco*RI and *Hind*III cut target vector pQE-80L was applied, a band at the expected size of ~5000 bp was visible. In lane 3, also a distinct band at the expected size of ~1500 bp was visible. Since the DNA fragments had the appropriate size and required purity, a Gibson assembly

was performed. Afterwards, the assembled DNA fragments were transformed into electrocompetent *E. coli* TOP10F' cells for amplification. Positive clones were selected on ampicillin containing media plates, where intense growth was visible after incubation overnight. The plasmids were isolated applying standard procedures and checked for their correct assembly by restriction enzyme digestion as well as sequencing. Figure 18 shows an agarose gel where four vector preparations of pQE-80L_est8, after restriction enzyme digestion with *Eco*RI and *Hind*III, were applied (lanes 2- 5). When comparing to the marker (lane 1), clear bands can be seen at the sizes of ~5000 bp and ~1500 bp. Those are the expected bands, which account for the vector backbone (4751 bp) and the insert est8 (1386 bp). Two expression constructs were sent for sequencing: pQE-80L_est8, lane 3 and 5. According to the sequencing results, both clones had the correct sequence.

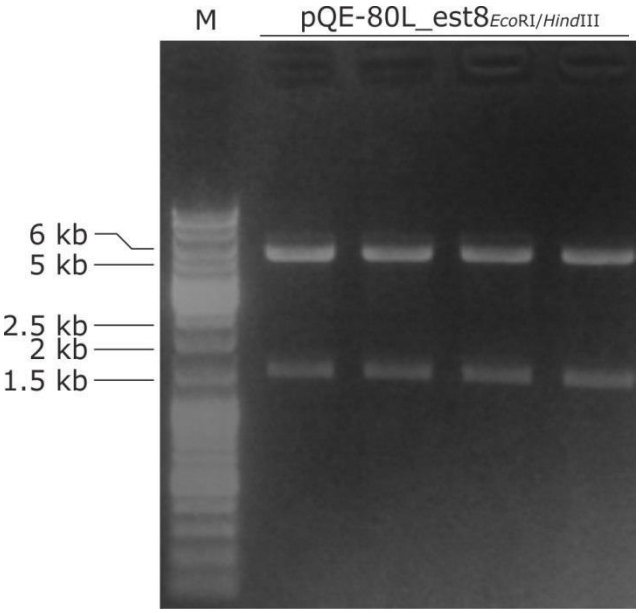


Figure 18: Restriction enzyme digestion of pQE-80L_est8 expression constructs after Gibson assembly.
4x 100- 200 ng plasmid preparation of the assembled DNA molecules were digested with *Eco*RI and *Hind*III. 10 µL of the cut fragments were loaded onto the gel. Marker: O' GeneRuler DNA Ladder Mix, 10 µL. 1% agarose gel.

3.3.2. Controlling growth arrest of BWEC47 in late log phase

The high cell density supplementation incorporation of Trp analogues into the target protein was performed in Trp auxotrophic *E. coli* BWEC47 cells. The incorporation protocol which was chosen for these experiments (Fladischer, 2013) utilizes a limiting concentration of yeast extract (YE) to control Trp dependent growth arrest in late log phase. Due to lack of prior knowledge, this optimal YE concentration to reach late log phase had to be determined first.

The first YE titration experiment was performed with Trp auxotrophic *E. coli* BWEC47 containing the pSTWS2 plasmid for the constitutive expression of the tryptophan synthase (TrpS). The experiment was performed as described in section 2.2.6. The overnight culture (ONC) was inoculated from freshly prepared electrocompetent cells of BWEC47 {pSTWS2}. After incubation the OD_{600} of the culture was 13.28, which was about twice as high as it is usually expected for an ONC. Nevertheless the ONC of BWEC47 {pSTWS2} was used to inoculate main cultures with minimal M9 medium containing the following YE dilutions: 1 g/L, 2 g/L, 3 g/L and 4 g/L. The positive control contained 1 g/L YE and an excess amount of 10 mM Trp, to see how the strain behaved in the presence of unlimited amino acid supply. For each flask containing a different YE concentration, the OD_{600} was monitored and a growth curve was compiled (Figure 19).

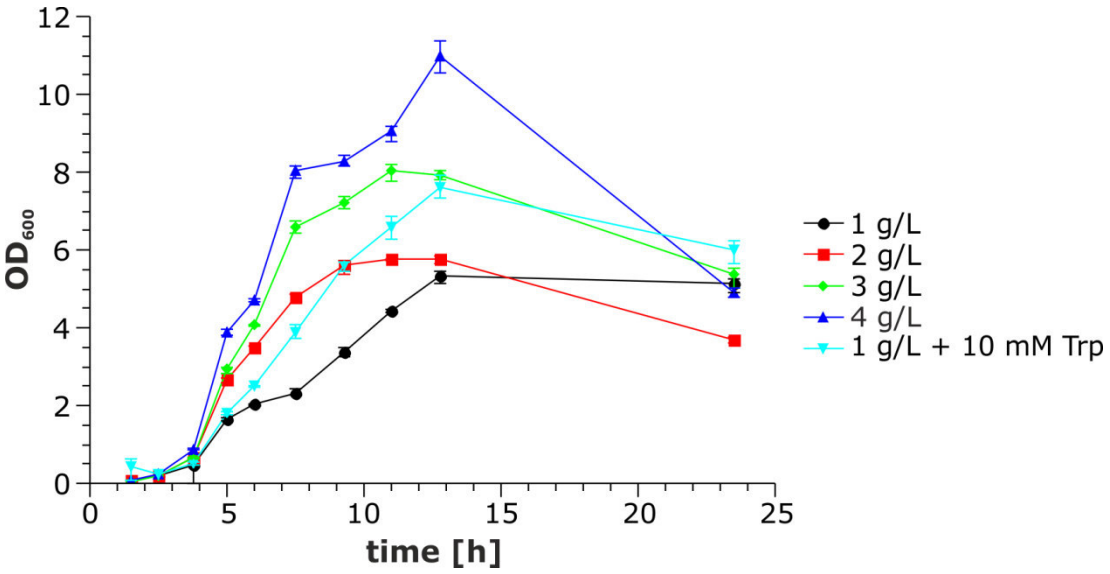


Figure 19: Progression of OD_{600} over time for Trp auxotrophic *E. coli* BWEC47 {pSTWS2} in cultures supplemented with different YE concentrations.
1.1 X M9 medium was prepared and supplemented with different amounts of yeast extract (YE), as outlined in Table 12 and described in section 2.2.6. Final YE concentrations in the cultures were: 1 g/L, 2 g/L, 3 g/L and 4 g/L. The positive control contained 1 g/L YE + 10 mM Trp. Cultures were inoculated out of the same pre-culture of *E. coli* BWEC47 {pSTWS2}, incubated at 37 °C, 140 rpm and OD_{600} was measured after 1.5 h, 2.5 h, 3.75 h, 5 h, 6 h, 7.5 h, 9.25 h, 11 h, 12.75 h and 23.5 h in triplicate determination. The ability of the strain to resume growth after Trp depletion was tested in the culture containing 1 g/L YE, by adding 10 mM Trp after 6.7 h. Finally the average OD_{600} values were plotted over time. Error bars indicate the difference from the mean value to the highest and the lowest measured value of a triplicate determination.

From the collected data, it was not possible to detect a correlation between the YE in the medium and the final OD_{600} of the cultures. According to expectations the cultures should show uniform growth in the first phase followed by a stagnation of the OD_{600} at a certain value, depending on the amount of YE in the medium. Cultures with high YE concentrations should therefore reach higher final OD_{600} values than those with lower YE concentrations. Unfortunately this was not the case. Almost none of the cultures showed a stagnation of the OD_{600}; the cultures containing 4 g/L, 3 g/L, 2 g/L YE and the positive control even showed a decrease of the OD_{600} between 12.75 hours and 23.5 hours of incubation. Only the culture which contained 1 g/L YE behaved as expected. Around 5 hours, slowed growth accompanied by a stagnation of the OD_{600} was observed. To control the ability of the strain to resume growth after Trp depletion, 10 mM Trp were added after 6.7 hours. The strain behaved as anticipated and resumed growth until a final OD_{600} of ~5 was reached.

Concluding from these results it was assumed that the chosen YE concentrations were possibly too high for the Trp auxotrophic strain. A further aspect which was taken into account was the possible exploitation of unknown indole sources in the YE by the constitutively expressed TrpS. Following this thought, Trp would be synthesized from indole by the TrpS consequently preventing Trp depletion. It was decided to repeat the YE titration experiment of Trp auxotrophic *E. coli* BWEC47 {pSTWS2} with lowered YE concentrations. Interactions of the TrpS with possible indole sources in the YE should also become apparent in the course of these experiments.

The second YE titration experiment of Trp auxotrophic *E. coli* BWEC47 {pSTWS2} was performed with lowered YE concentrations of 0.25 g/L, 0.5 g/L and 1 g/L as described in section 2.2.6. Again the OD_{600} of the ONC was rather high, with a measured value of 18.02. Upon monitoring of the OD_{600} of the main cultures, initially different growth behaviour, as expectable from the different amounts of YE added, was observable (data not shown). Although none of the cultures exhibited Trp depletion, which was indicated by a continuous increase of the OD_{600} over 11.25 hours. After 24 hours of incubation the cultures reached the same high final OD_{600} of ~6, independent from the added amount of YE. This result further confirmed the assumption that the constitutively expressed TrpS may exploit unknown indole sources in the YE to synthesize Trp. It was therefore decided to try a medium without YE to control Trp dependent growth arrest in late log phase.

The titration of the optimal Trp concentration was performed as outlined in section 2.2.7. M9 medium (Table 13) was supplemented with 15 µM or 30 µM Trp as indicated in Table 20. Those values were derived from previous experiments, in which 7 µM cAA was found to be the limiting concentration for the multi-auxotrophic BWEC1 to reach an OD_{600} of 1 (personal communication

P. Fladischer, ACIB Graz). Since the aim was to deplete the cultures at an OD_{600} of 3, an optimal Trp concentration was presumed between 15 µM and 30 µM. The OD_{600} of the ONC was yet again quite high with a value of 21. After induction, the main cultures exhibited a greatly expanded lag phase of over 9 hours (data not shown). Only after 23.7 hours of incubation a significant growth was observe- as well as measureable. During the further incubation, the cultures behaved as expected. The OD_{600} was increasing varyingly strong, depending on the amount of Trp. After ~3 hours of Trp depletion, 1 mM Trp or 1 mM indole were added. Subsequent to that, the cultures resumed growth.

Due to the very high OD_{600} values which were determined for the ONCs by then and the extremely expanded lag phase, it was decided to elaborate on finding causes for this unusual behaviour. Therefore, titration experiments with lowered Trp concentrations, empty strains and with other plasmids were conducted. The results of those experiments (data not shown) led to the assumption that the *E. coli* BWEC47 {pSTWS2} glycerol stocks from which the experiments described in section 2.2.6 and 2.2.7 were started, were contaminated.

To be on the safe side, the plasmids of interest, pSTWS2, pSTWS1a and pQE-80L_est8 were retransformed into electrocompetent Trp auxotrophic *E. coli* BWEC47 cells for a new round of growth tests. The experiments were performed as described in section 2.2.8. Since the previous experiments led to the conclusion that a reduction of the aimed OD_{600} for depletion from OD_{600} 3 down to OD_{600} 2 would be suitable, the initial Trp concentration was set at 18 µM Trp. This concentration was again derived from the multi-auxotrophic BWEC1, which needs 7 µM cAA to reach an OD_{600} of 1, and from titration experiments which are not outlined in this work. This time, the OD_{600} of the ONCs was in a reasonable range between 3.5 and 4.7. Upon inoculation of the main cultures, the OD_{600} was monitored and a growth curve was compiled (Figure 20). The strain, carrying the constitutive expression construct pSTWS2 for the TrpS (curve 3, Figure 20) showed slowed growth between 6 and 9 hours of incubation. Although, no real growth arrest was observable 1 mM indole was added after 8.8 hours. The culture resumed growth and reached the same final OD_{600} as the positive control (curve 2, Figure 20). The negative control (curve 1, Figure 20) showed almost no change in the OD_{600}, but grew over night.

The strain carrying the inducible expression construct pSTWS1a for the TrpS was cultivated once with (curve 4, Figure 20) once without 19 cAA supplementation (curve 5, Figure 20). The culture with 19 cAA supplementation (curve 4, Figure 20), showed growth arrest at OD_{600} ~2 between 6 and 9 hours of incubation. After Trp supplementation the culture restarted to grow and reached a final OD_{600} of ~4. The culture without 19 cAA supplementation (curve 5, Figure 20) levelled out at an OD_{600}

value of 0.5- 0.6, after inoculation to an initial OD_{600} value of ~0.0930. The strain carrying the pQE-80L_est8 construct was also cultivated once with (curve 6, Figure 20) once without 19 cAA supplementation (curve 7, Figure 20). Both cultures showed expectably similar growth behaviour as the BWEC47 {pSTWS1a} cultures, with a reduced final OD_{600} of ~3 after Trp supplementation. The BWEC47 {pQE-80L_est8} culture without 19 cAA supplementation (curve 7, Figure 20) levelled out at an OD_{600} value of 1, after inoculation to an initial OD_{600} value of ~0.106. The differences of the growth curves, measured for the strains carrying the pSTWS1a plasmid compared to the ones carrying the pQE-80L_est8 plasmid, can obviously be traced back to the difference in the initial OD_{600} values.

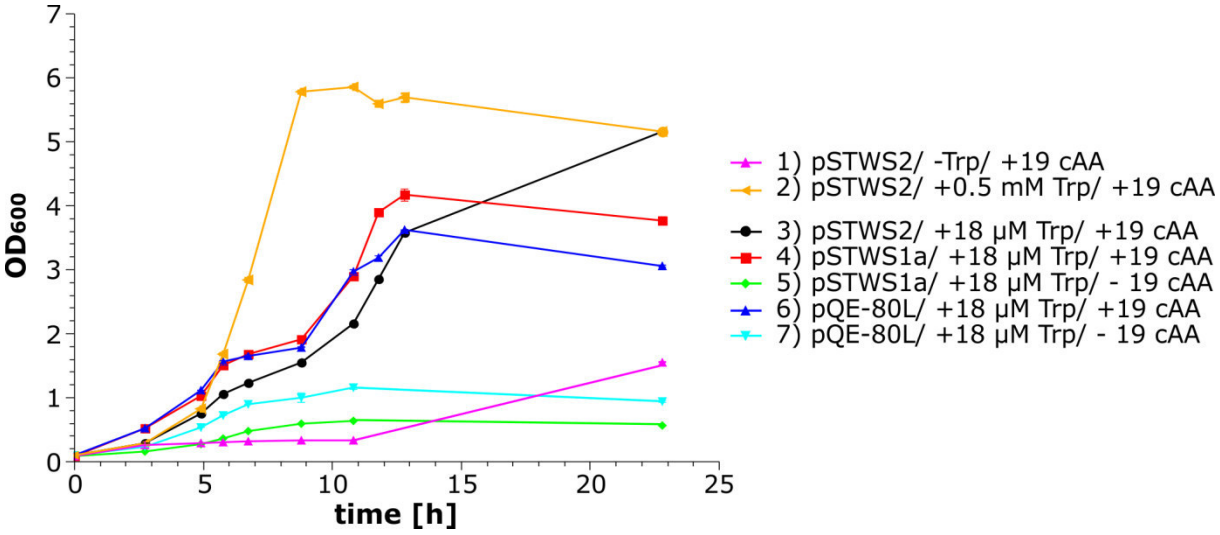


Figure 20: Progression of OD_{600} over time for Trp auxotrophic *E. coli* BWEC47 under different conditions and with different plasmids.

Plasmids of interest, pSTWS2, pSTWS1a and pQE-80L_est8 were transformed into electrocompetent Trp auxotrophic *E. coli* BWEC47 cells. Transformed cells were directly used to inoculate ONCs in LB medium. M9 medium for the main cultures was prepared as outlined in Table 12 and described in section 2.2.8, supplemented with different amounts of Trp and 19 cAA stock. For the strain carrying the pSTWS2 plasmid, a negative control (1) without Trp and a positive control (2) with an excess amount of 0.5 mM Trp were prepared. Cultures were inoculated to an initial OD_{600} of 0.1, incubated at 37 °C, 140 rpm shaking and OD_{600} was measured after 2.75 h, 4.92 h, 5.75 h, 6.75 h, 8.8 h, 10.8 h, 11.8 h, 12.8 h and 22.8 h in duplicate determination. The ability of the strains to resume growth after Trp depletion was tested in the cultures 3, 4 and 6. 1 mM indole was added to culture 3, while 1 mM Trp was added to cultures 4 and 6 after 8.8 h. Finally the average OD_{600} values were plotted over time. Error bars indicate the difference from the mean value to the highest and the lowest measured value of a duplicate determination. At time points 11.8 h, 12.8 h, OD_{600} was only determined for the cultures 2, 3, 4 and 6.

Concluding from these results, the expression construct pSTWS2 for the constitutive expression of the TrpS was ruled out. The incomplete depletion and the rapid increase in OD_{600} overnight imposed the educated guess that the constitutively expressed TrpS may somehow have an effect on the growth behaviour or may exploit some source in the medium for Trp synthesis. Since the BWEC47 strains carrying the pSTWS1a and the pQE-80L_est8 constructs, behaved as expected in a rather similar way, it was decided to use the inducible expression construct pSTWS1a for further experiments. From the available data (Figure 20), an optimal Trp concentration of 18 µM to reach an OD_{600} of 2 can be assumed for BWEC47 carrying inducible expression plasmids.

Due to the fact that yeast extract is a simple and convenient cAA source, a YE titration experiment was performed for the Trp auxotrophic *E. coli* BWEC47 strain carrying the pSTWS1a plasmid. The experiment was performed as described in section 2.2.6; M9 medium was prepared as outlined in Table 12 containing the following YE dilutions: 0.1 g/L, 0.25 g/L, 0.50 g/L, 0.75 g/L and 1.0 g/L. The positive control contained 1 g/L YE and an excess amount of 10 mM Trp, to see how the strain behaved in the presence of unlimited amino acid supply. For each flask containing a different YE concentration, the OD_{600} was monitored and a growth curve was compiled (Figure 21). Initially the cultures grew varying strongly, depending on the amount of YE. Nevertheless all cultures, including the positive control, levelled out a final OD_{600} around 4.8 between 12.25 and 23.75 hours. These results showed that YE was not suitable to control Trp dependent growth arrest in BWEC47 {pSTWS1a}. For the further expression of enzymes containing Trp analogues, it was therefore decided to go back to the medium with cAA supplementation and a limiting concentration of 18 µM Trp, which was found to be to optimal concentration to deplete the cultures at an OD_{600} of 2.

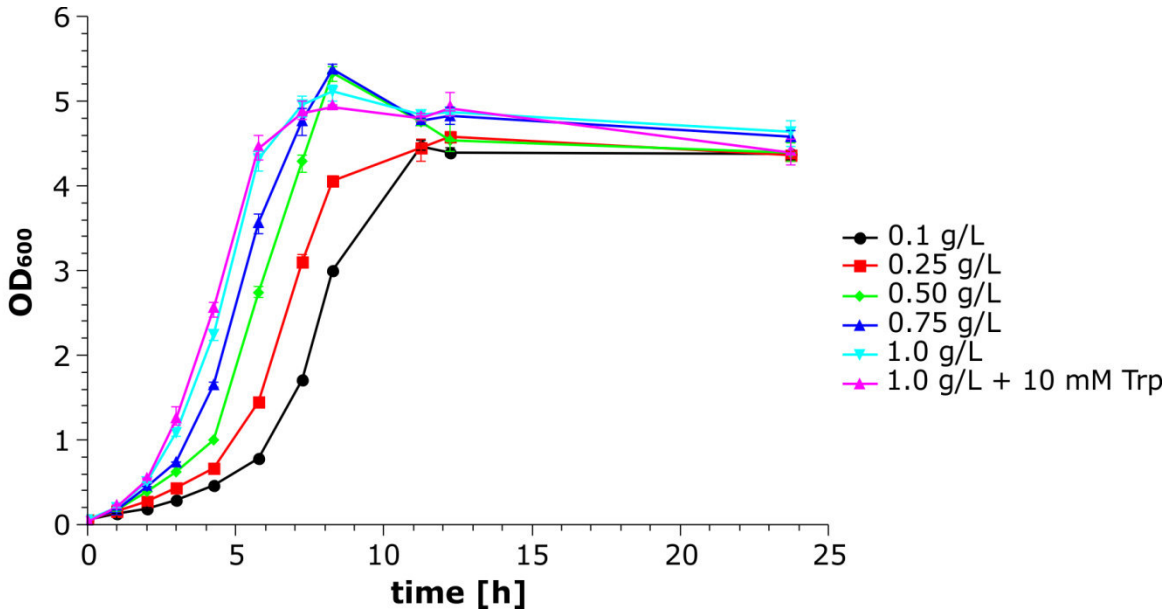


Figure 21: Progression of OD_{600} over time for Trp auxotrophic *E. coli* BWEC47 {pSTWS1a} in cultures supplemented with different YE concentrations.
1.1 X M9 medium was prepared and supplemented with different amounts of yeast extract (YE), as outlined in Table 12 and described in section 2.2.6. Final YE concentrations in the cultures were: 0.1 g/L, 0.25 g/L, 0.5 g/L, 0.75 g/L and 1 g/L. The positive control (1 g/L+10 mM Trp) contained 1 g/L YE and an excess amount of 10 mM Trp. The cultures were inoculated out of the same pre-culture of *E. coli* BWEC47 {pSTWS1a}, incubated at 37 °C, 140 rpm shaking and OD_{600} was measured after 1 h, 2 h, 3 h, 4.25 h, 5.75 h, 7.25 h, 8.25 h, 11.25 h, 12.25 h and 23.75 h in triplicate determination. Finally the average OD_{600} values were plotted over time. Error bars indicate the difference from the mean value to the highest and the lowest measured value of a triplicate determination.

3.3.3. *In-vivo* synthesis and incorporation of Trp, 4NW and 7FW with TrpS

After verification of suitable parameters to control growth arrest of Trp auxotrophic *E. coli* BWEC47 in late log phase (section 3.3.2), a supplementation incorporation of *in-vivo* synthesized Trp and Trp analogues into Est8 was performed. In the used strategy, the *in-vivo* synthesis of Trp analogues is catalysed by the tryptophan synthase (TrpS) from *Salmonella typhimurium*. In this way, indole and indole analogues are converted to the corresponding Trp analogue in a non-reversible condensation of indole and serine. For the following incorporation experiments, the indole analogues 4-aminoindole and 7-fluoroindole were selected.

Trp auxotrophic *E. coli* BWEC47 cells carrying the inducible expression construct for the TrpS (pSTWS1a) as well as the pQE-80L_est8 vector construct, were grown in minimal M9 medium (for composition see Table 15) containing a limiting concentration of 18 µM Trp. In late log phase, the cultures were depleted of Trp which was accompanied by growth arrest at OD_{600} ~1.4. According to the growth experiments which are described in section 3.3.2, growth arrest was expected at a higher OD_{600} of 2. To make sure that the cultures were completely depleted of Trp, the OD_{600} was monitored over a short starvation phase of ~30 minutes. After no significant change in the OD_{600} was measureable, the cultures were supplemented with indole or one of the indole analogues, expression of the TrpS as well as target protein expression was induced and the cultures were incubated for 21 hours. 4 hours, 16 hours and 21 hours after induction, an increase of the OD_{600} in all three cultures was observable, reaching a final OD_{600} of 4.3 in case of the parent culture. The cultures, in which the protein was expressed in the presence of indole analogues, reached lower final OD_{600} values between 1.7 and 2.7. This resumption of growth was quite unexpected for the cultures supplemented with indole analogues, because the strain should theoretically not be able to grow on ncAAs. However, the biomass which was gained from the experiments was with ~2.6 g CWW in each of the variant cultures significantly lower than the CWW of the parent culture with ~6 g.

Target protein expression was verified by SDS-PAGE analysis. The corresponding gel image is depicted in Figure 22. Tight regulation of the inducible expression systems was checked by applying a sample of the Est8[Trp] parent culture, which was taken right before induction (Figure 22, lane 1). Two distinct bands slightly above the 40 kDa and the 25 kDa marker were visible, accounting for the α- and β- subunits of the tetrameric TrpS. This leakiness of the trc promoter may even be beneficial, since low levels of TrpS are available upon addition of indole and indole analogues and induction of target protein expression. In this way indole and indole analogues can be promptly converted to the corresponding Trp analogues. The pQE-80L vector construct, carrying the gene of interest, showed tight regulation. No over-expression band at the expected size of ~52 kDa was visible

(Figure 22, lane 1). The samples that were taken in the course of protein expression showed clear bands at the expected size of ~52 kDa (Figure 22, lanes 3- 11). For the Est8[Trp] parent culture where indole was added, the expression band was most significant, increasing in intensity from the 4 hour to the 21 hour sample (Figure 22, lanes 3, 6, 9). The samples which were taken of the variant cultures, Est8[4NW] and Est8[7FW] also showed clear expression bands at the expected size of ~52 kDa, being notably weaker than the expression bands of the parent protein. The α- and β-subunits of the co-expressed TrpS accounted for the most prominent bands at the sizes of ~40 kDa and ~25 kDa in lanes 3- 11. From these observations it can be concluded that the simultaneous expression of the TrpS and the target protein Est8 in the presence of indole and indole analogues was successful.

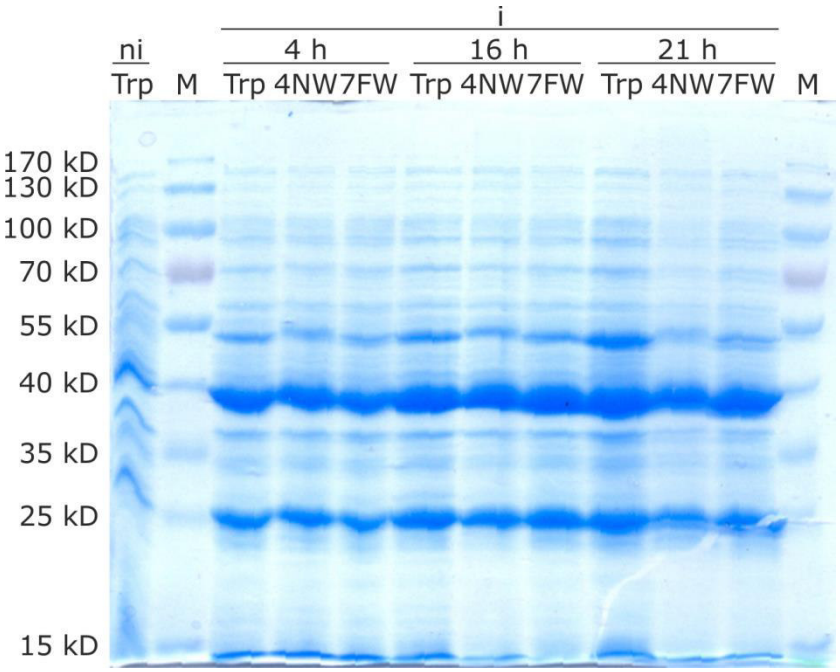


Figure 22: Expression of Est8 in the presence of Trp and Trp analogues, which were *in-vivo* synthesized from the corresponding indole and indole analogues by the co-expressed tryptophan synthase.
0.1 OD_{600} units were loaded of the non-induced (ni) sample, taken right before induction and of the induced (i) samples, taken after 4 h, 16 h and 21 h expression. Marker: Page Ruler^{TM} Prestained Protein Ladder, 5 µL. 10% SDS gel, Coomassie stain. Abbreviations: 4-aminotryptophan (4NW), 7-fluorotryptophan (7FW).

Despite the low levels of protein expression, the variants as well as the parent protein were purified by Ni-chelate chromatography. Samples were taken from the different fractions of the purification process and analysed by SDS-PAGE (Figure 23). Figure 23a shows the SDS gel of the purification of the Est8[Trp] parent protein. The lanes where the lysate, pellet and flow through samples (Figure 23a, lanes 1, 2, 3) were applied were massively overloaded. Nevertheless a clear over-expression band at the expected size of ~52 kDa was visible in the lysate fraction (Figure 23a, lane 1), accompanied by two other massive bands at the sizes of ~40 kDa and ~25 kDa belonging to the TrpS. The lane, where the pellet sample was applied (Figure 23a, lane 2), was accidently overloaded with the 6.25 x

amount, making a profound statement about the solubility behaviour impossible. While comparably low amounts of the protein were found in the flow through (Figure 23a, lane 3), significant amounts of the target protein were removed from the column during the washing step. This is indicated by a significant band at the expected size of ~52 kDa in the wash fraction (Figure 23a, lane 4). The eluate of the purification of Est8[Trp] showed a significant over-expression band at the expected size of ~52 kDa, accompanied by further unspecific bands of larger and smaller size (Figure 23a, lane 5).

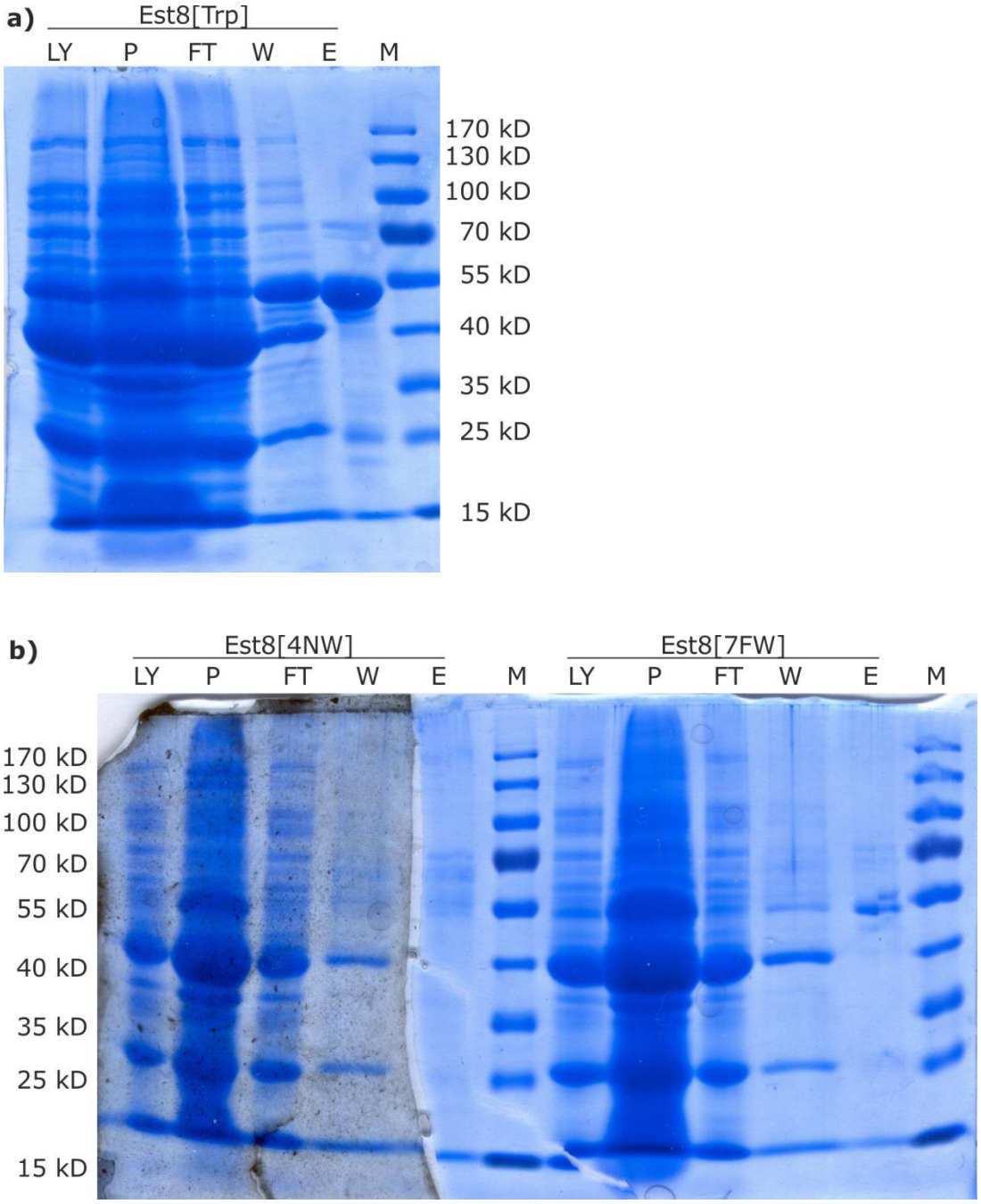


Figure 23: SDS-PAGE analysis of cell disruption and 6xHis-tag purification of Est8[Trp] and Trp variants.
2 µL lysate (LY), 10 µL pellet solution (P), 2.5 µL flow through (FT), 10 µL wash (W) and 2 µL eluate (before buffer exchange) (E) were applied. Marker: Page Ruler^{TM} Prestained Protein Ladder, 5 µL. 10% SDS gel, Coomassie stain. Abbreviations: 4-aminotryptophan (4NW), 7-fluorotryptophan (7FW). (a) Est8[Trp]. (b) Est8[4NW]. (c) Est8[7FW].

In Figure 23b, the SDS gel of the purification of the variant proteins is depicted. The lysate fractions (Figure 23b, lanes 1, 7) exhibited in both cases only slight to no bands at the expected size of ~52 kDa. Again, the lanes where the pellet samples (Figure 23b, lanes 2, 8) were applied were massively overloaded with the 6.25 x amount. Significant bands at the expected size were found in these fractions, indicating that the proteins were expressed in insoluble form or that the cell rupture by sonication did not work properly. This assumption was further reinforced by the fact that no significant expression band was visible in the eluate in case of Est8[4NW] (Figure 23b, lane 5). In case of Est8[7FW] a band was visible at the expected size of ~52 kDa (Figure 23b, lane 11).

After purification, the eluates were desalted, concentrated and the total protein concentration was determined. For the parent protein Est8[Trp] a total protein concentration of 11.6 mg was determined. The total protein concentrations which were determined for the variants Est8[4NW] and Est8[7FW] were significantly lower yielding 0.335 mg and 1.08 mg, respectively.

3.3.4. Protein expression in the presence of 1MeW

Based on the structure of the Est8, Trp was identified as attractive target for incorporation studies (outlined in more detail in chapter 3.1). The *in-vivo* synthesis of Trp analogues and subsequent incorporation into target proteins resulted in low protein yields (section 3.3.3). Since sample-intensive activity measurements on diverse substrates were planned, it was decided to skip the *in-vivo* synthesis and add the Trp analogue directly. Choosing this approach, the set of possible Trp analogues was rather limited. Only a few tryptophan analogues are commercially available. Most of them are fairly expensive, making a routine application very costly. Based on availability, the relatively cheap Trp analogue 1-methyltryptophan (1MeW) was chosen for incorporation studies.

The high cell density supplementation incorporation of 1MeW into the target protein Est8 was performed in Trp auxotrophic *E. coli* BWEC47 cells. The cultivation was conducted in minimal M9 medium (for composition see Table 15) containing a limiting concentration of 18 µM Trp. The cultures showed exponential growth behaviour until Trp depletion, which was reached at the expected OD_{600} value of 2. Subsequent to that, the cultures were supplemented with 1MeW; protein expression was induced and conducted for 4 hours. The final OD_{600} values of the cultures were in the range of 3.3- 3.6, indicating a resumption of cell growth upon addition of the ncAA.

The expression and solubility of the target protein was verified by SDS-PAGE analysis (Figure 24a). Comparing the lanes of the non-induced (ni) and induced (i) samples (Figure 24a, lanes 1, 3) a proper regulated promoter function and a successful expression of the target protein can be assumed. The

distribution between the soluble and insoluble fraction after treatment with CelLytic™ B, showed a clear band at the expected size of ~52 kDa in the soluble (sol) and no band in the insoluble (ins) fraction (Figure 24, lanes 4, 5). Based on these results it was concluded that the protein was well expressed under the chosen conditions and that it can be found in the soluble fraction. In further consequence, the protein was purified by Ni-chelate chromatography. Samples were taken from the different fractions of the purification process and analysed by SDS-PAGE (Figure 24b). The lysate (Figure 24b, lane 2), showed a clear over-expression band at the expected size of ~52 kDa, while only a very slight band was visible at this size in the insoluble, pellet fraction (Figure 24b, lane 3). Significant amounts of target protein were found in the flow through and wash (Figure 24b, lanes 4, 5), which indicates that the column was overloaded. The eluate before and after buffer exchange were applied in lanes 6 and 7, showing distinct bands at the expected size only accompanied by one slight band at the size of ~50 kDa.

Finally the total protein concentration was determined with the Bio-Rad protein assay. In the eluate pool, which was gained from the purification of a total of 7.32 g CWW, a total protein concentration of 2 mg/mL was determined, accounting for a protein yield of 1.9 mg protein/g CWW. Hence a total amount of 14 mg protein was available for the following activity measurements.

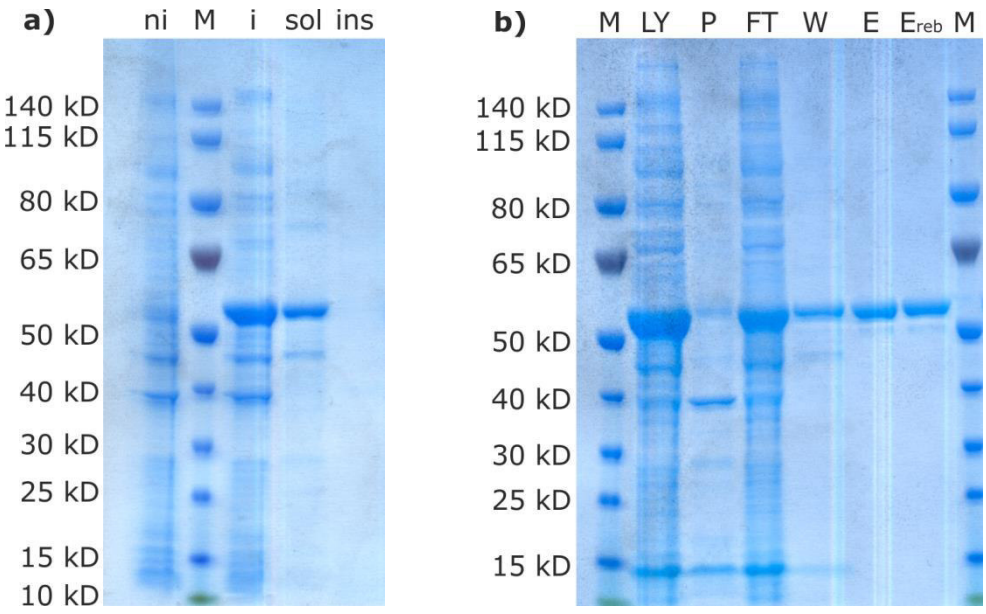


Figure 24: SDS-PAGE analysis of expression, solubility and 6xHis-tag purification of Est8, expressed in the presence of the Trp analogue 1MeW.
(a) Expression and solubility of Est8, in the presence of 1MeW. 0.1 OD_{600} units were loaded of the non-induced (ni) sample, taken right before induction and of the induced (i) sample, taken after 4 h expression. 0.0325 OD_{600} units were loaded of the soluble (sol) and insoluble (ins) fractions after treatment with CelLytic™ B. (b) Migration patterns of cell disruption and purification samples of Est8, expressed in the presence of 1MeW. 6 µL lysate (LY), 1 µL pellet solution (P), 6 µL flow through (FT), 10 µL wash (W), 1 µL eluate (before buffer exchange) (E) and 1 µL eluate (after buffer exchange) (E_{reb}) were applied. Marker: Page Ruler™ Prestained Protein Ladder, 3 µL. 4- 12% NuPAGE® precast SDS gels, Coomassie stain. Abbreviations: 1-methyl-L-tryptophan (1MeW).

3.4. Activity assays and kinetic characterization of the esterases on *p*NPB

The esterase activity of the different protein variants was determined spectrophotometrically following the hydrolysis of *para*-nitrophenyl butyrate (*p*NPB). *p*NPB is a fatty acid ester of *p*-nitrophenol and represents a suitable substrate for the kinetic characterization of esterases, as it has been shown previously (Ribitsch et al., 2012, 2011). Hydrolysis of *p*NPB results in the release of colourless butyric acid together with yellow coloured *p*-nitrophenol which can be detected spectrophotometrically at 405 nm. The reaction principle is depicted in Figure 25.

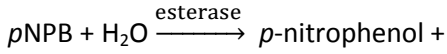


Figure 25: Principle of the esterase catalysed conversion of *p*NPB in the presence of H_2O to *p*-nitrophenol and butyric acid.

The activity assays were carried out under steady-state conditions as described in section 2.2.13. The increase in absorbance at 405 nm was recorded for 5 minutes. Using the maximum linear rate, the change of absorbance per minute (ΔE) in mAU/min was obtained. Enzyme activity was subsequently calculated applying Lambert-Beer's law (Formula 2).

$$\frac{U}{mL} = \frac{\Delta E}{\varepsilon \cdot d} \cdot \frac{V_{total}}{V_{enz}} \cdot f$$

Formula 2: Lambert-Beer's law used for the calculation of the enzyme activity in U/mL.
The parameters and the corresponding units are listed in Table 25. (Ɛ*p*-nitrophenol = 9031.7 L mol^{-1} cm^{-1}; d = 0.6509 cm).

The values for the molar extinction coefficient, Ɛ of *p*-nitrophenol and the film thickness were provided by Armin Baumschlager (2013a).

Table 25: Description of the parameters, their abbreviations and corresponding units, applied for the calculation of the enzyme activity using Lambert-Beer's law.

abbreviation	description	unit
U/mL	change of concentration per minute	µmol min^{-1} mL^{-1} ≙ mM min^{-1}
ΔE	change in absorbance per minute	mAU min^{-1}
Ɛ	molar extinction coefficient	M^{-1} cm^{-1}
d	thickness of film	cm
V_{total}	total volume	µL
V_{enz}	volume of sample applied	µL
f	dilution factor	-
$c_{protein}$	protein concentration	mg mL^{-1}

Using the determined protein concentrations in mg/mL, the specific enzyme activity in U/mg was calculated for each substrate concentration using Formula 3.

$$\frac{U}{mg} = \frac{\Delta E}{\varepsilon \cdot d} \cdot \frac{V_{total}}{V_{enz}} \cdot f \cdot \frac{1}{c_{protein}}$$

Formula 3: Modified Lambert-Beer's law used for the calculation of the specific enzyme activity in U/mg.
The parameters and the corresponding units are listed in Table 25. ($\varepsilon_{p\text{-nitrophenol}}$ = 9031.7 L mol^{-1} cm^{-1}; d = 0.6509 cm).

Subsequently the kinetic constants were determined. The mean specific enzyme activities (n = 6) were plotted over the corresponding *p*NPB concentrations and fitted to the Michaelis-Menten equation using nonlinear regression. Hanes-Woolf plots were compiled by plotting the ratio of the initial substrate concentration [S] to the initial rate of reaction v, against [S]. Regression analysis was performed using the software QtiPlot. The corresponding diagrams are depicted in Figure 26. The kinetic parameters calculated based on these nonlinear and linear regression analyses, are summarized in Table 26.

Table 26: Kinetic parameters obtained by nonlinear and linear regression analysis of kinetic data of Est8[Met], Est8[Nle] and Est8[1MeW] on *p*NPB.

kinetic parameter	plot	Est8[Met]	Est8[Nle]	Est8[1MeW]
V_{max}	Michaelis-Menten	63.5	36.1	66.0
[U mg^{-1}]	Hanes-Woolf	66.2	36.8	69.0
K_m	Michaelis-Menten	0.385	0.475	0.378
[mM]	Hanes-Woolf	0.583	0.588	0.586
k_{cat}	Michaelis-Menten	54.8	31.1	56.9
[s^{-1}]	Hanes-Woolf	57.1	31.7	59.4
k_{cat}/ K_m	Michaelis-Menten	142	65.5	151
[s^{-1} mM^{-1}]	Hanes-Woolf	97.9	53.9	101

The Michaelis-Menten plots of the parent protein Est8[Met] and the variants Est8[Nle] and Est8[1MeW] (Figure 26a, c, e) showed saturation curves close to ideal enzyme activity curves. The initial rates of reaction per milligram protein [U/mg] increased with increasing substrate concentrations [S], asymptotically approaching the maximum specific enzyme activity, V_{max} [U/mg]. By fitting the data to the Michaelis-Menten equation, a V_{max} of 63.5 ± 1.8 U/mg was calculated for the parent protein Est8[Met]. Est8[1MeW] exhibited a similar value for V_{max} of 66.0 ± 2.2 U/mg. The expression in the presence of Nle reduced the maximum specific enzyme activity by a factor of 1.8, to a value of 36.1 ± 1.1 U/mg. Similar values were determined upon linear regression analysis applying the Hanes-Woolf plot (Figure 26b, d, f). In this graphical representation of the Michaelis-Menten equation, V_{max} is given by the reciprocal of the slope (k = 1/ V_{max}).

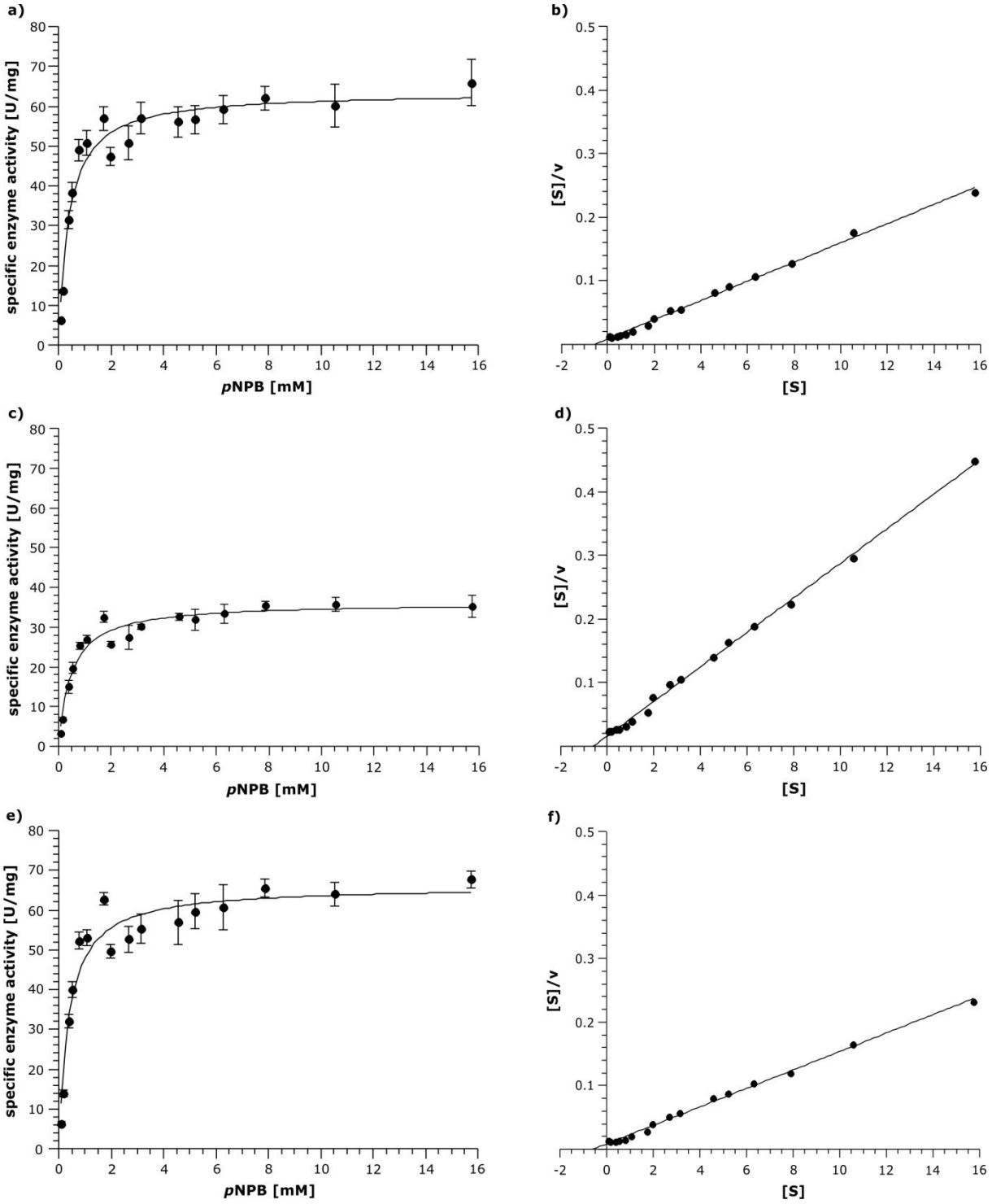


Figure 26: Nonlinear and linear regression analysis of kinetic data obtained for Est8[Met], Est8[Nle] and Est8[1MeW] on *p*NPB.

Substrate solutions containing different concentrations of *para*-nitrophenyl butyrate (*p*NPB) were prepared and dispensed in 96-well plates as described in section 2.2.13. 20 µL of enzyme dilution in 50 mM Tris-HCl, pH 7 were added per 200 µL substrate solution and the change in absorbance due to release of yellow coloured *p*-nitrophenol was recorded for 5 min at 405 nm. 20 µL 50 mM Tris-HCl, pH 7 were used as blank. From the maximum linear rates, the protein concentrations and the dilution factor, the specific enzyme activities in U/mg for each substrate concentration were determined applying Lambert Beer's law. Kinetic constants were determined by plotting the specific enzyme activities over the corresponding *p*NPB concentrations and fitting them directly to the Michaelis-Menten equation using nonlinear regression. Hanes-Woolf plots were compiled by plotting the ratio of the initial substrate concentration [S] to the initial rate of reaction v, against [S]. Regression analysis was performed using QtiPlot. (a) Michaelis-Menten kinetics of Est8[Met]. (b) Hanes-Woolf plot of Est8[Met]. (c) Michaelis-Menten kinetics of Est8[Nle]. (d) Hanes-Woolf plot of Est8[Nle]. (e) Michaelis-Menten kinetics of Est8[1MeW]. (f) Hanes-Woolf plot of Est8[1MeW]. Abbreviations: norleucine (Nle), 1-methyl-L-tryptophan (1MeW).

The Michaelis constant, K_m [mM] represents the substrate concentration at which the reaction rate corresponds to the half of the maximum rate of reaction ($K_m = V_{max}/ 2$). It describes the binding-affinity of the substrate for the enzyme. For example a small value for K_m indicates a high affinity of the substrate for the enzyme. Obviously the K_m value is dependent on the substrate, the enzyme as well as the other reaction conditions such as temperature and pH (Chmiel, 2011). Upon nonlinear regression analysis of the kinetic data, a K_m value of 0.385 ± 0.06 mM was calculated for Est8[Met]. Est8[1MeW] was again in the same range, showing a K_m value of 0.378 ± 0.07 mM. A higher K_m value, indicating a lower affinity of the substrate to the enzyme, was determined for the variant Est8[Nle] with 0.475 ± 0.07 mM. K_m values were also determined based on the Hanes-Woolf plot. Here, the K_m can be derived from the y-intercept, which is given by K_m / V_{max}. The hereby determined K_m values were quite similar among them but differed significantly from the K_m values determined using nonlinear regression.

When looking at the Hanes-Woolf plots (Figure 26b, d, f), a major drawback of this graphical representation of the Michaelis-Menten equation becomes obvious. The point of intersection of the line and the y-axis is always located near the origin, which results in serious inaccuracies when determining K_m. Therefore it seems advisable to use mainly nonlinear regression analysis for the determination of the kinetic parameters, since no transformation of the measured values is needed and the original Michaelis-Menten equation can be applied. As it is also described in the literature, graphical methods like the Hanes-Woolf plot, the Lineweaver-Burk plot or the Eadie-Hofstee diagram may be used to further support and confirm the calculated values (Chmiel, 2011).

The turnover number, k_{cat} is the rate constant for the conversion of the enzyme-substrate complex to the product. It is defined as the maximum number of substrate molecules that can be converted by a single enzyme molecule or rather by an active site per unit of time. Therefore k_{cat} is given in s^{-1} and can be calculated using Formula 4 (Nelson et al., 2004). For the parent protein, Est8[Met] a turnover number of 54.8 s^{-1} was calculated. The Est8[1MeW] variant showed a slightly higher value of 56.9 s^{-1}, while the turnover number for Est8[Nle] reached 31.1 s^{-1}. Concluding from that it can be said that the proteins Est8[Met] and Est8[1MeW] were able to convert around 55 substrate molecules per active site per second, while Est8[Nle] converted 31 substrate molecules per active site per second.

$$k_{cat} = \frac{V_{max}}{[E_t]}$$

Formula 4: Calculation of the turnover number k_{cat} [s^{-1}].
V_{max}: Maximum specific enzyme activity, [M min^{-1}]; E_t: applied enzyme concentration based on the molecular weight of the enzyme [M]. Est8: 51700 g mol^{-1}.

The Michaelis constant, K_m as well as the turnover number, k_{cat} are of little significance in themselves. A high k_{cat} value indicates a high working speed of the enzyme but provides no information about the binding of the substrate which is essential for the conversion. In contrast the K_m value describes the binding-affinity of the substrate for the enzyme, but neglects the quantity of the conversion (Chmiel, 2011). A parameter which combines these two features is the catalytic efficiency, k_{cat}/ K_m [s^{-1} mM^{-1}]. The catalytic efficiency interrelates the reaction rate with the concentration of free enzyme and substrate. It indicates how efficiently a substrate is converted into the product by a certain enzyme (Palmer, 1995). The values for the catalytic efficiency ranged from 142 s^{-1} mM^{-1} for Est8[Met], to 151 s^{-1} mM^{-1} for Est8[1MeW] down to 65.5 s^{-1} mM^{-1} for Est8[Nle].

Concluding from these results it can be said that the parent protein Est8[Met] as well as the Est8[1MeW] variant, which was expressed in the presence of the tryptophan analogue 1MeW, showed similar activity on the soluble substrate *p*NPB. The determined maximum specific enzyme activities were in the range of ~65 U/mg. The values for the Michaelis constants, K_m came to ~0.38 mM. The expression in the presence of Nle obviously reduced the specific activity of Est8 by a factor of 1.8, to a value of 36.1 U/mg. The determined Michaelis constants, K_m was with 0.475 mM higher than for the other two proteins, indicating a lower binding affinity of the substrate to the enzyme.

In summary it can be stated that, functional parent and variant proteins of Est8 were expressed in the Met auxotrophic strain, BWEC50 as well as in the Trp auxotrophic strain, BWEC47 under the chosen conditions. The alteration in activity due to the expression in the presence of Nle may already be a hint that the supplementation incorporation of Nle into the target protein worked. Although the activity was reduced on the short-chain model substrate *p*NPB, it does not necessarily mean that this is the case for the polymeric target substrates. Since the kinetic parameters for the parent Est8[Met] and the Est8[1MeW] variant were almost identical, it was speculated that the possible incorporation of the Trp analogue 1MeW may not have such a drastic effect on the hydrolysis behaviour of the enzyme on short-chain substrates. Another reason may be that the supplementation incorporation was not successful or that only partially labelled target protein was expressed. However this should be revealed by the analysis of the incorporation efficiency using MALDI-MS.

3.5. Testing the hydrolytic activity towards polymeric substrates

Subsequent to the kinetic characterization on the small, soluble substrate *p*NPB, the parent protein Est8[Met] as well as the variants Est8[Nle] and Est8[1MeW] were tested for their potential to hydrolyse the synthetic polymer PBAT. Poly(butylene adipate co-terephthalate) which is commercialised by BASF under the trade name ecoflex®, is a fully biodegradable aliphatic-aromatic copolyester from terephthalic acid, adipic acid and 1,4-butanediol. The amount of the aromatic carboxylic acid in the polymer is crucial for the degradability of the same (Müller, 2005) and accounts for 47 mol%. The possible soluble release products after hydrolysis, which are detect- and quantifiable are adipic acid (Ada), terephthalic acid (Ta), mono(4-hydroxybutyl)terephthalate (BTa) and bis(4-hydroxybutyl)terephthalate (BTaB) (personal communication, V. Perz, ACIB, Tulln). A schematic representation of the degradation mechanism is provided in Figure 27.

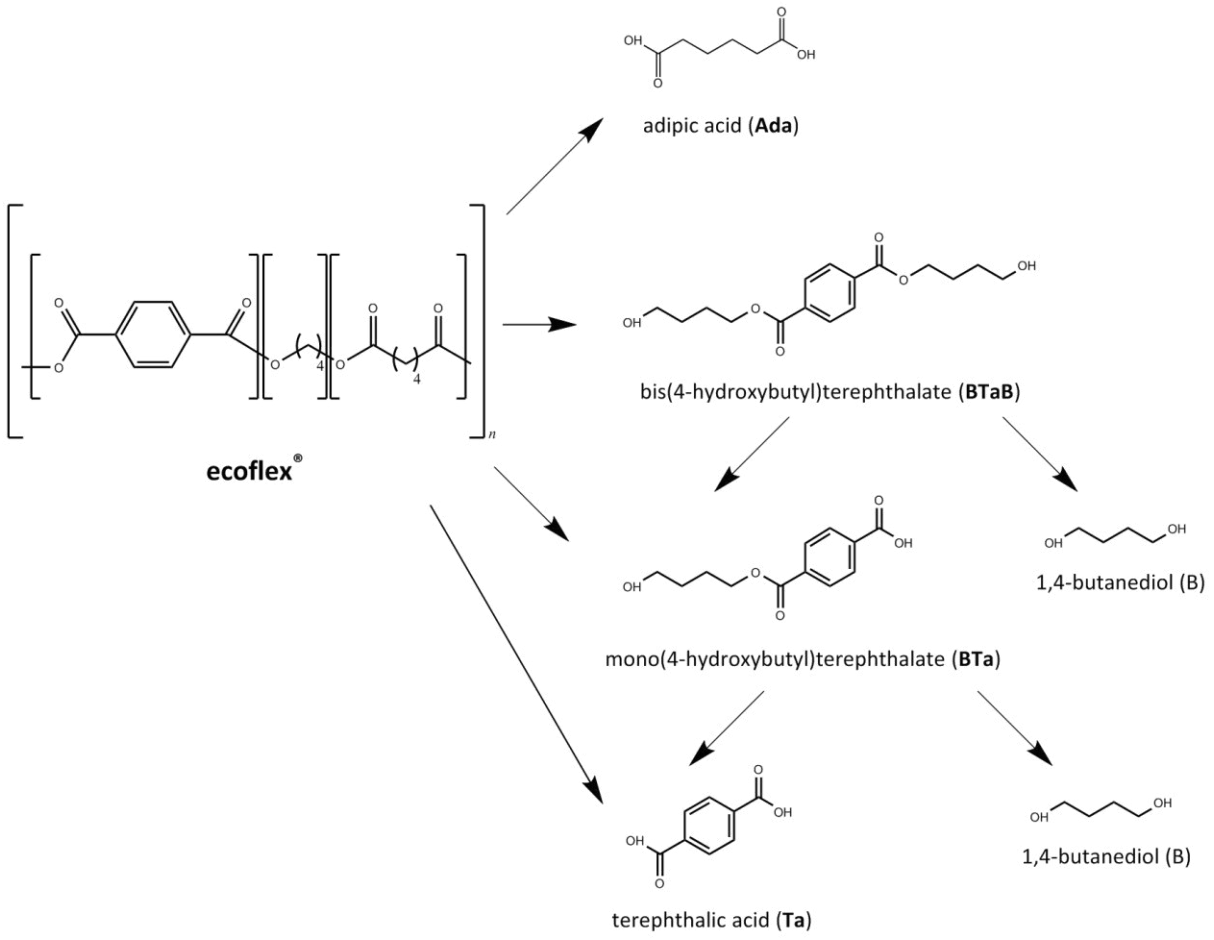


Figure 27: Structure of the aliphatic-aromatic copolyester ecoflex® and the possible degradation products upon enzymatic hydrolysis.
ecoflex® is a copolyester of terephthalic acid (Ta), adipic acid (Ada) and 1,4-butanediol (B). Random distribution of the monomers along the polymer chain allows the formation of different aliphatic and aromatic degradation products upon enzymatic hydrolysis. The possible soluble release products, which are detect- and quantifiable with the HPLC method used include: adipic acid (Ada), bis(4-hydroxybutyl)terephthalate (BTaB), mono(4-hydroxybutyl)terephthalate (BTa) and terephthalic acid (Ta). BTa and Ta can either be formed in the course of ecoflex® hydrolysis or by further processing of BTaB. 1,4-butanediol (B) is not detected with the method used. (Prepared based on Baumschlager, 2013a: Figure 30).

The degradation assays were performed at the two pH values of 6 and 8, which were determined in a previous study by Baumschlager (2013a) to be the pH-optima of the parent protein Est8[Met]. As Est8[Met] had already shown a high temperature stability in earlier studies (Baumschlager, 2013a, Schöffmann, 2013) it was decided to perform the degradation assays at 50 °C. This elevated temperature should reflect conditions similar to a biogas process, since the main goal was to find enzymes which are able to degrade ecoflex® under anaerobic conditions (personal communication, D. Ribitsch, ACIB, Graz). The hydrolytic activity of Est8[Met], Est8[Nle] and Est8[1MeW] was determined after 24 h, 48 h and 72 h. The chromatographic peaks were integrated and converted into concentrations [µM] with calibration curves kindly provided by Veronika Perz (ACIB, AG Gübitz). In Figure 28 the time profiles of the detected ecoflex® hydrolysis products upon incubation with Est8[Met], Est8[Nle] and Est8[1MeW] are summarized.

For the parent protein, Est8[Met] the ecoflex® hydrolysis products BTaB, BTa and Ta were detected; Ada was not detected in any of the samples. The degradation profile of Est8[Met] at pH 6 (Figure 28a) differed significantly from the degradation profile which was determined upon incubation at pH 8 (Figure 28b). At pH 6, the largest hydrolysis product of ecoflex®, BTaB was decreasing over the three time points measured, from 71.7 µM to 57.7 µM. In parallel, the amounts of BTa and Ta increased. Especially for BTa a distinct increase from 79.8 µM after 24 h up to 142 µM after 72 h was detected. The decrease of BTaB may indicate that the reaction from ecoflex® to BTaB is not as fast as the follow-up processing from BTaB to BTa. Although, it seemed as if the formation and consumption of BTaB levelled out at ~60 µM between 48 h and 72 h, which would indicate an equilibrium between formation and consumption of BTaB. Due to the accumulation of BTa and the slow increase of Ta, it seems that the last hydrolysis step is least favoured by the enzyme under these conditions. At pH 8 the amounts of BTaB were also decreasing over the three time points measured, but were significantly lower ranging from 27.7 µM after 24 h down to 4.68 µM after 72 h. The amounts of BTa were accordingly higher. Interestingly, the amounts of Ta were significantly higher at pH 8, with a final Ta concentration of 64.4 µM after 72 h compared to a Ta amount of 4.49 µM upon incubation at pH 6 for 72 h. Concluding from that it can be assumed that the processing of BTaB to BTa and further to Ta is performed much faster at pH 8, resulting in a higher amount of the end product of hydrolysis, Ta.

Also for the Est8[Nle] variant the hydrolysis products BTaB, BTa and Ta were detected; Ada was again not detected in any of the samples. At pH 6, the Est8[Nle] variant exhibited a slightly different degradation pattern than the parent protein (Figure 28c). The largest hydrolysis BTaB also decreased over the three time points measured, from 94.8 µM to 78 µM.

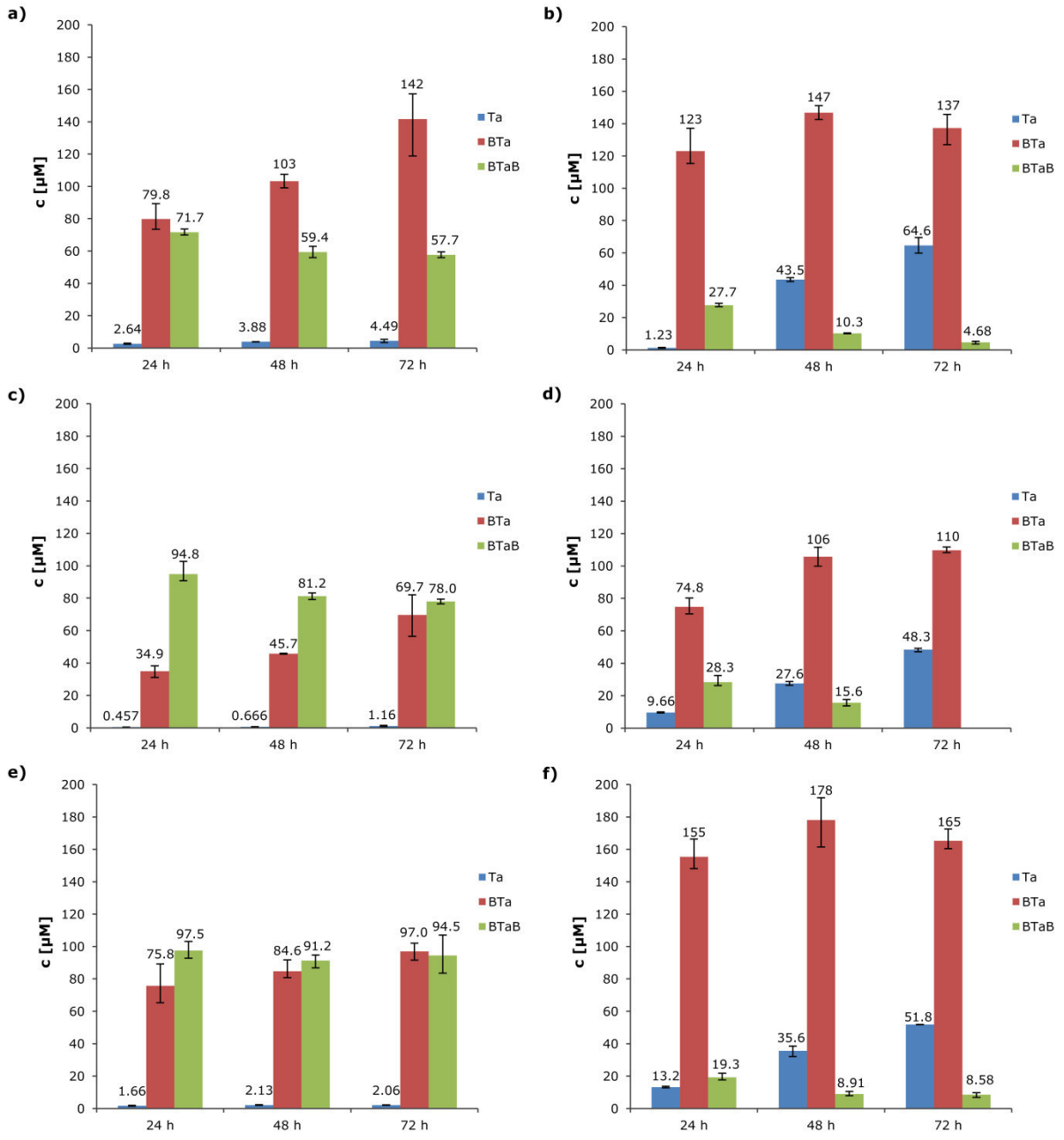


Figure 28: Time profiles of soluble ecoflex® hydrolysis products upon incubation with Est8[Met], Est8[Nle] and Est8[1MeW].

0.01 ± 0.0002 g ecoflex® were weighed into 1.5 mL Eppendorf tubes and supplemented with 1 mL of citrate-phosphate buffer, pH 6 or phosphate buffer, pH 8 containing 0.6 µM enzyme. As blank, 1 mL buffer without enzyme was added to the substrate. The tubes were incubated at 50 °C and 100 rpm for the times indicated. Upon supplementation with ice-cold methanol (1:1) and precipitation for 15 minutes on ice, the samples were centrifuged. Afterwards the supernatant was adjusted to pH 4 by adding formic acid, followed by another precipitation step for 1 hour at 4 °C. 5 µL were injected into HPLC (column: X-Terra RP18, 3.0 x 150 mm, 3.5 µm particle size, eluents: multi-step gradient flow starting with 72% (v/v) ddH_2O, 8% acetonitrile (ACN), 20% CH_2O_2 (0.1 %) and increasing the ACN to 70% while lowering the percentage of ddH_2O to 10%, flow: 0.5 mL/min, time: 25 min, oven temperature: 25 °C). Soluble hydrolysis products terephthalic acid (Ta), mono(4-hydroxybutyl)terephthalate (BTa) and bis(4-hydroxybutyl)terephthalate (BTaB) were detected with a photodiode array detector at 241 nm. Time profile is depicted for each enzyme at pH 6 and pH 8. Error bars indicate the difference from the mean value to the highest and the lowest measured value of a triplicate determination after subtracting the mean value of the blank, which was determined in duplicates. (a) Est8[Met], pH 6; (b) Est8[Met], pH 8; (c) Est8[Nle], pH 6; (d) Est8[Nle], pH 8; (e) Est8[1MeW], pH 6; (f) Est8[1MeW], pH 8.

However, the relatively high BTaB concentrations accompanied by the low but increasing BTa amounts, led to the assumption that the processing of BTaB is not as fast as with the parent protein. Therefore higher BTaB amounts could accumulate in the reaction. Only low amounts of Ta were detected upon incubation with Est8[Nle], which may be a hint that the processing of BTa to Ta and B is less favoured by the enzyme. The total degradation products detected at each time point for Est8[Nle], were 15- 27% lower compared to the parent protein. The percentage of BTaB contributing to the total degradation products was between 46 and 28% for the parent protein. In case of the Nle variant, BTaB made up a larger portion of the total degradation products, ranging between 72 and 52%. At pH 8, the Nle variant showed a similar degradation pattern as the parent protein, with the amounts of degradation product always being distinctly lower (Figure 28d). Interestingly, no BTaB was detectable after 72 h. This may indicate that the hydrolysis of BTaB to BTa and B is much faster than the initial processing of ecoflex® to BTaB, leading to a shortage in BTaB. The total degradation products detected at each time point for Est8[Met] at pH 8, were 23- 25% higher compared to the Nle variant, ranging between 152 and 206 µM compared to 112 – 158 µM. Based on these results it can be also stated for the Nle variant, that the processing of BTaB to BTa and further to Ta is performed more efficiently at pH 8.

Incubation of ecoflex® with the Est8[1MeW] variant led to the detection of the hydrolysis products BTaB, BTa and Ta; Ada was not detected in any of the samples. The amounts of BTaB and BTa, which were detected after the three time points measured at pH 6, were always quite similar fluctuating around 90 µM (Figure 28e). This behaviour may be interpreted in a way, that the hydrolysis reaction of ecoflex® to BTaB was as fast as the follow-up processing of the formed BTaB to BTa. Although, when taking the low levels of Ta into account, a major accumulation of BTa should be observable to support this theory. But this was not the case. Besides minor fluctuations, it seems as if the enzyme exhibited most of its hydrolytic activity at pH 6 within the first 24 h of incubation. This is substantiated by the amount of total degradation products, which was 12% higher than the amount of Est8[Met] and 26% higher than the amount of Est8[Nle] after 24 h. The assessment of the hydrolytic activity of Est8[1MeW] on ecoflex® at pH 8 (Figure 28f) showed in general this shift towards the formation of Ta, which was also observable for the other two proteins. Between 24 h and 48 h, BTaB decreased from 19.3 µM to 8.9 µM but remained at this concentration also after 72 h. BTa fluctuated around 160- 170 µM, exhibiting no significant change. As observed at pH 6, it seemed that most of the hydrolysis of the ecoflex® and the larger intermediate BTaB, happened within the first 24- 48h of incubation. The only striking difference was the increase of Ta from 13.2 µM after 24 h to 51.8 µM after 72 h, which demonstrates that the enzyme exhibited hydrolytic activity on the small substrate BTa also after 72 h of incubation. The amount of total degradation products of

Est8[1MeW] after 24 h was again 19% higher than the amount of Est8[Met] and 40% higher than the amount of Est8[Nle]. This confirmed the assumption that Est8[1MeW] was most active on ecoflex® within the first 24 h of incubation.

Based on the described results, the following conclusions can be drawn. In general it seems that the degradation of ecoflex® is performed more complete at pH 8. For all three enzymes tested, the amounts of the smaller hydrolysis products BTa and Ta are distinctly higher at pH 8 than at pH 6. Especially in case of Ta, which is almost not present at pH 6, this difference becomes apparent. When looking at the differences in the degradation patterns of the enzymes, the most striking difference between the parent and Nle variant protein occurred at pH 6. Here the processing of BTaB is not as fast as with the parent protein, indicated by higher BTaB concentrations. At pH 8, the Nle variant showed a similar degradation pattern as the parent protein, with the amounts of degradation product always being distinctly lower. For Est8[1MeW] it seemed that the enzyme was most active on ecoflex® and the larger intermediate BTaB within the first 24 h. Although the enzyme exhibited hydrolytic activity on the small substrate BTa also after 72 h, which indicates that it was still active.

The so far described conclusions were based on the assumption that the enzymes gradually degrade ecoflex® to BTaB; BTaB to BTa (and B), and finally BTa to Ta (and B). Theoretically, the formation of BTa and Ta should also be possible by direct hydrolysis of ecoflex®, due to the fact that the aliphatic and aromatic building blocks are randomly distributed along the polymer chain. In this context the mode of cleavage, in particular if the enzymes attack the polymer exo- or endo-wise, would be of interest. To get a deeper insight into the degradation mechanism, the parent protein Est8[Met] as well as the variants Est8[Nle] and Est8[1MeW] were tested for their potential to hydrolyse the ecoflex® model substrate BaBTaBBa.

BaBTaBBa consists of bis(4-hydroxybutyl)terephthalate with a benzoic acid molecule esterified to either side. Theoretically, due to its symmetrical structure, an exo-wise and an endo-wise enzymatic cleavage mechanism can be postulated (Figure 29). If the enzyme attacks the substrate at the outer ester bonds to the benzoic acid molecules (Figure 29a), two molecules of benzoic acid (Ba) and one molecule of bis(4-hydroxybutyl)terephthalate (BTaB) would be formed. Undergoing further enzymatic hydrolysis, BTaB can be converted to mono(4-hydroxybutyl)terephthalate (BTa), terephthalic acid (Ta) and 1,4-butanediol (B). Consequently, the possible soluble release products upon exo-wise enzymatic hydrolysis which are detect- and quantifiable are BTaB, BTa, Ba and Ta. Assuming that the enzyme attacks the substrate exclusively at the inner ester bonds (Figure 29b), endo-wise cleavage may be present, causing the release of Ta and Ba.

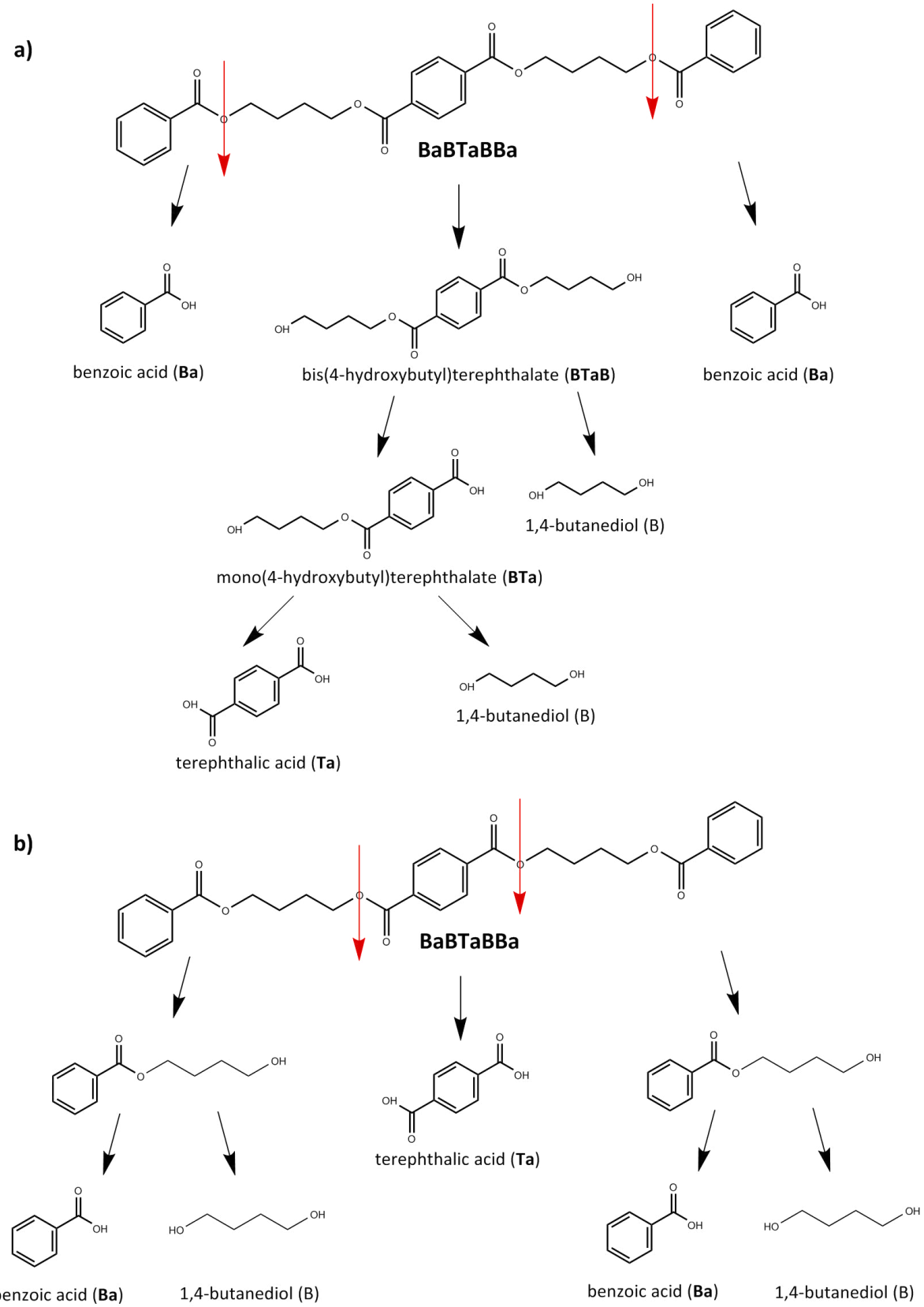


Figure 29: Structure of the ecoflex® model substrate BaBTaBBa and the possible degradation products upon exo- and endo-wise enzymatic cleavage.

BaBTaBBa consists of bis(4-hydroxybutyl)terephthalate with a benzoic acid molecule esterified to either side. (a) Upon exo-wise enzymatic cleavage starting from the outer ester bonds, the possible soluble release products which are detect- and quantifiable with the HPLC method used include: bis(4-hydroxybutyl)terephthalate (BTaB), mono(4-hydroxybutyl) terephthalate (BTa), benzoic acid (Ba) and terephthalic acid (Ta). (b) Possible release products upon endo-wise enzymatic cleavage, starting from the inner ester bonds include Ta and Ba.

The degradation assays on BaBTaBBa were also performed at the two pH values of 6 and 8 at 50 °C. The hydrolytic activity of Est8[Met], Est8[Nle] and Est8[1MeW] was determined after 24 h, 48 h and 72 h. The chromatographic peaks were integrated and converted into concentrations [µM] with calibration curves kindly provided by Veronika Perz (ACIB, AG Gübitz). In Figure 30 the time profiles of the detected hydrolysis products upon incubation with Est8[Met], Est8[Nle] and Est8[1MeW] are summarized.

For the parent protein, Est8[Met] all possible hydrolysis products, BTaB, BTa, Ba and Ta, were detected upon incubation at pH 6 (Figure 30a) and increased over the measured time points. The largest hydrolysis product BTaB was not detectable after 24 h. After 48 h a concentration of 13.3 µM and after 72 h a concentration of 394 µM was detected. When taking the mode of cleavage and the comparably high BTa concentrations into account, this fact is not surprising. According to the degradation scheme in Figure 29a, BTa is formed from BTaB. Obviously, the reaction from BTaB to BTa was faster than the initial hydrolysis of substrate BaBTaBBa. Therefore the formed BTaB was readily converted to BTa, which accumulated in the reaction. After 48 h and 72 h BTaB concentrations of 13.3 µM and 394 µM were detected, respectively. This could possibly indicate a deceleration of the conversion from BTaB to BTa.

Interestingly, the BTa concentrations which were detected upon incubation at pH 8 (Figure 30b), were significantly lower, ranging between 468 µM and 725 µM. The BTaB concentrations were higher, with 264 µM after 24 h and 463 µM after 72 h. After 48 h a BTaB concentration of only 17.8 µM was detected; but due to the 24 h and 72 h values, this value was classified as outlier. Based on these results, it seemed that the conversion of BTaB to BTa at pH 8 was not as favoured as at pH 6. When looking at the progression of the smaller hydrolysis products, Ba and Ta, Ba was the most prominent hydrolysis product, ranging between 1853 µM and 2744 µM at pH 6 and 1951 µM and 3139 µM at pH 8. The determined Ta concentrations at pH 8 were with 146 µM and 428 µM after 24 h and 48 h, higher than those at pH 6. Although after 72 h, a Ta concentration of 560 µM was determined at both pH values.

Obviously, a strict differentiation between exo- and endo-wise cleavages may not be applicable to analyse the obtained data. Although, the amounts of the degradation products when set in relation with each other allow to deduce a tendency, either towards exo- or endo-wise cleavage. While high amounts of BTaB and BTa would be indicative for exo-wise cleavage, endo-wise cleavage would be characterized by increased amounts of Ta. Following this thought, it seems that the parent protein Est8[Met] exhibits a stronger tendency towards exo-wise cleavage at pH 6 than at pH 8.

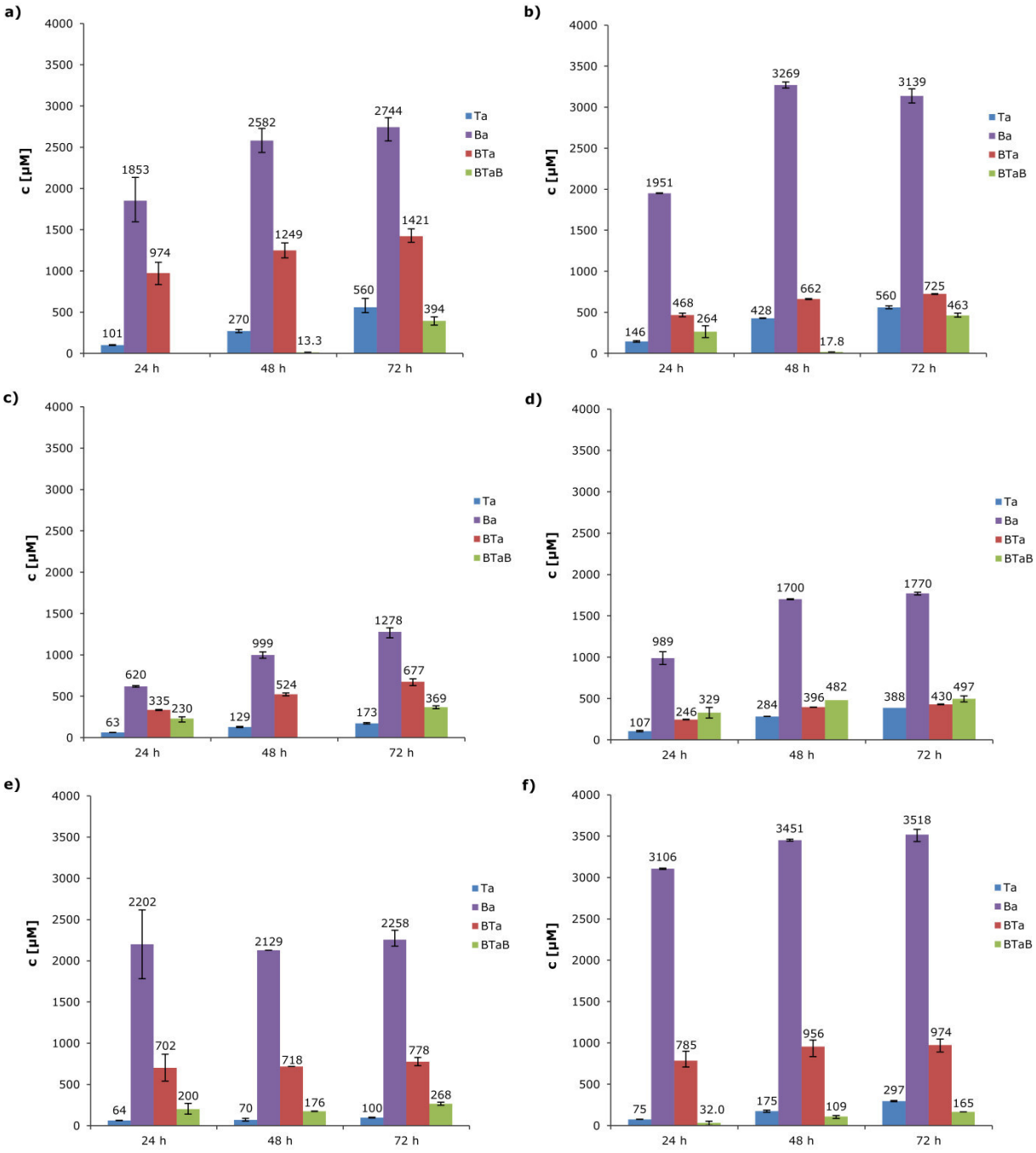


Figure 30: Time profiles of soluble BaBTaBBa hydrolysis products upon incubation with Est8[Met], Est8[Nle] and Est8[1MeW].

0.00709 ± 0.0002 g BaBTaBBa (bis(4-hydroxybutyl)terephthalate with a benzoic acid molecule on either side) were weighed into 1.5 mL Eppendorf tubes and supplemented with 1 mL of citrate-phosphate buffer, pH 6 or phosphate buffer, pH 8 containing 0.6 µM enzyme. As blank, 1 mL buffer without enzyme was added to the substrate. The tubes were incubated at 50 °C and 100 rpm for the times indicated. Upon supplementation with ice-cold methanol (1:1) and precipitation for 15 minutes on ice, the samples were centrifuged. Afterwards the supernatant was adjusted to pH 4 by adding formic acid, followed by another precipitation step for 1 hour at 4 °C. 5 µL were injected into HPLC (X-Terra RP18, 3.0 x 150 mm, 3.5 µm particle size, eluents: multi-step gradient flow starting with 72% (v/v) ddH_2O, 8% acetonitrile (ACN), 20% CH_2O_2 (0.1 %) and increasing the ACN to 70% while lowering the percentage of ddH_2O to 10%, flow: 0.5 mL/min, time: 25 min, oven temperature: 25 °C). Soluble hydrolysis products terephthalic acid (Ta), benzoic acid (Ba), mono(4-hydroxybutyl) terephthalate (BTa) and bis(4-hydroxybutyl)terephthalate (BTaB) were detected with a photodiode array detector at 241 nm. Time profile is depicted for each enzyme at pH 6 and pH 8. Error bars indicate the difference from the mean value to the highest and the lowest measured value of a triplicate determination after subtracting the mean value of the blank, which was determined in duplicates. (a) Est8[Met], pH 6; (b) Est8[Met], pH 8; (c) Est8[Nle], pH 6; (d) Est8[Nle], pH 8; (e) Est8[1MeW], pH 6; (f) Est8[1MeW], pH 8.

The Est8[Nle] variant exhibited a different degradation profile on BaBTaBBa than the parent protein. At pH 6 (Figure 30c), the total degradation products were in general between 50% and 60% lower than for Est8[Met]. When looking at the progression of the largest hydrolysis product, BTaB, an increase from 230 µM after 24 h to 369 µM after 72 h, was observable. This is of particular interest, because similar observations were made upon incubation of Est8[Nle] with ecoflex® at pH 6. There it was speculated that the processing of BTaB was not as fast as with the parent protein, resulting in an accumulation of this degradation product. With BaBTaBBa as substrate, this also seemed to be the case. The incubation of Est8[Nle] with BaBTaBBa at pH 8, resulted in a distinctly different degradation pattern (Figure 30d). At all three time points, the concentration of BTaB was higher and those of BTa lower, than determined at pH 6, indicating an increased accumulation of the largest degradation product. Interestingly, the smaller degradation products Ba and Ta exhibited in all cases significantly higher values than at pH 6. Concluding from that, it seems that also the Est8[Nle] variant, exhibits a stronger tendency towards exo-wise cleavage at pH 6 than at pH 8.

The incubation of the Est8[1MeW] variant with BaBTaBBa led to the detection of the hydrolysis products BTaB, BTa, Ta and Ba at pH 6 (Figure 30e) and pH 8 (Figure 30f). What was already observed on ecoflex®, seemed to be the case again: The enzyme exhibited most of its hydrolytic activity within the first 24 h of incubation. Minor fluctuations were also observed after 48 h and 72 h, indicating that the enzyme was still active. When comparing the amounts of BTa, the differences between the two pH values were not as distinct as determined for the other two enzymes. Especially for the parent protein, the BTa values determined at pH 6 differed significantly from those at pH 8. Concluding from those results, no real tendency towards exo- or endowise cleavage could be determined for Est8[1MeW].

Based on the current results it can be stated that a strict differentiation between exo- and endo-wise cleavage as outlined in Figure 29 is not possible. Although, it is possible to deduce a tendency towards exo- or endo-wise cleavage by analysing and comparing the degradation patterns of the enzymes on BaBTaBBa. Accordingly, the parent protein as well as the Est8[Nle] variant, exhibited a stronger tendency towards exo-wise cleavage at pH 6. At pH 8 the results were indicative for endo-wise cleavage. For the Est8[1MeW] variant no real tendency towards exo- or endo-wise cleavage could be determined.

3.6. Analysis of analogue incorporation

In order to determine the incorporation efficiency of the amino acid analogues into the target protein Est8, a matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was performed at the ACIB Core Facility Metabolomics (Medical University of Graz). The samples were prepared as described in section 2.2.15 and subsequently in-gel digested with three enzymes. The resulting peptides were analysed by MALDI-MS. The mass analysis results of Est8[Nle] showed a protein sequence coverage of 84%. This means that 84% of the whole protein sequence was found in the matched peptides (Figure 31). Furthermore, all 9 Met residues (without the start codon) which are contained in the structure of Est8 are located within these matched peptides. Then it was analysed, how many of these peptides, corresponding to one Met position contained Nle instead of Met.

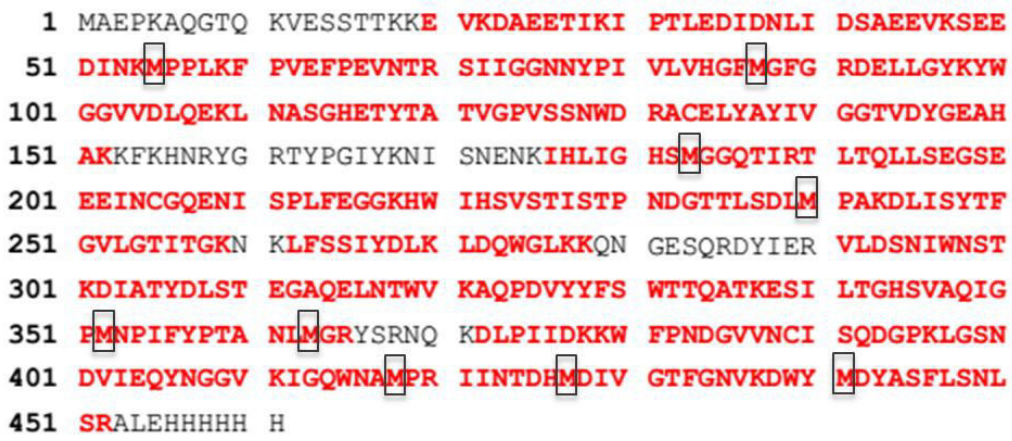


Figure 31: Amino acid sequence of Est8 and the matched peptides found in the MS analysis of Est8[Nle].
The matched peptides covered 84% of the protein sequence and are shown in bold red. All 9 Met residues (without the start codon) are located within these matched peptides. Met residues are marked with squares.

For example in case of Met55, 9 peptides were found covering this position. In 8 of these 9 peptides, Met was replaced by Nle (Figure 32).

Query	Start – End	Peptide
583	48 – 59	K.SEEDINKMPPLK.F + Met-->Nle (M)
584	48 – 59	K.SEEDINKMPPLK.F + Met-->Nle (M)
585	48 – 59	K.SEEDINKMPPLK.F + Met-->Nle (M)
1683	48 – 70	K.SEEDINKMPPLKFPVEFPEVNTR.S + Met-->Nle (M)
1300	55 – 70	K.MPPLKFPVEFPEVNTR.S + Met-->Nle (M)
1301	55 – 70	K.MPPLKFPVEFPEVNTR.S + Met-->Nle (M)
1302	55 – 70	K.MPPLKFPVEFPEVNTR.S + Met-->Nle (M)
1303	55 – 70	K.MPPLKFPVEFPEVNTR.S + Met-->Nle (M)
1351	55 – 70	K.MPPLKFPVEFPEVNTR.S + Oxidation (M)

Figure 32: Nine peptide sequences were found upon MS analysis of Est8[Nle], covering the position Met55.
Start – End indicate the amino acid positions in the Est8 sequence. The sequence of the found peptide is given in the right column, with underlined Met55. If Met was replacd by Nle, this is indicated by “Met --> Nle”. In 8 out of 9 peptides, Met was replaced by Nle. In one peptide, Met was found in its oxidized form.

On other positions, like Met352 all the six peptides which were found, contained Nle. To sum up, at every Met position at least 50% of the corresponding peptides contained Nle instead of Met. Based on these results, successful incorporation of Nle into Est8 can be assumed. Clearly, these are only qualitative statements, which allow no statement about the homogenity of the protein preparation. This would only be possible by analyzing the intact protein. Unfortunately, MS analysis of intact proteins is currently only possible for proteins up to 40 kDa. The relatively large size of the protein (~52 kDa), made it necessary to perform an in-gel digestion and analyse the resulting peptides.

The parent protein, Est8[Trp] as well as the variants, Est8[7FW] and Est8[4NW], which were expressed using the developed protocol for supplementation incorporation of *in-vivo* synthesized Trp and Trp analogues, were also sent for MS analysis. For the parent protein and the Est8[7FW] variant, a protein sequence coverage of 86% was determined. In both cases, all 10 Trp residues were located within the matched peptides. For Est8[4NW], too little protein was available for reliable MS analysis. Analysis of the matched peptides found for the parent protein revealed that Trp was found on all Trp positions. For the Est8[7FW] variant, at least 50% of the corresponding peptides contained 7FW instead of Trp. Based on these results it can be assumed that the biosynthesis and incorporation of Trp and Trp analogues with the developed protocol was successful.

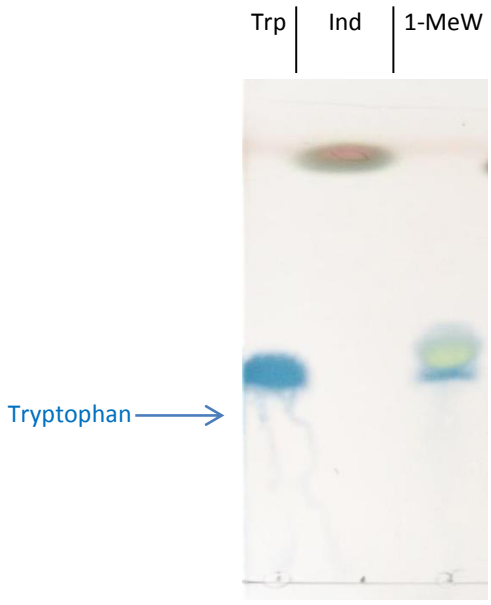


Figure 33: TLC of 10 µmol of tryptophan (Trp), indole (Ind), 1-methyltryptophan (1MeW).
Results of the TLC analysis and the depicted image of TLC plate were kindly provided by Barbara Scherz (ACIB, Graz).

MS analysis of the protein, which was expressed in the presence of the Trp analogue 1MeW, resulted in a high protein sequence coverage of 97%. However, the analysis of the matched peptides revealed, that the protein contained Trp at every Trp position. Thus, the supplementation

incorporation of 1MeW was not successful. One of the first reasons which came into mind was the possible impurity of the analogue, 1MeW. Protein yields which were as high as for the parent protein (1.9 mg/g CWW), comparable kinetic parameters and similar behaviour on polymeric substrates already aroused suspicion.

Therefore, 1MeW was analysed via TLC by Barbara Scherz (ACIB, Graz) to evaluate the purity of the used chemical. TLC showed that the substance 1MeW, which was used for the experiments, was not pure (Figure 33). Partial impurities were expected, due to the fact that the substance is sold with a purity of only 95%. Although the present TLC revealed that the substance exhibited a distinctly higher impurity than stated by the manufacturer. Based on these findings it was concluded that this substance cannot be used for further incorporation experiments.

4. CONCLUSION

The present study describes the engineering of a potential polymer degrading enzyme with the tools of synthetic biology and the subsequent assessment of the polymer degradation capability of the generated enzyme variants.

Analysis of the crystal structure of the target protein Est8 identified methionine and tryptophan as suitable targets for incorporation studies. Protein expression in the presence of the methionine (Met) analogue norleucine (Nle) yielded sufficient amounts of protein (0.609 mg protein/g CWW). In case of tryptophan, the supplementation incorporation was not as straight forward. Poor availability and the relatively high price of tryptophan (Trp) analogues, made it necessary to synthesize the Trp analogues by co-expressing the tryptophan synthase (TrpS) from *Salmonella typhimurium*. In the presence of serine, this enzyme converts indole analogues to the corresponding Trp analogues which can then be incorporated into the target proteins. Expression of TrpS and the target protein Est8 in the presence of indole and indole analogues was successfully confirmed by SDS-PAGE analysis. However the yields of purified protein were too low for any kind of kinetic characterization or activity measurement. Consequently, a classical supplementation incorporation experiment using the relatively cheap Trp analogue 1-methyltryptophan (1MeW) was performed. The protein yield was rather high, accounting for 1.9 mg protein/g CWW.

Kinetic characterization of the parent protein Est8[Met] and the variants Est8[Nle] and Est8[1MeW] was performed on the small, soluble substrate *para*-nitrophenyl butyrate (*p*NPB), where it could be demonstrated that functional parent and variant proteins were expressed under the chosen conditions. The expression in the presence of Nle reduced the maximum specific activity by a factor of 1.8. Est8[1MeW] exhibited a similar value compared to the parent protein. In case of Est8[Nle] it was speculated that the alteration in activity may already be a hint that the supplementation incorporation of Nle into the target protein worked, while for Est8[1MeW], similar behaviour to the parent protein was interpreted to be indicative for the expression of non- or partially labelled target protein. Due to the fact that the results of the analysis of the analogue incorporation by MALDI-MS were not yet available, the hydrolytic activity towards polymeric substrates was tested for the parent protein and both variants.

On the aliphatic-aromatic copolyester ecoflex® all three enzymes exhibited degradation activity in varying amounts. Although the degradation patterns were shifted, a significant improvement of the activity due to the incorporation of ncAA was not detectable. To get a deeper insight into the

degradation mechanism, the enzymes were tested for their potential to hydrolyse the ecoflex® model substrate BaBTaBBa. Exo-wise and endo-wise cleavage mechanisms were postulated and compared to the degradation patterns gained from the experiments. The results showed that rather a tendency towards exo- or endo-wise cleavage than a strict differentiation can be deduced from the degradation patterns.

Analysis of analogue incorporation by MALDI-MS revealed that successful incorporation of Nle into Est8 can be assumed. For the proteins, which were expressed in the presence of the Trp analogue 1MeW no successful incorporation could be confirmed. TLC analysis of the 1MeW revealed that the substance exhibited a relatively high impurity. Therefore it can be assumed that the used protein formulation of Est8[1MeW], may have been a mixture of non - and partially labelled protein.

To sum up, it could be successfully demonstrated that functional proteins can be expressed by supplementation incorporation of ncAAs. Although a reduction in the specific activity by a factor of 1.8 was observed upon kinetic characterization on *p*NPB. Activity tests on polymeric substrates revealed that the hydrolytic activity and the degradation pattern were altered but not significantly improved with respect to more complete degradation of the polymer. Some tendencies and trends could be deduced from the results, but have to be confirmed by further degradation experiments. A major drawback was the relatively large size of the protein (~52 kDa). To determine the incorporation efficiency more precisely, MS analysis of intact proteins must be performed. Currently this is only possible for proteins up to 40 kDa. Therefore it was necessary to perform an in-gel digestion and analyse the resulting peptides. The thereby gained results are only qualitative, which allow no statement about the homogeneity of the protein preparation. Additionally, it should be considered for further experiments to analyse the analogue incorporation in timely manner to prevent the use of non- or partially labelled protein for kinetic characterization and activity measurements.

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6. APPENDIX

6.1. Vector maps and sequences

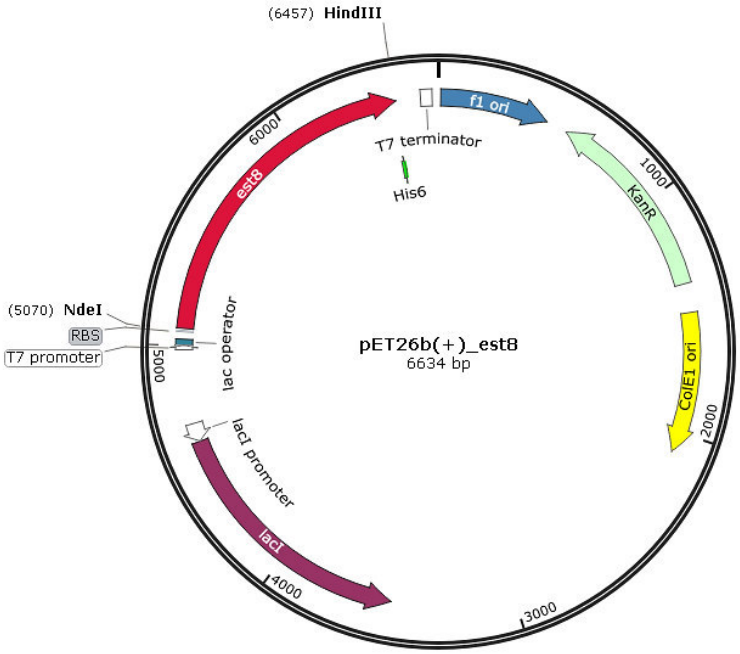

Figure 34: Vector map of pET26b(+)_est8.
A description of the features is provided in section 2.1.2. Relevant restriction sites *Nde*I (5070) and *Hind*III (6457) are indicated.

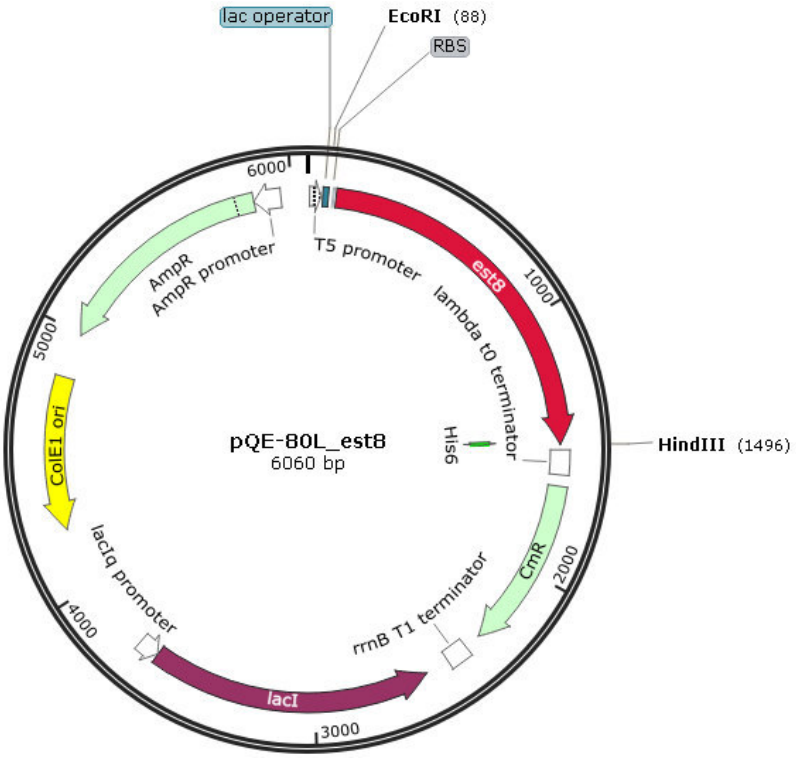

Figure 35: Vector map of pQE-80L_est8.
A description of the features is provided in section 2.1.2. Relevant restriction sites *Eco*RI (88) and *Hind*III (1496) are indicated.

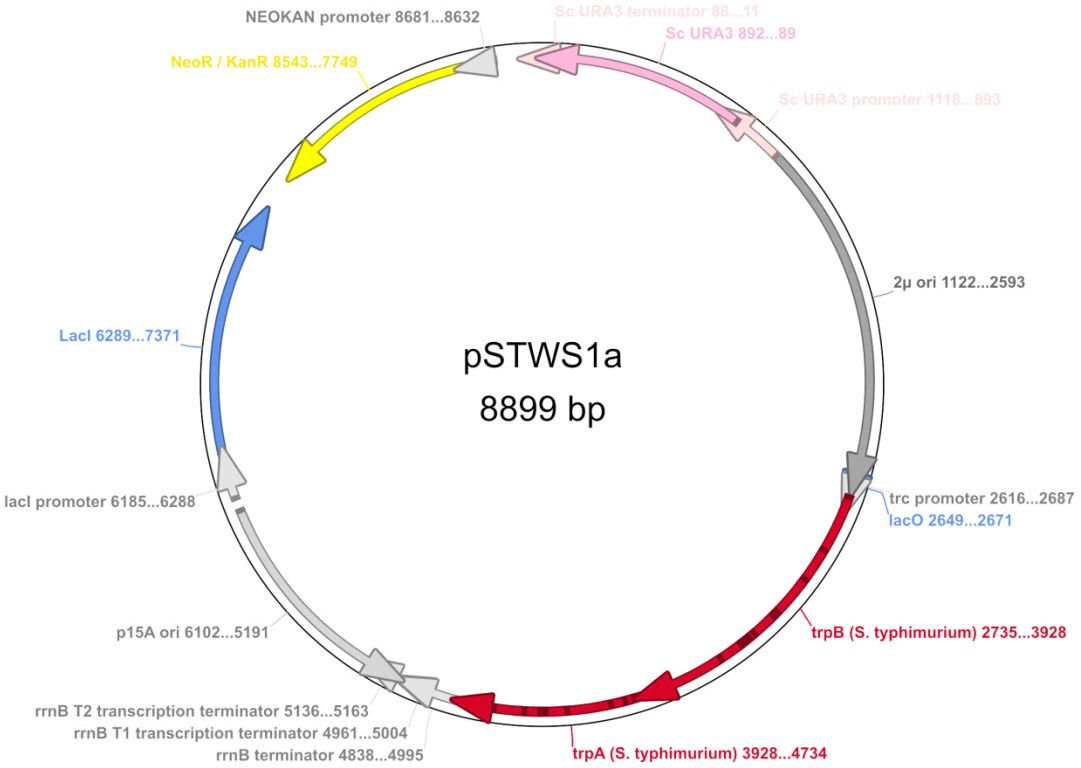

Figure 36: Vector map of pSTWS1a.
A description of the features is provided in section 2.1.2.

Figure 37: Vector map of pSTWS2.
A description of the features is provided in section 2.1.2.

atggcagaaccgaaagcacagggcacccagaaagttgaaagcagcaccaccaaaaaagaagttaaaga
cgcagaggaaaccatcaaaattccgaccctggaagatattgataacctgattgatagcgcagaagaag
tgaaatcagaagaggacattaacaaaatgcctccgctgaaatttccggttgaatttccggaagttaat
acccgtagcattattggtggtaacaactatccgattgttctggtgcatggttttatgggttttggtcg
tgatgaactgctgggttacaaatattggggtggtgttgttgatctgcaagaaaaactgaatgcaagcg
gtcatgaaacctataccgcaaccgttggtccggttagcagcaattgggatcgtgcatgtgaactgtat
gcatatattgttggtggcaccgttgattatggtgaagcacatgccaaaaaattcaaacataatcgtta
cggtcgtacctatccgggtatctataaaaacattagcaacgagaacaaaatccacctgattggtcata
gcatgggtggtcagaccattcgtaccctgacccagctgctgagcgaaggtagcgaagaagaaattaac
tgcggtcaagaaaacatcagtccgctgtttgaaggtggtaaacattggattcatagcgttagcaccat
tagcaccccgaatgatggcaccaccctgagcgatctgatgcctgcaaaagatctgattagctatacct
ttggtgttctgggcaccattaccggcaaaaacaaactgtttagcagcatctatgatctgaaactggat
cagtggggtctgaaaaaacagaatggtgaaagccagcgcgattatattgaacgtgttctggatagcaa
tatctggaacagcaccaaagatattgcaacctatgacctgagcaccgaaggtgcacaagaactgaata
cctgggttaaagcacagccggatgtttattactttagctggaccacccaggcaaccaaagaaagcatt
ctgacaggccatagcgttgcccagattggtccgatgaatccgatcttttatccgaccgcaaatctgat
gggtcgttatagccgtaatcagaaagatctgccgatcatcgacaaaaaatggtttccgaacgatggtg
tggttaattgtattagccaggatggtccgaaactgggtagcaatgatgttattgaacagtataatggt
ggcgtgaaaattggtcagtggaatgcaatgcctcgtattatcaataccgatcacatggatattgtggg
caccttggtaatgtgaaagattggtatatggattacgccagctttctgagcaatctgagccgtgccc
tggaa**catcatcatcaccatcattga**

Figure 38: Nucleotide sequence of est8 (1386 bp).
The N-terminal 6xHis-tag and the following stop codon are marked in green.

M A E P K A Q G T Q K V E S S T T K K E V K D A E E T I K I P T L E
D I D N L I D S A E E V K S E E D I N K **M** P P L K F P V E F P E V N
T R S I I G G N N Y P I V L V H G F **M** G F G R D E L L G Y K Y **W** G G
V V D L Q E K L N A S G H E T Y T A T V G P V S S N **W** D R A C E L Y
A Y I V G G T V D Y G E A H A K K F K H N R Y G R T Y P G I Y K N I
S N E N K I H L I G H S **M** G G Q T I R T L T Q L L S E G S E E E I N
C G Q E N I S P L F E G G K H **W** I H S V S T I S T P N D G T T L S D
L **M** P A K D L I S Y T F G V L G T I T G K N K L F S S I Y D L K L D
Q **W** G L K K Q N G E S Q R D Y I E R V L D S N I **W** N S T K D I A T Y
D L S T E G A Q E L N T **W** V K A Q P D V Y Y F S **W** T T Q A T K E S I
L T G H S V A Q I G P **M** N P I F Y P T A N L **M** G R Y S R N Q K D L P
I I D K K **W** F P N D G V V N C I S Q D G P K L G S N D V I E Q Y N G
G V K I G Q **W** N A **M** P R I I N T D H **M** D I V G T F G N V K D **W** Y **M** D
Y A S F L S N L S R A L E H H H H H H

Figure 39: Amino acid sequence of Est8 (51.7 kDa).
The amino acids methionine (Met, M) and tryptophan (Trp, W), which were used as targets for the supplementation incorporation of non-canonical amino acids, are marked in the sequence in red and blue, respectively.