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Studies on the role of EasC and EasE in ergot alkaloid biosynthesis

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Abstract

Ergot alkaloids are a group of secondary metabolites found in different fungi. In the past, their chemical and physiological properties had led to severe epidemics, causing death of many people. Due to their high structural similarity to serotonin, dopamine and epinephrine scientists are mainly interested in using them in treatment of different neurological diseases.

As total organic synthesis of alkaloids is rather complex and time consuming, the goal of biochemists and biocatalysts is the biocatalytic generation of at least some alkaloid pathway intermediates.

The aim of this master thesis was the expression and characterization of the enzymes involved in the first four steps of ergot alkaloid biosynthesis, leading to the formation of chanoclavine I aldehyde. As characterization of DmaW, EasF and EasD was successfully achieved by Metzger *et al.* 2009, Rigbers *et al.* and Wallwey *et al.* 2010, the main focus was set on the expression and characterization of EasC and EasE. Previous studies on those two enzymes had shown, that EasC is a catalase, which can easily be expressed in *E. coli*, whereas sequence analysis of EasE revealed a high sequence similarity to BBE-like enzymes. Until now, soluble expression of EasE could not be achieved.

Expression studies in *Escherichia coli* and *Komagataella pastoris* were performed, suggesting that *Komagataella pastoris* was the more suitable expression host for EasE. The protein obtained from expression in a 7 L bioreactor was further used in activity assays, helping to study its role in chanoclavine I synthesis.

EasC was expressed in *Escherichia coli* to allow biotransformations with both enzymes (EasC and EasE), which are assumed to work together for chanoclavine I formation. With our assays we were able to suggest that 4-dimethylallyl-tryptophan is converted to the corresponding "diene" when EasE is present, while the role of EasC remained unclear.

Zusammenfassung

Ergot-Alkaloide sind eine Gruppe von sekundären Metaboliten, die im Mittelalter durch den Genuss von mit Mutterkorn infizierten Roggenpflanzen zu schweren Epidemien und in weiterer Folge zum Tod vieler Menschen führten. Analysen der Ergot-Alkaloide zeigten, dass sie auf dieselben Rezeptoren wirken wie die strukturverwandten Neurotransmitter Dopamin, Epinephrin (Adrenalin) und Serotonin, weshalb Wissenschafter daran interessiert sind diese Alkaloide in der Behandlung neurologischer Erkrankungen einzusetzen.

Da die organische Totalsynthese dieser interessanten Biomoleküle sehr zeitaufwändig und arbeitsintensiv ist, wird versucht möglichst viele Stoffwechselintermediate biokatalytisch herzustellen.

Das Ziel dieser Masterarbeit war die Expression und Charakterisierung verschiedener Enzyme der Ergot-Alkaloid Biosynthese, wobei der Fokus vor allem auf die Enzyme EasC und EasE gerichtet wurde, die an der Bildung von Chanoclavin I beteiligt sind.

Bereits durchgeführte Studien haben gezeigt, dass EasC eine hohe Sequenzidentität zu Katalasen aufweist und in *Escherichia coli* leicht exprimiert werden kann, wohingegen Bemühungen EasE löslich zu exprimieren erfolglos blieben.

Während EasC in *Escherichia coli* exprimiert wurde, um es für später geplante Biotransformationen bereitzustellen, schien *Komagataella patoris* das für die Expression von EasE geeignetere Expressionssystem zu sein. Die Fermentation im 7 L Bioreaktor erlaubte die Isolierung von EasE, welches im Anschluss für Biotransformationen eingesetzt wurde. Die Ergebnisse dieser Assays deuten darauf hin, dass 4-dimethylallyl-tryptophan durch EasE zum dazugehörigen „Dien“ umgewandelt werden kann, die Rolle von EasC in der Chanoclavin I Synthese bleibt jedoch weiterhin ungeklärt.

Abbreviations

AA	acrylamide
A. nidulans	*Aspergillus nidulans*
BBE	berberine bridge enzyme
Bis	*N, N'*-methylenebisacrylamide
BMD	basal medium dextrose
BMM	basal medium methanol
BSM	basal salt medium
δ-ALA	δ-aminolevulinic acid
dH_2O/ ddH_2O	deionized water / extra pure water
DMAT	4-dimethylallyl-tryptophan
DNA	desoxyribonucleic acid
E. coli	*Escherichia coli*
fwd	forward
HIC	hydrophobic interaction chromatography
His	histidine
his_6-/ his_8-tag	hexahistidine-/octahistidine-tag
IPTG	isopropyl-*β*-D-thiogalactopyranosid
kDa	kilo Dalton
Km^r	kanamycin resistance
K. pastoris	*Komagataella pastoris*
LB	Luria-Bertani broth
MALDI-TOF	matrix assisted laser desorption ionization – time of flight
Ni-NTA	nickel-nitrilotriacetic acid-*N,N*-bis(carboxymethyl)glycine
OD_{600}	optical density measured at a wavelength (λ) of 600 nm
ONC	overnight culture
PCR	polymerase chain reaction
Ph	phenyl
pI	isoelectric point
pO_2	oxygen partial pressure
PTM	*Pichia* trace minerals
rev	reverse
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*

SDS-(PAGE)	sodium dodecyl sulphate-(polyacrylamide gel electrophoresis)
SEC	size exclusion chromatography
TBS / TTBS	Tris-buffered saline / Tris-buffered saline + Triton X-100
TEMED	*N, N, N', N'*-tetramethylethylenediamine
Trp	tryptophan
V	Volt
v/v	volume per volume
YPD	yeast peptone dextrose
Zeo^r	zeocin resistance

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Chapter 1: Introduction

1 Introduction

1.1 Alkaloids

Alkaloids are a widespread group of secondary metabolites, found in bacteria, fungi, plants and animals (Aniszewski *et al.*, 2007), known to have strong effects on human metabolism. Synthesis of those naturally occurring compounds usually starts off from amino acids, affecting that many of the alkaloids contain basic nitrogen atoms (Facchini *et al.*, 2005). The type of amino acid involved in scaffold formation strongly influences the structure of the metabolites, allowing the distinction of different classes of alkaloids: pyrroles and pyrrolidines, pyridines and piperidines, pyrrolizidines, tropanes, quinolones, isoquinolines, aporphines, indoles, imidazoles, norlupines, purines and steroids (Facchini *et al.*, 2005).

In plants alkaloids are assumed to mainly function in defense mechanisms. They are produced to prevent growth of parasites as well as they make sure that insects and animals do not try to eat the latter. Animals are also known to synthesize those secondary metabolites, but in contrast to plants they use alkaloids in their own metabolic processes (Hesse *et al.*, 2002). Many alkaloids are expected to be mainly involved in signal transduction, since famous members of that group of molecules like serotonin, histamine, dopamine and epinephrine act as important neurotransmitters (Hesse *et al.*, 2002).

The physiological importance is the main reason for scientists to further investigate that group of biomolecules. Deeper understanding of their chemical properties and better knowledge about their mode of action should allow the design of synthetic molecules, acting on the same receptors as the naturally occurring compounds, hence allowing the treatment of diseases, which are related to defects in signal transduction (Schiff, 2006; Schardl *et al.*, 2006).

Even though much is known about alkaloids, their medical application is still difficult as total organic synthesis of those molecules is not possible (Minami *et al.*, 2008) and knowledge on enzymes that could be used for biocatalytic production of the drugs is too little.

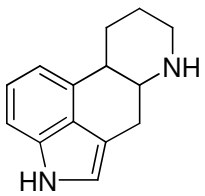
1.2 Ergot

Ergot, also called *Secale cornutum*, owes its name to its interesting structure, as French people used the term "argot" (engl. spur) when talking about that unknown species. These dark brown pegs can be found on ears of rye and were identified as a fungus in the 18th century (Schiff, 2006).

The reasons for botanists and scientists to characterize the ergot fungus were severe epidemics, being observed multiple times since the 9th century and causing death of many people. As it was expected that the fungus was responsible for that catastrophe, the disease was given the name "ergotism". One believed that the reason for the epidemics were the higher amounts of rye flour consumed by the population since the Middle Ages, but it was unknown why those ergots were causing so many troubles (Schiff, 2006; Schardl *et al.*, 2006). Many years later, it was found out that ergots produce alkaloids (metabolites) responsible for the fungus' toxicity.

1.3 Ergot alkaloids (EA)

Ergot alkaloids are a group of secondary metabolites derived from L-tryptophan and therefore belong to the group of indole derivatives. Having a look at their structure, ergot alkaloids can be classified upon their ergoline scaffold (structure of the basic ergoline scaffold see Figure 1).



ergoline ring system

Figure 1: basic ergoline scaffold

The three different classes resulting from this consideration are clavines, ergotamines and ergopeptines (Figure 2).

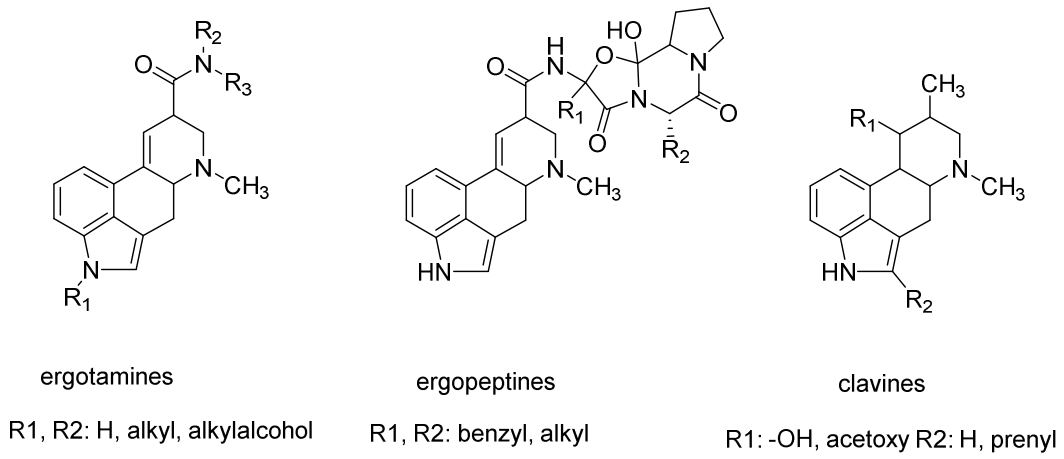


Figure 2: General structures of ergotamines, ergopeptines and clavines, representing the main products of ergot alkaloid biosynthesis

Although all of those products look rather different, the first part of their biosynthetic pathways is the same. It starts off from L-tryptophan and leads via dimethylallyltryptophan (DMAT), *N*-methyl-dimethylallyltrpytophan (N-Me-DMAT) and chanoclavine I to chanoclavine I aldehyde (see Figure 3).

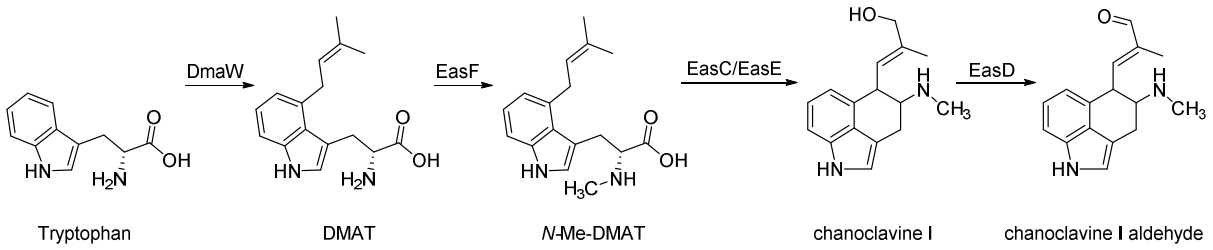


Figure 3: "Conserved part" of ergot alkaloid biosynthesis, leading to the formation of chanoclavine I aldehyde

All genes encoding for the enzymes required for the conserved part of ergot alkaloid biosynthesis are organized in a gene cluster (Goetz *et al.*, 2011).

The gene cluster of *Aspergillus nidulans* is given in the figure below (Figure 4)

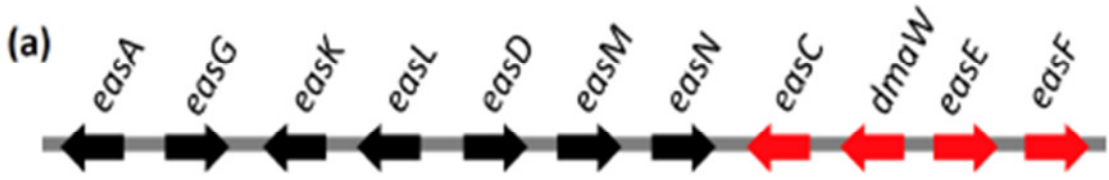


Figure 4: Gene cluster of ergot alkaloid biosynthesis, as it is found in *A. nidulans* (taken from Ryan *et al.*, 2013)

For the biosynthesis of chanoclavine I aldehyde, *dmaW*, *easF*, *easC*, *easE* and *easD* are required. *dmaW* encodes for a prenyl transferase and catalyzes the formation of DMAT from L-Trp and dimethylallylpyrophosphate (Metzger *et al.*, 2009). *easF* is the gene corresponding

to a SAM-dependent methyltransferase that methylates the amino group of DMAT, leading to *N*-Me-DMAT (Rigbers *et al.*, 2008). The subsequent chanoclavine I formation is likely to be catalyzed by two different enzymes encoded by *easC* and *easE*. It could be shown that expression of *easC* leads to the formation of a catalase (Goetz *et al.*, 2011), whereas *easE* shows high sequence similarity to BBE (Lorentz *et al.,* 2010), but the function of either of the enzymes in chanoclavine I synthesis is still unclear. The enzyme required for the oxidation of chanoclavine I, leading to chanoclavine I aldehyde is a short chain alcohol dehydrogenase encoded by *easD* (Wallwey *et al.*, 2010). All other genes of the cluster (see Figure 4) encode for enzymes involved later in the pathway (Figure 5) (Goetz *et al.*, 2011; Ryan *et al.*, 2013; Young *et al.*, 2015).

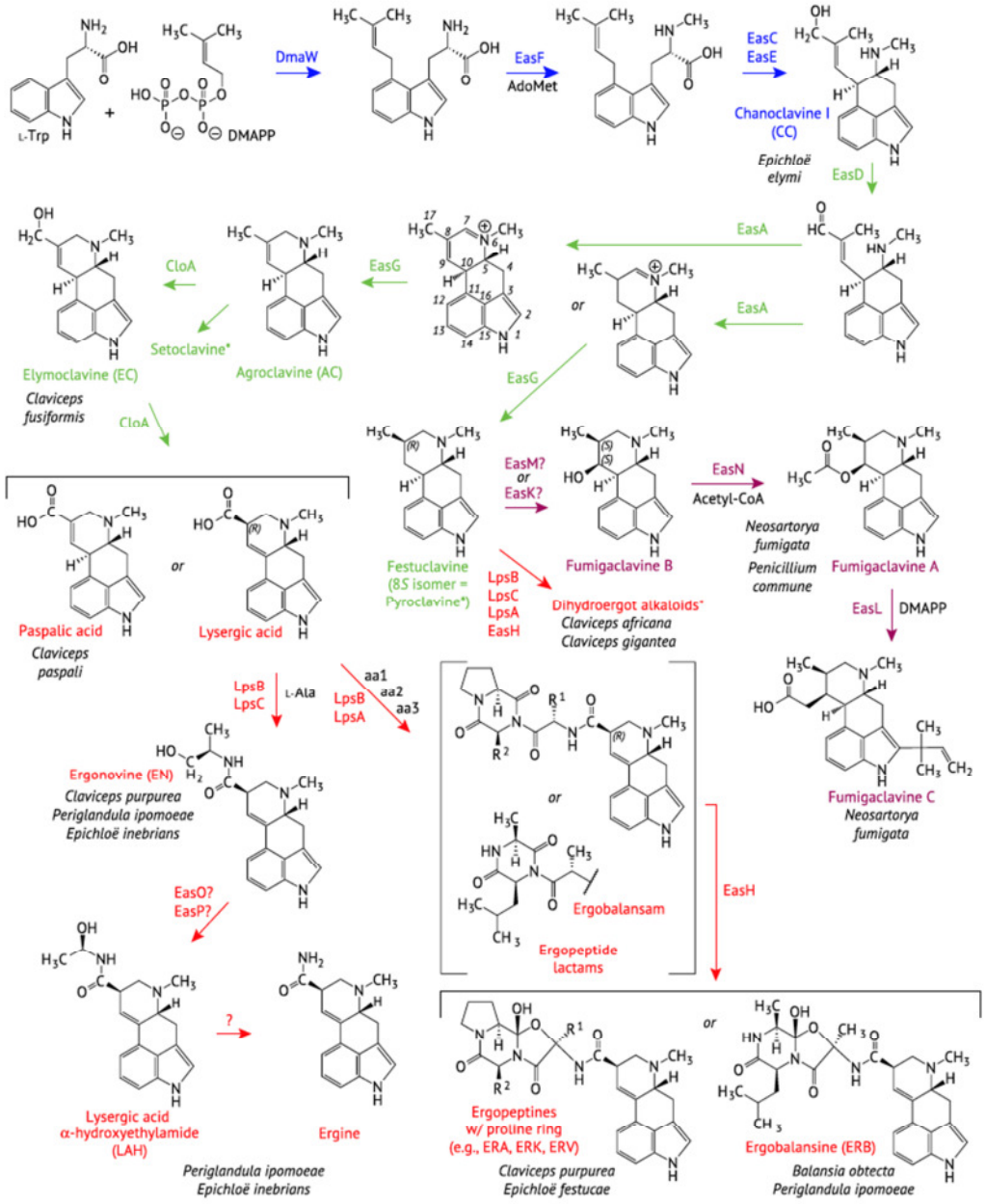


Figure 5: Overview of ergot alkaloid biosynthesis: the conserved part of that pathway is presented in blue, while green, red and purple were used to indicate the branches leading to clavines, ergopeptines and ergotamines.(taken from Young *et al.*, 2015)

1.3.1 Medical and pharmaceutical importance

Besides their toxic effects on human health if they are consumed in too high amounts, ergot alkaloids have some other chemical and physiological properties, which scientists are interested in. Their structural similarity to various neurotransmitters, especially to dopamine and serotonin, draws attention on this group of metabolites, as researchers are trying to find more and better drugs in treatment of neurological diseases (Haarmann *et al.*, 2009; Wallwey *et al.*, 2011; Nielsen *et al.,* 2014).

In addition, it could be shown that especially ergotamines interact with tryptaminergic and α-adrenergic receptors in blood vessels. As this group of ergot alkaloids has an activating effect on those receptors, it is likely that ergotamines could have positive effects on people suffering from migraine or other vascular caused headaches (Schiff, 2006).

The ability of various ergot alkaloids to act on serotonin receptors in an activating manner suggests that there is also potential use of those compounds as hallucinogens (Schardl *et al.*, 2006).

1.4 Flavin cofactors

Basically, two different flavin cofactors are distinguished – flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are both formed from vitamin B_2, also called riboflavin (Macheroux *et al.*, 2011).

While yeasts, plants and microorganisms are able to synthesize riboflavin on their own (Bacher *et al.,* 2000; Fischer *et al.,* 2008), mammals do not have biosynthetic routes for vitamin B_2, which forces them to take up riboflavin by dietary alimentation. FMN and FAD are then formed in ATP-dependent reaction steps, involving the riboflavin kinase and the FAD synthase (Macheroux *et al.*, 2011).

Flavin cofactors are mainly used by enzymes catalyzing biochemical reactions that require one- and/or two-electron transfer. The reason for that is that the flavin is able to change its oxidation state easily by shifting chemical bonds (Macheroux *et al.*, 2011). Just a relatively low amount of flavin dependent enzymes (about 10%) is involved in non-redox processes, such as DNA-repair, bioluminescence and circadian time keeping.

Studies also revealed that flavin cofactors do not just play catalytic roles, but that they strongly affect protein expression and protein stability (Winkler *et al.*, 2009).

1.5 Flavoproteins

Flavoproteins are proteins, containing either FMN or FAD as a cofactor – either covalently or non-covalently bound. (Macheroux *et al.,* 2011)

Although non-covalent linkage seems to be dominant, almost 10% of all flavoproteins contain a covalently linked cofactor (Macheroux *et al.*, 2011). Within the group of covalently flavinylated proteins one can further distinguish proteins using just one covalent bond to bind the cofactor and proteins binding the flavin bicovalently (Singer *et al*., 1956, Huang *et al*., 2005).

The first covalently linked flavin was discovered in the 1950s by Singer *et al*. (1956) upon their study on enzymes involved in the electron transport chain. In this enzyme, the so-called Succinate dehydrogenase, the flavin cofactor is linked to the protein via a mono-covalent bond. The latter is formed between the methyl group of the isoalloxazine ring (8α-position) and a histidine of the enzyme (Salach *et al.*, 1972).

By the time, studies revealed more and more enzymes with a covalently attached flavin cofactor. It was found out that mono-covalent linkage is sometimes also present in position six of the aromatic ring system. In that particular case, not a histidine but a cysteine is involved in bond formation. Interestingly, cysteines were also found out to interact with the 8α-methyl group, in the same way, tyrosines are known to form a covalent bond to the cofactor (Kearney *et al.*, 1972; McIntire *et al.*, 1981; Heuts *et al.,* 2009). The structure of the isoalloxazine ring system and its correct numbering is depicted in Figure 6:

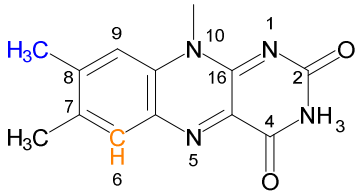


Figure 6: Numbering of the isoalloxazine ring, indicating the C-atoms involved in covalent flavinylation: Histidines, cysteines and tyrosines are all able to form a covalent bond to the 8α-methyl group, while linkages to position six are just known to be formed by cysteines (Heuts *et al.,* 2009).

In 2005, the first enzyme containing a bicovalently linked flavin was discovered (Huang *et al.*). This biocatalyst, called glucooligosaccharide oxidase (GOOX), binds the cofactor via a cysteine in position six and a histidine in position 8α. First, it was thought that this bi-covalent linkage was unique, but sequence alignments revealed that this type of cofactor attachment could be observed in all kinds of plants, fungi and bacteria. Blast searches also showed that the special protein folding, required for bicovalent cofactor binding is neither present in archaea nor in animals.

Right after the discovery of bicovalent cofactor linkage the reason for enzymes to use that way of cofactor attachment was unknown. So scientists carried out different experiments to find out more about the advantage of binding the cofactor in that particular way. Decreased cofactor mobility, allowing substrates to enter the active site more easily, and an increased reduction (midpoint) potential, enhancing oxidation rates, are the strongest effects that could be detected (Heuts *et al.*, 2009).

1.5.1 Berberine bridge enzyme (BBE)

BBE is one of the best-characterized flavoproteins. It was identified binding FAD bi-covalently, allowing the enzyme to catalyze very specific oxidation reactions. The enzyme was found to be part of the benzylisoquinoline biosynthesis, controlling the conversion of (*S*)-reticuline to (*S*)-scoulerine (Figure 7) (Winkler *et al.*, 2008 and 2009; Kutchan *et al.,* 1998).

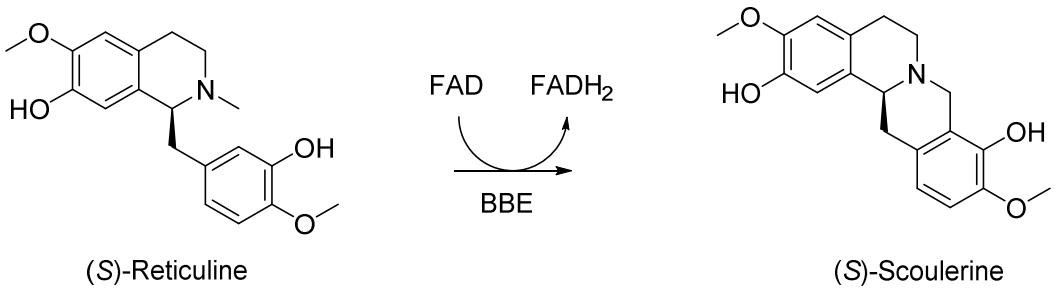


Figure 7: Conversion of (*S*)-Reticuline to (*S*)-Scoulerine catalyzed by BBE

The mechanism suggested for that particular reaction involves a nucleophilic attack of a glutamate on the hydroxyl group in position C3' of (*S*)-reticuline, triggering the formation of a carbonyl bond and due to the increased nucleophilicity of the neighboring C2' the formation of the fourth ring. At the same time, a hydride is abstracted form the *N*-methyl group and transferred onto the flavin cofactor, which is thereby reduced (Winkler *et al.*, 2008).

Since aromatic systems are very low in energy and are therefore chemically favorable, a proton is released from the C2' position, allowing the re-aromatization of the ring system. Re-oxidation of the flavin is achieved in an oxygen dependent process, leading to the release of hydrogen peroxide (Winkler *et al.*, 2008).

1.5.2 EasE

EasE seems to be the key enzyme catalyzing the formation of chanoclavine I (Lorenz *et al.*, 2010; Goetz *et al.*, 2011). Sequence alignments showed that the enzyme exhibits high sequence similarity to BBE (-like enzymes), but contains a far longer N-terminal domain. In addition, sequence analysis with ClustalP revealed a potential signal peptide sequence, involving the first thirty *N*-terminal amino acids (Nielsen *et al.*, 2014; Goetz *et al.*, 2011). Even though the *N*-terminus seems to be of great importance, its role could not yet be identified.

At the same time, the high sequence similarity of EasE to BBE-like enzymes suggests that the enzyme contains a bicovalently linked flavin, which strongly influences the enzyme properties. It allows the enzyme to act as an oxidoreductase, indicating that the reaction catalyzed by EasE is very likely to be an oxidative process. This affects that H_2O_2 is produced in that reaction step, giving rise to the assumption that the catalase EasC (see section 1.6.4) is required to remove the toxic side product (Nielsen *et al.*, 2014).

Until now, it was tried to express EasE in *S. cerevisiae* (Nielsen *et al.*, 2014), but just very low amounts of proteins – if at all – could be obtained, leading to the fact that *in vitro* studies did not allow the detection of any substrate conversion.

Having problems with *in vitro* studies, *in vivo* experiments were used to get further information on the properties of EasE. Nielsen *et al.* (2014) analyzed the expression, as well as the activity of EasE in presence of pdi1, which is known to help in disulphide bond formation. Co-expression of EasE and pdi1 increased chanoclavine I production by approximately 1.5-fold. Further the effect of co-expressing fad1 – an important enzyme in FAD-formation – on enzyme activity was studied. In that case chanoclavine I-production is increased by 2.5-fold, indicating that a higher co-factor supply has a positive effect on the ability of EasE to convert *N*-Me-DMAT.

Knockout studies also revealed that chanoclavine I formation does not only require EasE, but also a second enzyme called EasC. In the *easE* deficient mutant strains an accumulation of *N*-Me-DMAT could be observed (Lorenz *et al.*, 2010; Goetz *et al.*, 2011), suggesting that EasE is involved in the initial reaction step.

A potential reaction mechanism for chanoclavine I was already published in 1998 by Gröger and Floss, suggesting that “diene” formation is the first oxidation step (Figure 8) taking place in chanoclavine I synthesis (see also Schardl *et al.*, 2006; Lorenz *et al.*, 2010). After the

epoxidation, leading to the second proposed intermediate, a spontaneous S_N2 reaction is expected to trigger the loss of carbon dioxide, subsequently inducing the ring closure, leading to chanoclavine I (reaction intermediates see Figure 8).

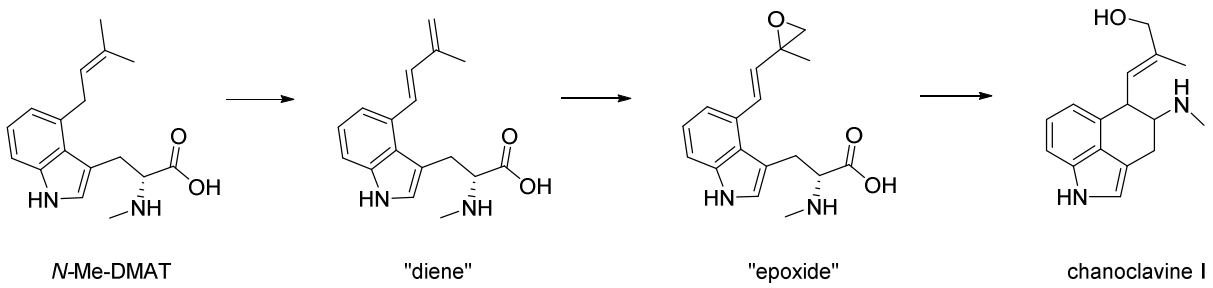


Figure 8: Reaction mechanism proposed for chanoclavine I formation (see also Lorenz *et al.*, 2010)

The role of EasE in that reaction is unknown, even though it is suggested that its BBE domain might be important in the cyclization step – either to catalyze the ring formation or to determine the stereo specificity (Lorenz *et al.,* 2010).

1.6 Catalases

Catalases are a biologically very important group of enzymes, as they are involved in the conversion of the toxic hydrogen peroxide to water and molecular oxygen. While the majority of those enzymes act in a heme-dependent manner, a small number uses manganese instead, making people call them manganese catalases (Zamocky *et al.*, 2008).

Based on the reaction mechanism as well as on the cofactor used, three different classes of catalases can be distinguished: Class I catalases, the "normal" catalases, are found in all organisms (from bacteria to animals), while class II catalases (catalase-peroxidases) are not present in plants and animals. Class III catalases, the manganese dependent catalases – also called pseudo-catalases, form to the smallest of the three groups and are just found in bacteria (Zamocky *et al.*, 2008).

1.6.1 Class I (monofunctional catalases)

Monofunctional catalases, also called "typical" catalases, are considered the largest group of peroxide degrading enzymes (Zamocky *et al.*, 2008). Phylogenetic analysis showed that within the family of catalases significant differences can be observed, allowing the distinction of three different clades: Clade I-enzymes are found in eubacteria, plants and algae and contain heme *b* as a prosthetic group. Within that class of catalases these enzymes are the smallest, with a subunit-size between 55 and 69 kDa. Clade II-enzymes are larger (75–84 kDa) and are just found in eubacteria and fungi. In contrast to clade I-enzymes the prosthetic group is not heme *b*, but heme *d*, which differs from heme *b* in modifications of the porphyrin ring system. The group of clade III-enzymes includes the majority of all known catalases and is found in bacteria, fungi, plants and animals. Their size can be rather different (43-75 kDa), but all enzymes of that class use heme *b* as a cofactor. Interestingly a second cofactor (NADPH) could be identified to also be used by clade III catalases, allowing them not only to perform the cleavage of the toxic hydrogen peroxide, but also to catalyze oxidation reactions. (Vetrano *et al.*, 2005; Zamocky *et al.*, 2008)

1.6.2 Class II- (catalase-peroxidases) and Class III- (manganese catalases) catalases

Catalase-peroxidases are bi-functional enzymes, found in prokaryotes as well as in eukaryotes. They are particularly interesting due to their high catalytic activity at neutral pH, which is not typical for catalases. As most of class I-catalases, catalase-peroxidases require

heme *b* as a cofactor, even though the reaction taking place to degrade hydrogen peroxide varies from the mechanism used by class I-catalases (Zamocky *et al.*, 2008).

Manganese catalases form the smallest class of catalases. This group of enzymes uses a dimanganese-cluster to cleave hydrogen peroxide in a rather unique way, which strongly differs from the reaction mechanism of class I and class II catalases (Zamocky *et al.*, 2008).

1.6.3 Physiological role of catalases

Catalases are physiologically important, as their main role is the control of hydrogen peroxide levels in different organisms, to prevent intoxication (Zamocky *et al.*, 2008). At the same time these enzymes have to make sure that the hydrogen peroxide concentration does not drop too much, as that would strongly influence signaling pathways in a negative way. Alterations in catalase expression levels might also lead to problems in targeting to peroxisomes (Terlecky *et al.*, 2006), as well as to mitochondria (Petrova *et al.*, 2004).

Regulation of catalase levels on erythrocytes seems to be particularly important, as many oxidases produce hydrogen peroxide, which has to be removed in the following. If the degradation is not efficient enough, hemoglobin is oxidized to methemoglobin, further leading to the hemolysis and/or aggregation of erythrocytes, as well as to the polymerization of hemoglobin (Masuoka *et al.*, 2006).

1.6.4 EasC

EasC is the second enzyme involved in chanoclavine I synthesis (Goetz *et al.*, 2011; Nielsen *et al.*, 2014). Sequence analysis showed that the enzyme exhibits high sequence similarity to catalases. Size and occurrence suggest that EasC belongs to class I catalases - to be more precise to clade 3 of that class. This means that the enzyme belongs to the family of “normal” catalases and uses heme *b* as a cofactor. At the same time it is likely that EasC can catalyze oxidation reactions as well, if NADPH is close enough to its binding site on the enzyme, but this property has not yet been shown. EasC can easily be expressed in *E. coli* and was shown to have catalase activity (Goetz *et al.*, 2011; Nielsen *et al.*, 2014). It was able to convert hydrogen peroxide to water and molecular oxygen, but addition of *N*-Me-DMAT to an enzyme solution did not lead to the modification of the substrate (Nielsen *et al.*, 2014). So, it is assumed that EasC has to interact with EasE, together catalyzing the formation of chanoclavine I. (Nielsen *et al.*, 2014)

1.7 Aim of the thesis

The aim of this master thesis was the expression and characterization of the enzymes involved in chanoclavine I aldehyde formation with special focus on EasE and EasC, which were expected to be the key enzymes catalyzing chanoclavine I formation.

At first, all genes corresponding to the enzymes involved in the different reaction steps ought to be cloned into *E. coli* vectors, to be able to express and analyze the proteins. As previous studies by Goetz *et al.* (2011) had shown that EasE exhibits high sequence similarity to BBE, which could successfully be expressed in *P. pastoris* but not in *E. coli* (Winkler *et. al*, 2008*)*, *easE* was additionally cloned into the *Pichia* vector pPICZα.

Studies on EasC mainly involved its expression and purification, trying to enhance cofactor saturation, as it was just important to make sure that active EasC was available for different biotransformations.

Since EasE was known to be difficult to express in soluble form, the main goal was to find expression conditions that allow expression and isolation of the protein in active form. Therefore, expression studies, co-expressing different solubility tags, were done in *E. coli*.
At the same time screening experiments, testing the secretory expression of EasE in *P. pastoris*, helped to obtain enough information to allow the large-scale expression of this flavoprotein, further enabling the purification of EasE. Finally, the enzyme was planned to be used in biotransformation assays, allowing the analysis of its role in chanoclavine I synthesis.

Chapter 2: Materials

2 Materials

2.1 General

All chemicals and media ingredients were purchased from Sigma-Aldrich (St. Louis, MO, USA), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland) or Becton, Dickinson and Company (Franklin, Lakes, NJ, USA).

The restriction enzymes used were ordered from Thermo Scientific/Fermentas (St. Leon-Rot, Germany) or NEB (Ipswich, MA, USA).

All DNA templates used were provided by Michael Naesby, Evolva SA (Switzerland). The genes were taken from *Aspergillus fumigatus* or *Aspergillus japonicas,* codon optimized for *S. cerevisiae* and delivered in yeast vectors.

2.2 Culture media

- LB-medium
  - 5 g/L Yeast Extract
  - 5 g/L NaCl
  - 10 g/L Tryptone/Peptone
  - **For plates**: additionally add 15g/L Agar (-Agar)

- YPD-medium
  - 10 g/L BD Bacto™ Yeast Extract
  - 20 g/L BD Bacto™ Peptone
  - 20 g/L D-Glucose (Dextrose): autoclaved separately in a 10x stock
  - **For plates**: additionally add 15g/L Agar (-Agar)

2.3 Expression media – *K. pastoris* screening

- BMD-medium
  - 20 mL 2M potassium-phosphate-buffer (pH 6 or pH 7)
  - 10 mL 10x YNB
  - 5 mL 10x (D-Glucose): end-concentration: 20g/L
  - 200 µL 500x biotin (end-concentration): 0.4 mg/L
  - 65 mL dH_2O

  ---

  100 mL in total

- BMM2-medium
  - 20 mL 2M potassium-phosphate-buffer (pH 6 or pH 7)
  - 10 mL 10x YNB
  - 1 mL MeOH abs.
  - 200 µL 500x biotin (end-concentration): 0.4 mg/L
  - 69 mL dH_2O

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  100 mL in total

- BMM10-medium
  - 4 mL 2 M potassium-phosphate-buffer (pH 6 or pH 7)
  - 2 mL 10x YNB
  - 1 mL MeOH abs.
  - 40 µL 500x biotin (end-concentration): 0.4 mg/L
  - 13 mL dH_2O

  ---

  20 mL in total

All media components except from biotin (sterile filtered) were autoclaved separately and mixed just before use.

2.4 Expression media – *K. pastoris* (Fermenter)

- BSM-medium (modified after Hartner/Winkler)
  - 0.52 g $CaSO_4 \cdot 2\ H_2O$
  - 6.96 g $MgSO_4 \cdot 7\ H_2O$
  - 8.58 g K_2SO_4
  - 6.00 g KOH
  - 0.66 g NaCl
  - 96.69 g glycerol
  - 50 mL 85% H_3PO_4
  - 2950 mL dH_2O

  3000 mL in total

- PTM1-medium (final volume 200 mL)
    - 0.016 g NaI
    - 0.04 g $Na_2MoO_4 \cdot 2\ H_2O$
    - 0.004 g H_3BO_3
    - 0.146 g $CoCl_2 \cdot 6\ H_2O$
    - 0.04 g biotin

    - 1.2 g $CuSO_4 \cdot 5\ H_2O$
    - 0.59 g $MnCl \cdot 4\ H_2O$
    - 4.0 g $ZnCl_2$
    - 13.0 g $FeSO_4 \cdot 7\ H_2O$
    - 1.0 g H_2SO_4 conc.

2.5 Buffer systems

- Lysis- (Binding-) buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10mM imidazole, pH 8
- Wash buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8
- Elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole, pH 8
- HIC start buffer: 50 mM potassium phosphate, 1 M $(NH_4)SO_4$, pH 7.5
- Gel filtration buffer: 100 mM Tris, 150 mM NaCl, pH 8

- Storage buffer
    - DmaW: 50 mM HEPES, 5% glycerol, before addition of glycerol pH 7.5
    - EasF: 50 mM HEPES, pH 7.5
    - EasC: 20 mM Tris, 50mM NaCl, pH 7.5
    - EasD: 20 mM Tris, 50mM NaCl, pH 7.5
    - EasE: 100 mM Tris, 150mM NaCl, pH 8

- Running buffer
    - Agarose-gel electrophoresis: TAE-buffer (50x): 2 M Tris/acetate, 0.05 M EDTA, pH 8.5
    - SDS-PAGE (5x): 15 g/L Tris, 71 g/L glycine, 2.5 g/L SDS, 1.68 g/L EDTA

2.5.1 Buffers and solutions for SDS-polyacrylamide gel electrophoresis

Table 1: Pipetting instructions for the preparation of SDS gels

	Separating Gel (12.5%)	**Stacking Gel (5%)**
acryl amide (40%) / 0.8% Bis[1]	3.12 mL	562.5µL
1.5 mM Tris-HCl pH 8.8	3.75 mL	-
0.5 mM Tris-HCl pH 6.8	-	625µL
20% SDS	50µL	25µL
dH_2O	2.99 mL	3.69 mL
10% APS (ammonium peroxodisulphate)	48µL	25µL
TEMED[2]	10µL	5µL

[1] *N, N'*-methylenebisacrylamide

[2] *N, N, N', N'*-tetramethylethylenediamine

- Staining solution
    - 75 mL/L acetic acid, 500 mL/L MeOH, 2.5 g/L Brilliant Blue R

- Destaining solution
    - 75 mL/L acetic acid, 200 mL/L MeOH

- Sample buffer (2x)
    - 1 mL Tris-HCl pH 6.8, 400 mg SDS, 300 mg DTT, 20 mg Bromphenol Blue, 2 mL glycerol, 10 mL H_2O

2.5.2 Buffers for dot blot and antibody detection

- TBS (1x): 50 mM Tris-HCl, 150 mM NaCl, pH 8
- TTBS (anti-his): 50 mM Tris-HCl, 150 mM NaCl, 0.2 % Triton X-100, pH 8
- Antibody 1 (anti-his): TTBS, 5 % BSA, dilution (1:5000)
- Antibody 2 (anti-rabbit): TTBS, a little bit of milk powder or 5% BSA, dilution (1:5000)

2.6 Standards

- DNA standard: Gene Ruler 1 kb DNA ladder (Thermo Scientific)
- Protein standard: LMW-standard (90 kDa, 66 kDa, 45 kDa, 30 kDa, 20 kDa, 14 kDa) (Amersham Biosciences)

2.7 Kits

- Plasmid purification:
  - Nucleospin® Plasmid Quick Pure (Macherey-Nagel)
  - NucleoBond® PC100 (Macherey-Nagel)

- Gel and PCR clean-up
  - Wizard SV Gel and PCR Clean-up System (Promega)

2.8 Bacterial strains

- *E. coli* TOP10 (Stratagene): for plasmid amplification
- *E. coli* BL21Star (DE3) (Stratagene): expression strain
- *K. pastoris* KM71H WT: expression strain
- *K. pastoris* KM71H [pPICK-PDI]: expression strain (co-expresses protein disulfide isomerase of *S. cerevisiae* → helps in protein folding)

2.9 Primers

Table 2: Primers for cloning of DmaW, EasF and EasD into the *E. coli* vector pET M11

DmaW	Forward Primer	5‘-TTGCCATGGGCACTGCTGGTCAAGGTATTAAGACTGG-3‘
	Reverse Primer	5’-TATGCGGCCGCCTAGTACTGGGTGATGACGCC -3’
EasF	Forward Primer	5‘-TTGCCATGGGCACAATTTCTGCTCCACCTATTATTG-3‘
	Reverse Primer	5’-TTAGCGGCCGCTTAGTTCAATCTTAGTCTCAATTGATAGAATGC-3’
EasD	Forward Primer	5‘-TTGCCATGGGCACCGTTGTCAACTCTAGAATTTTCG-3’
	Reverse Primer	5’-TATGCGGCCGCTTATGGAGTTGCAGCACCAAC-3’

Table 3: Primers for cloning of EasC and EasE into different *E. coli* vectors

EasC	Forward Primer (out)	5‘-TTGCCATGGCTCCAGATTCTAAGCCAAC-3‘
	Reverse Primer (mid)	5’-CCATGTTTTGGACCATCGTATCTTGGAATTCTTGACC-3’
	Forward Primer (mid)	5’-GGTCAAGAATTCCAAGATACGATGGTCCAAAACATGG-3’
	Reverse Primer (out)	5’-TATGCGGCCGCTCACAATCTAGCTGGAATAAAAGATTC-3’
$EasE_{30}$	Forward Primer (out)	5‘-TTGCCATGGTTGATGATGATAGACATGATTGCAG-3
	Reverse Primer (mid)	5’-CCGGCAGTACCGTGGTTGTTCAAGAATTGTAAAGCTCTC-3’
	Forward Primer (mid)	5’-GAACAACCACGGTACTGCCGGTCAATTCATTTTGAGAAACAC-3’
	Reverse Primer (out)	5’-TATGCGGCCGCTCAAACAACGGAAGCAGACAAAGAACC-3’
$EasE_{85}$	Forward Primer (out)	5‘-TTGCCATGGTTTTACAAGAATCCTTGGATTCTAAGTGGC -3’
	Reverse Primer (mid)	5’-CCGGCAGTACCGTGGTTGTTCAAGAATTGTAAAGCTCTC-3’
	Forward Primer (mid)	5’-GAACAACCACGGTACTGCCGGTCAATTCATTTTGAGAAACAC-3’
	Reverse Primer (out)	5’-TATGCGGCCGCTCAAACAACGGAAGCAGACAAAGAACC-3’

Table 4: Primers for cloning of EasE into the *K. pastoris* vector pPICZα

$EasE_{30}$	Forward Primer	5’-TCTCTCGAGAAAAGAGAGGCTGAAGCT GTTGATGATGATAGACATGATTGCAGATG-3’
	Reverse Primer	5‘-TATGCGGCCGCTTAGTGATGGTGATGATGGTGATGATG AACAACGGAAGCAGACAAAGAACCC-3‘
$EasE_{85}$	Forward Primer	5‘-TCTCTCGAGAAAAGAGAGGCTGAAGCT GTTTTACAAGAATCCTTGGATTCTAAG-3’
	Reverse Primer	5‘-TATGCGGCCGCTTAGTGATGGTGATGATGGTGATGATG AACAACGGAAGCAGACAAAGAACCC-3‘

Table 5: DNA constructs generated

plasmid	host	selection	properties	cloned genes
M11	*E. coli*	Km^r	*N*-terminal his_6-tag	*dmaW, easF, easC, easD,* $easE_{30}^*$, $easE_{85}^*$
GST	*E. coli*	Km^r	*N*-terminal his_6-tag and gene for C-terminal GST-tag	$easE_{30}^*$, $easE_{85}^*$
dsbA	*E. coli*	Km^r	*N*-terminal his_6-tag and gene for C-terminal bacterial disulfide oxidoreductase	$easE_{30}^*$, $easE_{85}^*$
dsbC	*E. coli*	Km^r	*N*-terminal his_6-tag and gene for C-terminal disulfide bond isomerase	$easE_{30}^*$, $easE_{85}^*$
nusA	*E. coli*	Km^r	*N*-terminal his_6-tag and gene for C-terminal nusA	$easE_{30}^*$, $easE_{85}^*$
MBP	*E. coli*	Km^r	*N*-terminal his_6-tag and gene for C-terminal maltose binding protein	$easE_{30}^*$, $easE_{85}^*$
GB1	*E. coli*	Km^r	*N*-terminal his_6-tag and gene for C-terminal immunoglobulin binding protein domain B_1	$easE_{30}^*$, $easE_{85}^*$
2TZ	*E. coli*	Km^r	*N*-terminal his_6-tag and gene for C-terminal 2TZ solubility tag	$easE_{30}^*$, $easE_{85}^*$
ZZ	*E. coli*	Km^r	*N*-terminal his_6-tag and gene for C-terminal ZZ solubility tag	$easE_{30}^*$, $easE_{85}^*$
pPICZα	*K. pastoris*	Zeo^r	*N*-terminal α-factor	$easE_{30}^*$, $easE_{85}^*$

$^*easE_{30}$: *easE* starting with Val30 of the amino acid sequence

$easE_{85}$: *easE* starting with Val86 of the amino acid sequence

2.10 Equipment

PCR devices:	2720 Thermal Cycler (Applied Biosystems)
	Primus 25 advanced (peqlab)
	Gene Amp® PCR System 9700
DNA electrophoresis equipment:	Sub-Cell® GT Agarose Gel Electrophoresis Systems (BIO-RAD)
Agarose gel documentation system:	Gel Doc 200 (BIO-RAD)
Electrophoresis power supply:	PowerPac™ 300 Cell (BIO-RAD)
Protein electrophoresis equipment:	Mini-PROTEAN® 3 Cell (BIO-RAD)
Incubation shaker:	Infors HT Multitron Standard
Centrifuges:	Heraeus Laborfuge 400R
	Heraeus Fresco™ 17 Centrifuge
	Sorvall® RC-5B Plus Du Pont
	Eppendorf Centrifuge 5810 R
	Avanti J-20-XP (Beckman Coulter)
Sonication device:	Labsonic U (B. Braun Biotech International)
FPLC:	ÄKTAexplorer 100 (GE Healthcare)
Purification column:	Ni-NTA agarose (Quiagen)
	Ni-sepharose™ 6 FastFlow (GE Healthcare)
	Ph-sepharose™ 6 FastFlow XK 50/20 (GE Healthcare)
	Superdex 200 prep grade XK 16/100 (GE Healthcare)
Spectrophotometer:	Specord 210 (analytikjena)
	Specord 200 Plus (analytikjena)
NanoDrop:	NanoDrop2000 Spectrophotometer (Thermo Scientific)
Various:	ISS110 SpeedVac® System (ThermoSavant)
	Centriprep® 10 kDa/30 kDa MWCO (Millipore, Pall)
	Eppendorf Thermomixer comfort
	Gene Pulser® II electroporator (BIO-RAD)
	Fermenter BBI Biostat CT5-2 (Sartorius)
	Bio-Dot™ Apparatus (BIO-RAD)

Chapter 3: Methods

3 Methods

3.1 Methods using *E. coli* as expression host

3.1.1 Transformation

The process of transformation was used to transfer all kinds of plasmid DNA into chemically competent bacteria cells (Top10, BL21*), later allowing plasmid amplification as well as protein expression.

Like known for standard heat-shock protocols, the competent cells frozen at -70 °C were thawed on ice. Afterwards, 50 to 100 µL of those cells were mixed with 0.5 to 5 µL of plasmid DNA and stored on ice for 30 minutes. Later a heat-shock was applied, incubating the cell-DNA-mixture at 42 °C for 2 minutes. After cooling down the samples again, 900 µL of LB-medium were added. To allow regeneration, the reaction tubes were incubated at 37 °C for 30-60 minutes (slight shaking: around 300 rpm), before centrifuging the latter for 1 minute at 13300 rpm to get a small pellet. Having poured off the supernatant, the pellet was re-suspended in the remaining amount of media and then plated on LB-agar plates, containing the type of antibiotic the plasmids do have a resistance gene for.

In addition, the same protocol was applied to a sample just containing competent cells, as negative control.

3.1.2 Vector preparation

An overnight culture (ONC) was prepared, which was further used to isolate DNA with the help of a "Midi-prep" (Nucleobond AX-Purification Kit).

Later on, this purified vector DNA was digested, using *NotI* and *NcoI*, incubating the reaction batches at 37 °C overnight. The next day, the enzymes were inactivated, placing the samples into a thermo cycler, which was preheated to 80 °C, for 20 minutes.

Afterwards, alkaline phosphatase was added to the batches, to hydrolyze terminal phosphates at the restriction sites, thus inhibiting vector re-ligation in the ligation process with the desired insert. Therefore the samples were again incubated at 37 °C for 1h.

Finally, loading dye was added and the DNA-mixture was separated on an agarose gel. The "vector-bands" were cut out and the DNA was isolated and cleaned up using the "Wizard SV Gel and PCR Clean-up System" provided by Promega.

3.1.3 PCR (Polymerase chain reaction)

PCRs were used to get a sufficient amount of DNA of the genes we were interested in. For DmaW, EasD and EasF amplification "normal" PCRs were performed, using Phusion DNA Polymerase, the corresponding 5x Phusion HF Buffer and primers as well as the DNA template. The components of the reaction batches and the corresponding volumes used are given in the table below (Table 6):

Table 6: Pipetting instructions for PCRs

Component	Volume (µL)
Phusion HF Buffer (5x)	10
dNTPs (2mM)	5
Primer (forward)	0.5 (final concentration: 0.5 µM)
Primer (reverse)	0.5 (final concentration: 0.5 µM)
DNA-template	1 (approx. 10 ng)
ddH_2O	32.5
Phusion DNA Polymerase	0.5
Total	**50**

The temperature program (cycling instructions) applied is provided in the table below (Table 7):

Table 7: Temperature program for PCRs

Cycle step	Temperature (°C)	Time (s)	Cycles
Denaturation	98	30	1
Denaturation	98	10	
Annealing	65	30	35
Extension	72	45	
Final Extension	72	10 min	1
Store	8	∞	

3.1.4 Overlap extension PCR

Overlap extension PCRs were used for the amplification of genes containing an unwanted restriction site, as well as to couple different genes. In step 1 two fragments were created, at the same time introducing a mutation in the particular restriction site or adding a spacing sequence to the genes of interest. Step two then involved the annealing of the two fragments, while step 3 is required to amplify the gene sequence formed in step 2. Pipetting schemes, as well as the PCR-programs applied, are summarized in the tables (Table 8 to Table 13) below:

Step 1

Table 8: Pipetting instructions for step 1 of overlap extension PCRs

Component	**Volume (µL)**
Phusion HF Buffer (5x)	10
dNTPs (2mM)	5
Primer (fwd/fwd mid)	0.5 (final concentration: 0.5 µM)
Primer (rev mid/rev)	0.5 (final concentration: 0.5 µM)
DNA-template	1 (approx. 10 ng)
ddH_2O	32.5
Phusion DNA Polymerase	0.5
Total	**50**

Table 9: Temperature program applied to the samples in step 1 of the overlap extension PCRs

Cycle step	**Temperature (°C)**	**Time (s)**	**Cycles**
Denaturation	98	30	1
Denaturation	98	10	
Annealing	60	30	35
Extension	72	45	
Final Extension	72	10 min	1
Store	8	∞	

Step 2

Table 10: Pipetting instructions for step 2 of overlap extension PCRs

Component	Volume (µL)
Phusion HF Buffer (5x)	10
dNTPs (2mM)	5
DNA-fragment 1	1-2 (x ng)
DNA-fragment 2	0.5-1 (x ng)
ddH_2O	x (fill up to 50 µL)
Phusion DNA Polymerase	0.5
Total	**50**

Table 11: Temperature program applied to the samples in step 2 of the overlap extension PCRs

Cycle step	Temperature (°C)	Time (s)	Cycles
Denaturation	98	30	1
Denaturation	98	10	
Annealing	58	30	20
Extension	72	45	
Final Extension	72	10 min	1
Store	8	∞	

Step 3 *(add mixture to batches of step 2)*

Table 12: Pipetting instructions for step 3 of overlap extension PCRs

Component	Volume (µL)
Phusion HF Buffer (5x)	10
dNTPs (2mM)	5
Primer (forward)	1
Primer (reverse)	1
ddH_2O	32.5
Phusion DNA Polymerase	0.5
Total	**50**

Table 13: Temperature program applied to the samples in step 3 of the overlap extension PCRs

Cycle step	Temperature (°C)	Time (s)	Cycles
Denaturation	98	30	1
Denaturation	98	10	
Annealing	68	30	25/30
Extension	72	45	
Final Extension	72	10 min	1
Store	8	∞	

3.1.5 (Preparative) agarose gels

Analytical agarose gels were used to analyze the size of DNA sequences created in PCRs or after digestion of plasmid DNA with restriction enzymes, while preparative agarose gels were used to purify various DNA samples.

For any of the applications mentioned above, 1% agarose gels were prepared, adding the desired amount of agarose to TAE-buffer and heating the suspension in the microwave until the agarose was dissolved completely. To later on allow UV-detection of the DNA-fragments, the viscous solution was cooled down to about 60 °C, before adding 5-8 µL Roti Safe (Roth) or HD Geen Plus DNA stain (Intas Science).

As reference "Gene Ruler" 1 kb DNA ladder (Thermo Scientific) was used.

3.1.6 PCR and gel clean-up

The band, corresponding to the DNA of interest, was cut out from the agarose gel, allowing DNA isolation using the "Wizard SV Gel and PCR Clean-up-System" provided by Promega.

The same "Clean-up-system" was also used to purify PCR-products as well as to concentrate DNA samples obtained in various isolation procedures.

3.1.7 Ligation

Ligation of vector and insert was performed, sticking to a typical ligation protocol. Vector, insert, T_4 DNA ligase, as well as its corresponding buffer were mixed (20 µL all together) and incubated at 22 °C for 1h. After inactivation of the enzyme, which was achieved by 10 min incubation at 65 °C, the whole ligation mixtures were transformed into *E. coli* Top10 cells (Transformation see also section 3.1.1).

3.1.8 Protein expression

Protein expressions were performed, using two different strategies.

***Strategy 1**: Protein expression at 37 °C*

This strategy was mainly used for screening (100 mL batches), to get an idea of whether a gene can be overexpressed or not.

At first, 5 mL of ONC were prepared. After overnight incubation at 37 °C on a shaker (130 rpm) for about 16 h, the ONC was used to inoculate the main culture. Therefore 1 mL of the ONC was transferred into a flask containing 100 mL of fresh LB-medium as well as the required antibiotic. Then the main culture was incubated on a shaker (at 37 °C; 130 rpm) until an OD_{600} (optical density at 600 nm) between 0.6 and 0.8 could be measured.

As soon as the OD_{600} was high enough, IPTG (0.1 mM end concentration) was added to induce protein expression. After three more hours of incubation on the shaker (at 37 °C; 130 rpm), the cells were harvested, using centrifugation (15 min; 4600 rpm).

***Strategy 2**: Protein expression at 20 °C*

As the protein expressions performed at 37 °C mainly showed inclusion bodies, it was decided to perform the upscaled protein expression at 20 °C. The lower temperature should decrease the speed of the protein expression, giving the proteins more time to fold properly – this is important as proper folding is the main requirement for a protein to be found in soluble form.

At first, 50 mL of ONC were prepared. After overnight incubation at 37 °C on a shaker (130 rpm) for about 16 h, the ONC was used to inoculate the main culture. Therefore 7 mL of the ONC were transferred into a flask containing 700 mL of fresh LB-medium as well as the required antibiotic. Then the main culture was incubated on a shaker (at 37 °C; 130 rpm) until an OD_{600} (optical density at 600 nm) between 0.6 and 0.8 could be measured.

As soon as the OD_{600} was high enough, IPTG (0.1 mM end concentration) was added to induce protein expression, while the temperature was decreased to 20 °C. After overnight incubation (130 rpm) the cells were harvested using centrifugation (15 min; 4600 rpm).

3.1.9 Sodium dodecyl sulphate-polyacrylamide (SDS) gel electrophoresis

For protein analysis SDS-gel electrophoresis was used (pipetting instructions see section 2.5.1).

Protein samples, which should be analyzed on the gel, were prepared as follows: 10-30 µL protein sample were mixed with equal amounts of 2x sample buffer (composition see section 2.5.1) and incubated at 95 °C for ten minutes. After short spinning of the samples about 5 µL were loaded onto the gel. In addition, 3-5 µL of the "Low Molecular Weight" (LMW) Standard (Amersham Biosciences) were applied to later be able to estimate the molecular weight of the expressed proteins.

Gels were run with 120 V in the beginning and 180 V as soon as the dye reached the separation gel and were afterwards stained with Coomassie Brilliant Blue R (composition see XX) for 5-10 min. Destaining of the gel, which was required to visualize the protein bands, was achieved by incubating the gel in destaining solution (composition see sectionxx) for at least 1 h.

3.1.10 Cell lysis and protein purification

For protein purification 5 mL Ni-NTA-columns were used. Prior to any purification, the columns were loaded with fresh Ni^{2+} using a 100 mM $NiSO_4$-solution and equilibrated using the binding-/lysis-buffer (50 mM NaH_2SO_4, 300 mM NaCl, 10 mM Imidazole; pH 8). The exact protocol is given below:

- loading and equilibration of a Ni-NTA-column (5 mL)
    - wash with about 150 mL of dH_2O
    - load Ni^{2+} onto the column using 20 mL of $NiSO_4$-solution
    - wash again with about 20 mL of dH_2O
    - equilibrate the column using 20 mL of binding-/lysis-buffer

Meanwhile cell pellets were thawed and re-suspended in lysis buffer. The cells were lysed by sonication with the Labsonic U device for 10 min (ice cooling) and afterwards the suspension was filled into centrifugation tubes and centrifuged at 18000 rpm and 4 °C for 30 min.

The supernatant was filtered immediately, making sure that just clear lysate was later slowly loaded onto the equilibrated Ni-NTA column. Right after washing with 30-50 mL of wash buffer (50 mM NaH_2SO_4, 300 mM NaCl, 20 mM Imidazole; pH 8), proteins were eluted

using the elution buffer (50 mM NaH_2SO_4, 300 mM NaCl, 300 mM Imidazole; pH 8) and collected in fractions of 3 mL each, which were kept on ice.
After SDS-PAGE analysis fractions containing the protein of interest were pooled.

As protein stability in the elution buffer is quite limited, the buffer was exchanged right after analysis of the protein fractions. Therefore two different techniques were used:

- ***Buffer exchange using a dialysis tube***
  At first, a 40 cm long dialysis tube was cut and left in dH_2O. Having closed one end of the tube, the merged protein fractions were transferred into the tube, which was then fixed at the wall of a beaker filled with buffer. Afterwards, the beaker was stored at 4 °C overnight and the buffer was stirred constantly to allow buffer exchange. The next day, the dialysis tube was removed and the protein solution was transferred into centripreps, for concentrating the protein.

- ***Buffer exchange using centripreps***
  The pooled protein fractions were first concentrated to a volume of about 2 mL. Then storage buffer was added and the protein was concentrated again (repeat 3 times).

Having concentrated the proteins to the desired final volume, the protein concentration was determined using a photometer. The absorption at 280 nm was measured and then the molar extinction coefficient as well as the Lambert-Beer's rule was used to calculate the final protein concentration.

3.1.11 In vitro hydrogen peroxide assay (Catalase *in vitro* by Aebi *et al.)*

This assay was performed to prove the activity of EasC. Therefore two different phosphate buffers – one sodium phosphate buffer and one potassium phosphate buffer (50 mM each, pH 7.5) – were prepared and mixed in a 1.5/1 v/v ratio. Further, a 30 mM hydrogen peroxide solution was generated by adding the required amount of 30 % hydrogen peroxide to the phosphate buffer mixture.

Then, different reference measurements were performed

- To determine the absorption of the 30 mM H_2O_2-solution at 240 nm, 666 µL of phosphate buffer were mixed with 333 µL hydrogen peroxide solution. Then the absorption was measured using just phosphate buffer as a blank
- Afterwards, the time dependence of the hydrogen peroxide absorption at 240 nm was analyzed

Analysis

- The enzyme activity was analyzed using different enzyme concentrations:
    - Two measurements with a final enzyme concentration of 1.6 µM*
    - Two measurements with a final enzyme concentration of 3.05 µM*

*hydrogen peroxide solution is included in that calculation

The measurement cuvette contained 666 µL of enzyme solution, as well as 333 µL of phosphate buffer + hydrogen peroxide, which was just added right before the measurement was started, while the reference cuvette also contained the enzyme solution, but just phosphate buffer instead if the H_2O_2 solution.
Having started the reaction with the addition of H_2O_2, the time dependent decrease in absorption at 240 nm was detected for 30 s.

3.2 Methods using *Komagataella. pastoris* as expression host

3.2.1 Cloning

To be able to express the genes in *Komagataella pastoris* at first a PCR was performed to amplify the gene of interest. Then a midi-prep DNA isolation was used to get a sufficient amount of *K. pastoris* vector (pPICZ-alpha). After overnight restriction digestion of the vector and the insert using *XhoI* and *NotI* the fragments were ligated and transformed into *E.coli* Top10 cells (procedures see upper section 3.1).

To check whether the ligation was successful an ONC was incubated at 37 °C and 130 rpm and a mini-prep DNA isolation was performed the next day. Isolated DNA was then sent for sequencing (LGC Genomics).

As the sequences were correct another ONC was prepared and incubated at 37 °C and 130 rpm to be able to isolate more DNA with a midi-prep. The DNA obtained in that step was re-suspended in 50 µL ddH_2O each. Then a restriction digestion using *SacI* and the corresponding buffer was used to linearize the plasmid DNA for electroporation. To properly digest the DNA the restriction batches were incubated at 37 °C overnight.

The following day, *SacI* was inactivated by incubating the samples at 65 °C for about 20 minutes. Later, the linearized DNA was desalted using membrane filters with pores of 0.025 µm in diameter. After 2 h of incubation, the desalted samples were removed from the filter and were concentrated in the Speed Vac. The resulting pellets were then re-suspended in 15 µL ddH_2O, each, and incubated at 37 °C for 10 min to allow better dissolving.

3.2.2 Electroporation (Electro transformation)

For the electro transformation, 5 µL DNA (approx. 1-5 µg) were mixed with 40 µL of competent cells (PDI/KM71H), transferred into electroporation cuvettes and incubated on ice for 2 min. Then the cells were pulsed (1500 V, about 5 ms). To enable regeneration, 1 mL of sorbitol 1M-YPD-mixture (1:1 v/v) was added. By inverting the cuvettes media and cells were mixed. The suspensions were transferred back to 1.5 mL reaction tubes and then incubated at 30 °C for 2.5 h.

In the end, 200 µL each were plated onto YPD-plates, containing different zeocin concentrations (100-500 µg/mL). The plates were incubated at 30 °C for three days.

3.2.3 Small-scale expressions in *K. pastoris*

Expression screenings were performed in 96-deep-well-plates. At first, 250 µL of BMD media were pipetted into each slot. Then different *K. pastoris* colonies, grown on YPD plates containing different Zeocin concentrations, were used to induce cell growth. As *Komagataella pastoris* grows best between 28 and 30 °C, the plates were incubated at 28 °C and 320 rpm for the next two days.

After 65 h, 250 µL BMM2-medium were added to all wells to induce protein expression. After eight more hours of incubation at 28 °C and 320 rpm 50 µL of BMM10-medium were pipetted to the expression solutions. The following two days 50 µL of BMM10 medium were added in the morning to keep the expression level high enough.

After 72 h of induction cells and supernatant were separated using centrifugation at 4000 rpm for 10 min.

3.2.4 Dot blot and antibody detection

Dot blotting was required in order to be able to analyze the supernatant obtained from the small-scale expressions.

Nitrocellulose membranes were cut into pieces of desired size and incubated in 1x TBS buffer for at least 10 min. Then, the dot-blot apparatus was arranged and one nitrocellulose membrane was placed onto the rubber pad. To equilibrate the membrane, each slot of the dot-blot apparatus was filled with 600 µL TBS-buffer, which was pulled through the membrane using a membrane vacuum pump. Later, the supernatants (see above) were pipetted into the different positions. After 10 minutes of incubation at RT, the supernatant was pulled through the membrane, too. Having washed each slot with 200 µL of TTBS buffer, the membrane was removed and used for immunoassays (antibody detection).

The nitrocellulose membranes were washed with TTBS buffer for about 10 minutes and incubated in 13% milk powder (suspended in TTBS buffer) for 1 h before washing them with the TTBS buffer again for 10 minutes. As the free positions should have been successfully saturated by then, the first antibody was applied overnight at 4 °C. Since the protein of interest contains a His-tag, an anti-his antibody was used (preparation of anti-body solution see section 2.5.2).

After incubation with the first antibody the membranes were washed three times with TTBS buffer (10 min each) and then incubated in a solution containing antibody 2 (see section 2.5.2)

for 1 h (also at 4 °C). To wash off unbound antibody, incubation with TTBS-buffer (3x10 min) was required again.
In the end the membranes were stored in 1x TBS-buffer.
For detection luminol and peroxidase solution were mixed 1:1 and applied to the membrane. As the secondary antibody is coupled with a peroxidase, luminol is converted to its oxidative state, at the same time emitting light, which in turn mediates the photoreaction when placing a film on the membrane.

3.2.5 Expression in shake flasks

Expression in shake flasks was used to optimize the expression of EasE in *Komagataella pastoris*, since low amounts of media and a simple expression protocol allows testing of different conditions at the same time.
At first, 50 mL of BMD medium were prepared for each flask. Using different phosphate buffers, the pH of the expression media could be varied easily. Then one *K. pastoris* colony was added to each medium and the flaks were incubated at 28 °C and 150 rpm.
After 64 h, 5mL of BMM10-medium were used to induce protein expression. To keep the MeOH-level in the media high enough to maintain protein production for the next 72 h, 50 µL of MeOH were added after 8 h, 24 h and 48 h of induction. In the meantime the expression batches were incubated in a shaker (at 28 °C and 180 rpm).
In the end, the pH of the suspension was set to 8 and cells were harvested using centrifugation (4600 rpm, 15 min). To be able to analyze the supernatant as well as the cell pellets, they were separated from each other.

3.2.6 Analysis of the supernatants

Supernatant analysis was done by trying to purify EasE with the help of Ni-sepharose, which should have been possible due to the his_8-tag attached to the protein. Afterwards, the fractions obtained were analyzed on an SDS-PAGE. The bands, which were likely to correspond to the protein of interest, were cut out and analyzed with MALDI-TOF.

3.2.7 Large scale expression in a 7 L bioreactor

The large scale expression was performed as described by Schrittwieser *et al.* (2011). The first pre-culture (50 mL of YPD-medium + zeocin (100 µg/mL) was grown to an OD_{600} of about 2 and was then used to inoculate 300 mL of a fresh YPD medium. The culture was then incubated at 30 °C and 130 rpm for 8 h until an OD_{600} of 2.5 to 10 was reached.

Subsequently, all of this second pre-culture was used to inoculate the fermenter, which was filled with 3 L of *in situ* sterilized BSM-medium (see section 2.4). In addition, 14 mL of trace element solution (PTM1) were added. Later on the batch was stirred overnight, controlling pO_2, airflow, temperature and pH. The fermenter was run at 30 °C, minimum of 30 % pO_2 and pH 5.

The next morning the cells started to starve, which resulted in a fast increase of pO_2. Therefore, the fed-batch phase was started. As mentioned in the publication by Schrittwieser *et al.* (2011), the phase was induced by adding about 15 g glycerol per hour. The feed-rate was then slowly increased up to 44 g glycerol/h to be able to feed the cells 1000 g glycerol within 24 h.

As soon as the cells had consumed all the glycerol, a sample was taken (reference) and 5 g of MeOH were added to start the induction phase. At the same time the glycerol-feed rate was reduced to 15 g glycerol/h within 1:59 h and after that to zero within one minute. Then the pH was set to 7 and MeOH-feed was started. In the beginning a feed-rate of about 3 g MeOH/h was used, which was slowly increased to 8 g MeOH/h so that the cells were treated with around 600 g MeOH in the expression time of 96 h.

In the end NaOH was used to set the pH to 8 and the cells were harvested using centrifugation (4000 rpm, 30 min, 4 °C). As the protein of interest was supposed to be found in the supernatant, the latter was separated from the cells and stored at 4 °C before applying it to a Ph-sepharose column.

3.2.8 Purification via a Ph-sepharose column

For that purification step, a 120 mL Ph-sepharose column was packed and equilibrated with HIC start buffer (50 mM potassium phosphate, 1 M $(NH_4)SO_4$, pH 7.5; buffer A). $(NH_4)SO_4$ was added to the fermentation supernatant until an end concentration between 0.75 and 1 M was reached. Then the supernatant was loaded onto the column and weakly bound proteins were washed off using 300-400 mL buffer A. As no protein was washed from the column

anymore elution of the specifically bound proteins was started with a buffer gradient (up to 100 % buffer B – 20 % ethanol) to the column. The gradient chosen was generated as follows:

- 0 % - 50 % buffer B in 30 min
- 50 % - 100 % buffer B in 60 min

For all eluting fractions (12 mL each) the absorption at 280, 375 and 450 nm was measured, allowing the identification of the fractions containing flavoproteins. The latter were analyzed on an SDS-PAGE and the fractions, in which the desired protein was found, were merged and concentrated.

3.2.9 Purification using gel filtration (SEC)

Gel filtration – size exclusion chromatography – was performed, since purification with hydrophobic interaction chromatography did not lead to a pure protein at all. After equilibrating the column (Superdex 200 prep grade) with the desired buffer (100 mM Tris, 150 mM NaCl, pH 8), the protein sample was loaded onto the column.
Elution fractions of 3 mL were collected and the absorption at 280, 375 and 450 nm was detected. All fractions showing a characteristic absorption at 375 and 450 nm were analyzed using SDS-PAGEs. After pooling the fractions with similar impurities the latter were concentrated using centripreps.

3.2.10 Activity assays

Activity assays were mainly performed to gain information on the reactivity of EasE and EasC. At the same time they were also used to make sure that no other enzymes co-existing in the reaction solution were able to alter the substrate (DMAT). DMAT was provided by Jörg Schrittwieser, who introduced the prenyl group in position four of commercially available L-Trp.
Therefore different reactions were set up (pipetting instructions see Table 14) and incubated at 37 °C for 3 h. Then the biotransformations were stopped by adding 100 µL of acetonitrile to the reaction mixtures. In addition, one set of assays was incubated for 72 h, to be able to study the effect of longer incubation times on substrate conversion:

Table 14: Components of the different biotransformation assays

	EasC* (µL)	EasE* (µL)	fermentation sup. (no EasE) (µL)	Buffer (µL)	DMAT* (µL)	NADPH* (µL)	t (h)
1	-	25	-	50	25	-	3
2	12,5	12,5	-	50	25	-	3
3	-	-	-	75	25	-	3
4	-	25	-	50	25	-	72
5	12,5	12,5	-	50	25	-	72
6	-	-	-	75	25	-	72
7	25	-	-	50	25	-	3
8	25	-	-	37,5	25	12,5	3
9	-	-	25	50	25	-	3
10	-	-	-	62,5	25	12,5	3

* conc.: EasC: 20 µM; EasE: unknown; DMAT: 1 mM; NADPH: 10 mM

Buffer: 100 mM Tris, 150 mM NaCl, pH 8

Chapter 4: Results

4 Results

4.1 Expression trials in *E. coli*

4.1.1 Cloning of the genes into *E. coli* vectors

All genes of the previously discussed part of the ergot alkaloid biosynthesis pathway were cloned into *E. coli* vectors as they were delivered form Evolva in *S. cerevisiae* vectors.
The *E. coli* vector system chosen in that case was M11 containing a kanamycin resistance gene as well as a coding sequence for an *N*-terminal his-tag. EasE was additionally cloned into other *E. coli* vectors for the co-expression of different solubility tags since a low solubility of that protein was expected due to its high sequence similarity to BBE.

In addition, the EasE gene was cloned into the vectors in two different lengths. One construct contained the nucleotide sequence, starting right after the signal peptide sequence, which was predicted using SignalP (cleavage site between Gly29 and Val30) and ending with the original C-terminus (will further be called $EasE_{30}$). This sequence length was chosen, as *E. coli* is not able to secrete proteins and can therefore not make use of the additional amino acids. If the signal peptide is not cut off correctly, this might even lead to the formation of improperly folded proteins.

The second construct also ended with the natural C-terminus but had a shorter *N*-terminal part. In that case, the *N*-terminus was set to Val86 (will further just be called $EasE_{85}$) to be able to study the importance of that part of the protein, as BBE and other BBE-like enzymes are lacking that sequence.
All constructs generated are summarized in Table 5 (p. 22).

The plasmids with the correct nucleotide sequence were finally transformed into *E.coli* Top10 and BL21* competent cells, further allowing plasmid isolation as well as expression of the genes.

4.1.2 Protein expression

Expression was successful for all genes, but while DamW, EasF, EasC and EasD could be solubly expressed in satisfying concentrations in an overnight fermentation process, $EasE_{30}$ and $EasE_{85}$ were not obtained in soluble form.

Figure 9 and Figure 10 below show the SDS-PAGE demonstrating the successful expression of all genes:

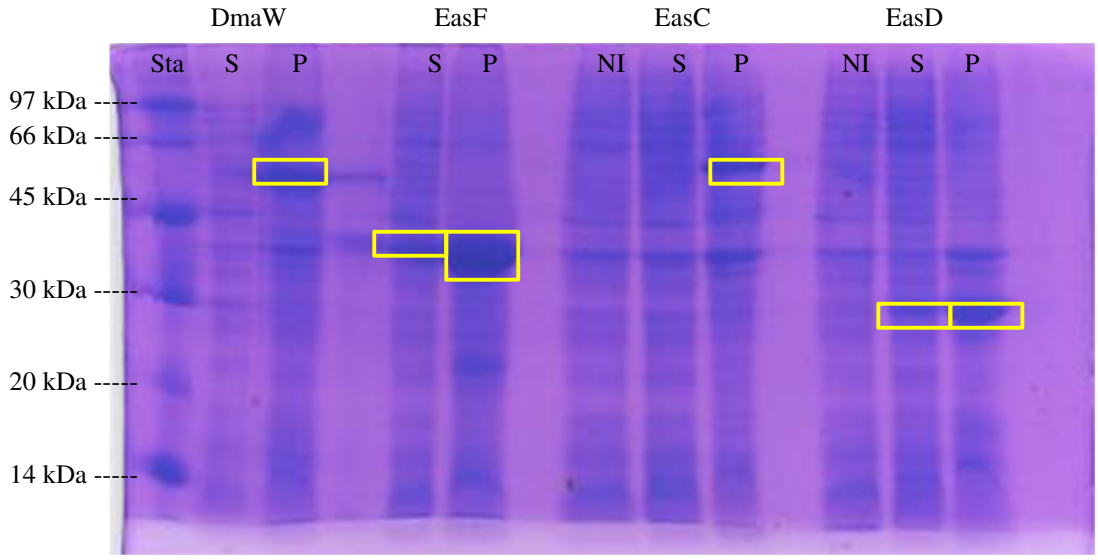

Figure 9: Screening of protein expression: SDS-PAGE analysis of samples taken from the supernatant (S) and the pellets (P) obtained right after the fermentation process. NI-(non-induced) samples show the bands of all proteins found in the pellet prior induction with IPTG.

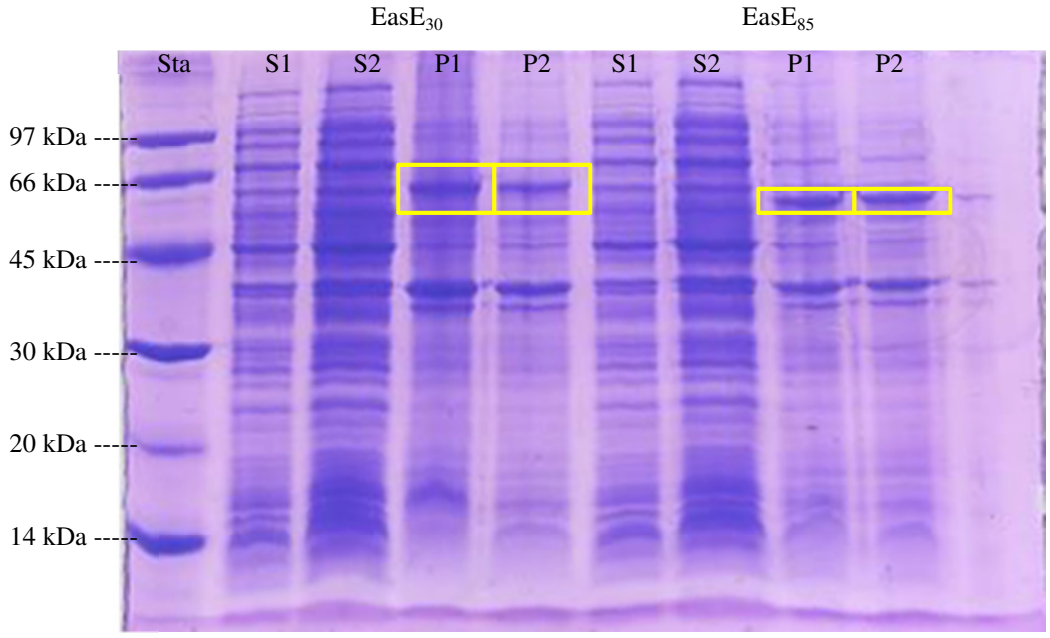

Figure 10: SDS-PAGE analysis of different protein samples obtained from the expression of $EasE_{30}$ and $EasE_{85}$: Lane 1 shows the LMW-Standard and lane 2 and 3 as well as lane 6 and 7 contain different concentrations of expression supernatant, while lane 4 and 5 and 8 and 9 show pellet samples, which were diluted in different ways.

4.1.3 Protein purification

Protein purification was performed as described in chapter 3 section 3.1.10 using a FastFlow Ni-NTA column, which was possible, as all of the expressed proteins contained an N-terminal hexahis-tag (his_6-tag) that specifically interacts with the Ni^{2+}-ions. To check the purity of the collected protein fractions they were analyzed using an SDS-PAGE. A typical SDS-PAGE obtained in those purification processes is shown in Figure 11:

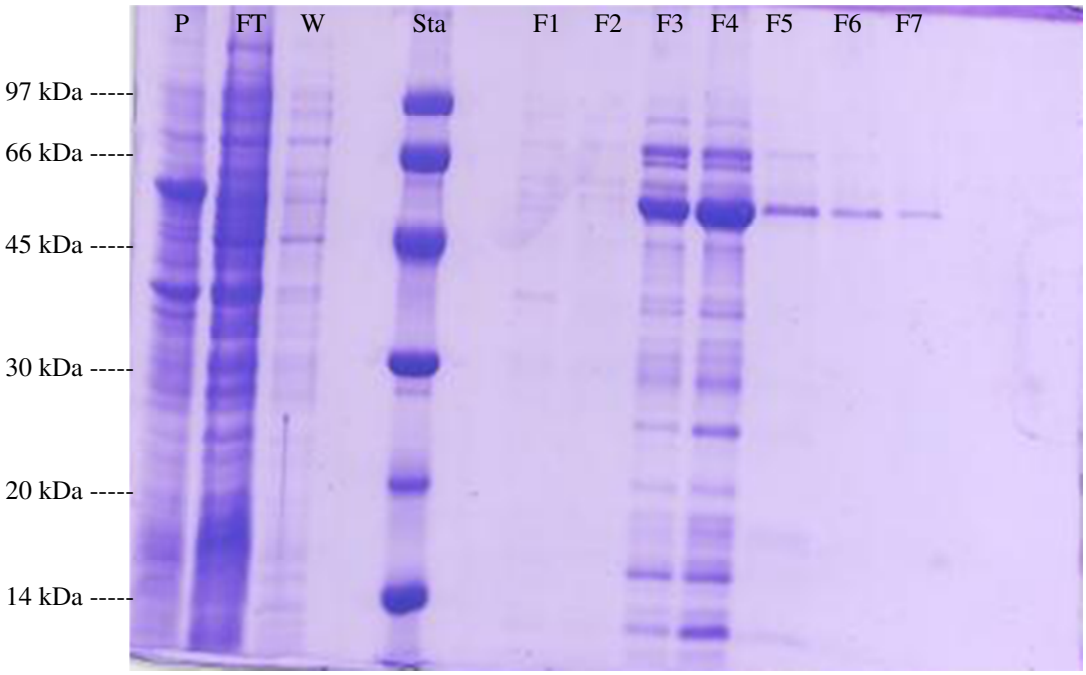

Figure 11: Analysis of the different fractions collected during protein purification of DmaW: P (pellet), FT (flowthrough), W (wash), Sta (LMW-standard), F1-F7 (elution fractions)

As characterization of DmaW, EasF and EasD was successfully achieved by Metzger *et al.* (2009), Rigbers *et al.* (2008) and Wallwey *et al.* (2010), expression and purification of those proteins was just performed to be able to use them in biotransformations. So, the following sections focus on the expression and characterization of EasC and EasE.

4.1.4 Further studies on EasC

EasC was previously described to be a heme-dependent catalase (Goetz *et al.*, 2011), but in the UV-VIS absorption spectrum just a very small peak at 410 nm, which is typical for the heme system, could be detected (Figure 12, panel A). This led to the assumption that EasC might just be loaded with a very low amount of cofactor, encouraging us to try different methods to increase cofactor saturation.

Therefore EasC was again expressed in a classical fermentation process, but 1 mL of saturated hemine solution was added to the LB-medium. Right after the purification process, the UV-VIS spectrum seemed to show a slightly higher absorption at 410 nm (Figure 12, panel B), but after dialysis no difference to the originally obtained spectrum could be observed (Figure 13, panel A).

As it was unclear in which way or whether at all *E. coli* can metabolize hemine to achieve a higher cofactor saturation of the catalase, it was decided to slightly adapt the strategy. The expression protocol remained the same, but δ-aminolevulinic acid (δ-ALA), a precursor of heme, was used instead of hemine. The idea was that *E. coli* might prefer naturally occurring metabolites to synthetic molecules as hemine allowing the bacteria to more easily metabolize them in the desired way. Unfortunately, this strategy only led to a marginal increase of cofactor saturation (see Figure 13, panel B).

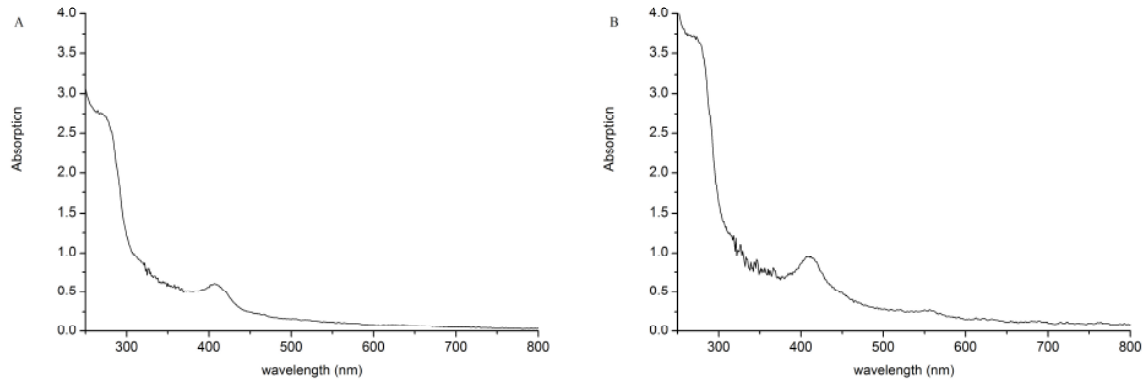

Figure 12: EasC absorption spectra: panel A: absorption spectrum of EasC after purification using a Ni-NTA column, **panel B:** absorption spectrum of EasC, which was expressed adding 1 mL of hemine to the LB-medium and purified via a Ni-NTA column.

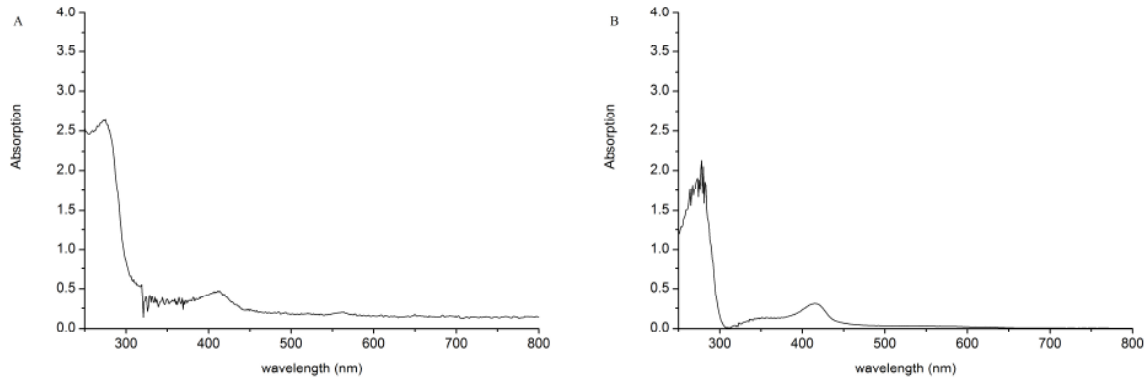

Figure 13: A: absorption spectrum of EasC, which was expressed adding 1 mL of hemine to the LB-medium, purified via a Ni-NTA column and dialyzed for buffer exchange. **B:** absorption spectrum of EasC, which was expressed adding 1 mL δ-aminolevulinic acid to the LB-medium and purified via a Ni-NTA column.

To prove the activity of the catalase EasC, a hydrogen peroxide assay was performed as described by Aebi *et al.* (1984).

It could be shown that EasC is able to metabolize H_2O_2, but the high reactivity of the enzyme did not allow the determination of any kinetic parameters. Starting the measurement just a few seconds earlier or later led to very different results, but still the activity of the enzyme could be confirmed. A typical time dependent absorption spectrum, demonstrating the decrease in absorption at 240 nm (characteristic for hydrogen peroxide) is depicted in Figure 14.

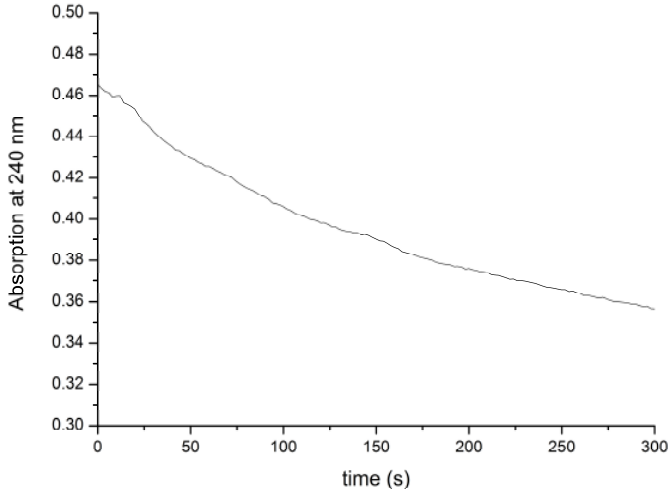

Figure 14: EasC catalyzed conversion of H_2O_2 to H_2O and O_2, shown by the time dependent decrease in absorption 240 nm

4.1.5 Homology model of EasC

The homology model was generated using the peroxisomal catalase of *Hansenula polymorpha* as a template and enabled the analysis of EasC's active site. Figure 15 shows the overall topology of EasC (A) and provides a deeper insight into the active site (B). The amino acids depicted in panel B may serve in substrate binding and cofactor stabilization as well as in the enzymatic reaction catalyzed by EasC.

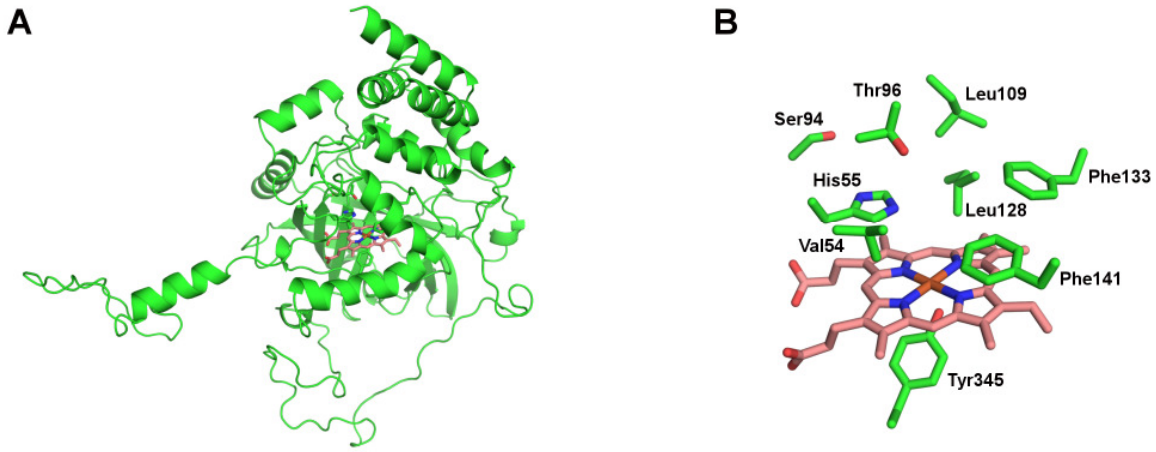

Figure 15: Analysis of the homology model of EasC, which was created using the peroxisomal catalase of *Hansenula polymorpha* as a template **A:** Overall topology of EasC with the heme-cofactor depicted in brown sticks. **B:** close-up view of the amino acids found in close vicinity of the cofactor.

4.2 *Komagataella pastoris*

4.2.1 Cloning

Since the soluble expression of EasE was not possible in *E.coli*, the corresponding nucleotide sequence was cloned into the *K. pastoris* vector pPICZα. The vector was chosen as it contains a coding sequence for an α-factor allowing the secretory expression of proteins. Again two truncated constructs were created ($EasE_{30}$ and $EasE_{85}$) and an octahis-tag (his_8-tag) was added to the C-terminus.

The resulting expression plasmids were transformed into WT KM71H and KM71H [pPICK-PDI] *K. pastoris* cells for gene expression.

4.2.2 Small scale expression in deep-well plates

To check protein expression small-scale expressions in deep-well plates were performed as described in section 3.2.3. The supernatants obtained were analyzed by dot blot (see section 3.2.4) to allow detection of EasE with the help of an anti-his anti-body. Figure 16 below shows the blots obtained in the first screening round:

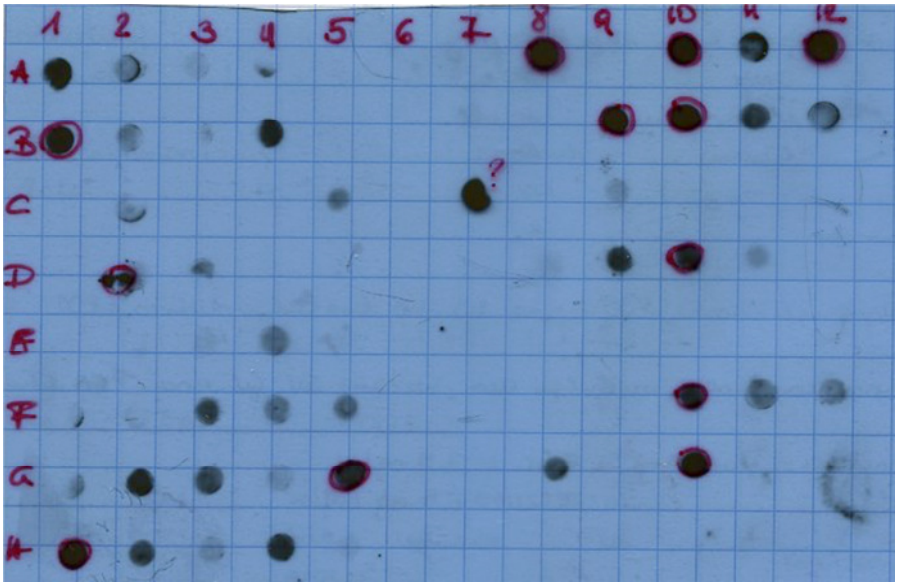

Figure 16: Dot blot obtained from supernatants of the first screening round: the red circled spots were used for further screening (lanes 1-5 show proteins expressed by WT KM71H-cells, while lanes 8-12 show proteins expressed by KM71H [pPICK-PDI]-cells

The circled spots (Figure 16) show the colonies with the highest expression levels, which were thus used for further screening. The second screening revealed the colonies with the highest and most stable expression level. Those colonies were then used for further expression studies (see Figure 17).

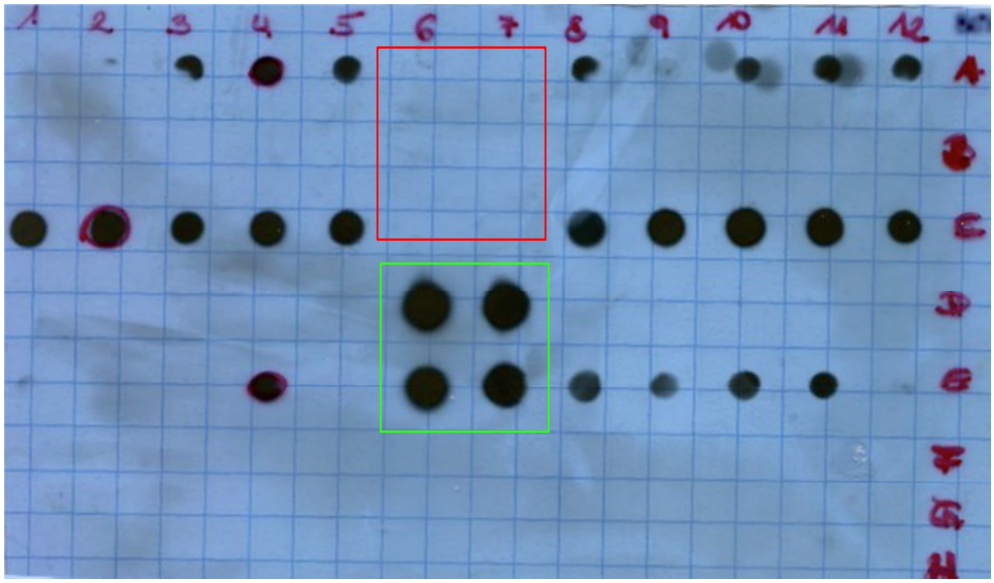

Figure 17: Dot blot obtained from supernatants of the second screening round: lanes 1-5 and lanes 8-12 all show proteins expressed by KM71H [pPICK-PDI]-cells, while D_{6-7} and E_{6-7} represent the positive controls (green box) and A_{6-7} to C_{6-7} the negative controls (red box). Colonies B_4 and C_2 were used in the expression experiments performed in shake flasks.

4.2.3 Expression in 300 mL shake flasks

Expression in shake flasks was used to optimize the expression conditions for EasE. As it is known that protein expression in *K. pastoris* is strongly pH dependent, different pH values were tested. For one screening the pH was set to 6, slightly below the pI predicted for EasE – this is the pH where other BBE-like enzymes are usually expressed. The second screening was tried at pH 7, above the pI of EasE since the deep-well-plate expression at pH 7 seemed to work really well for the protein.

The figure below shows the SDS-PAGE of quick purifications of $EasE_{30}$ at the two different pH-values (Figure 18).

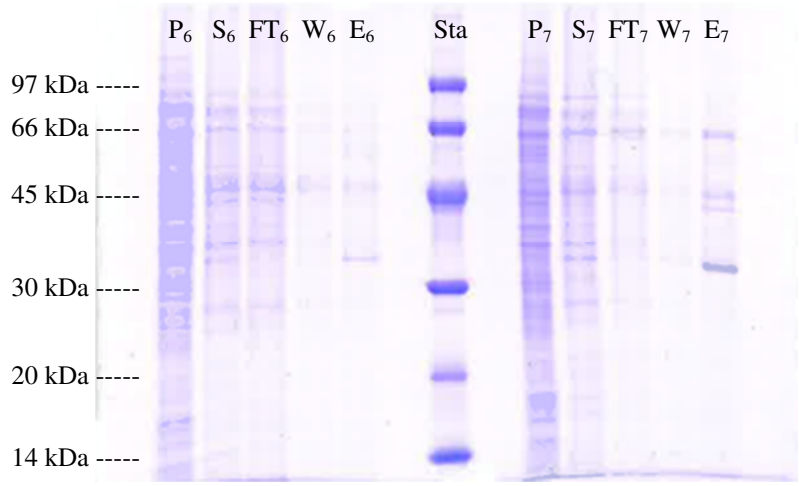

Figure 18: SDS-PAGE analysis of samples taken from different fractions collected during purification of the expression supernatants via Ni-sepharose columns: P (pellet), S (supernatant), FT (flowthrough), W (wash), E (elution), Sta (LMW-standard); the indices (6 and 7) indicate the pH-value of the expression medium the sample was taken from

Interestingly, the SDS-gels indicate that EasE is expressed more strongly at pH 7 compared to pH 6, even though pH 7 is not in the recommended pH-range for *K. pastoris* expressions (pH 5 to pH 6) according to the Invitrogen manual.

4.2.4 Expression in a 7 L bioreactor

Having identified the best expression conditions a large-scale expression in a bioreactor was performed. In Figure 19 the trend of different parameters (pO_2, amount of base required, airflow, stir rate, etc.) is depicted.

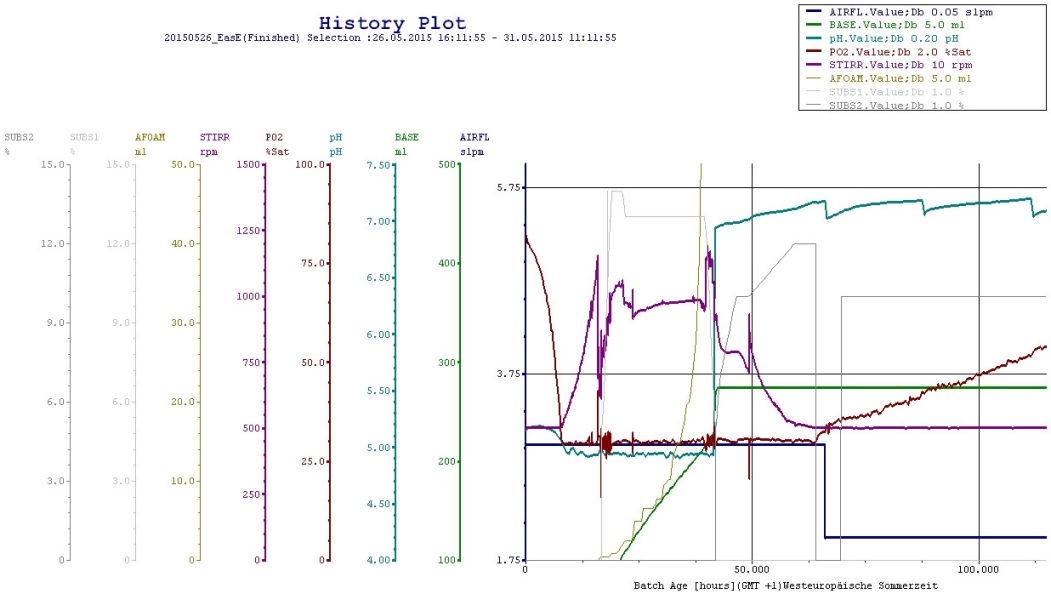

Figure 19: Trend of important parameters controlled during fermentation of $EasE_{30}$: the stir rate is shown in purple, while the pO2 and the pH are labeled in red and cyan. Information on the amount of base used in the process, as well as on the airflow can be obtained from the green and the blue graph.

The selected expression strain behaved as expected and fermentation was therefore performed according to our standard protocol (see section 3.2.7) at pH 7.

4.2.5 Hydrophobic interaction chromatography (HIC)

Purification via a Ph-sepharose column was performed after the fermentation process as a first purification step to remove major impurities and to concentrate the protein. This purification strategy is less efficient than a Ni-sepharose purification, but since EasE seemed to have lost its his-tag affinity chromatography could not be used for purification.

Figure 20 shows the purification protocol using the Ph-sepharose column as well as the absorption of the elution fraction at 280, 375 and 450 nm.

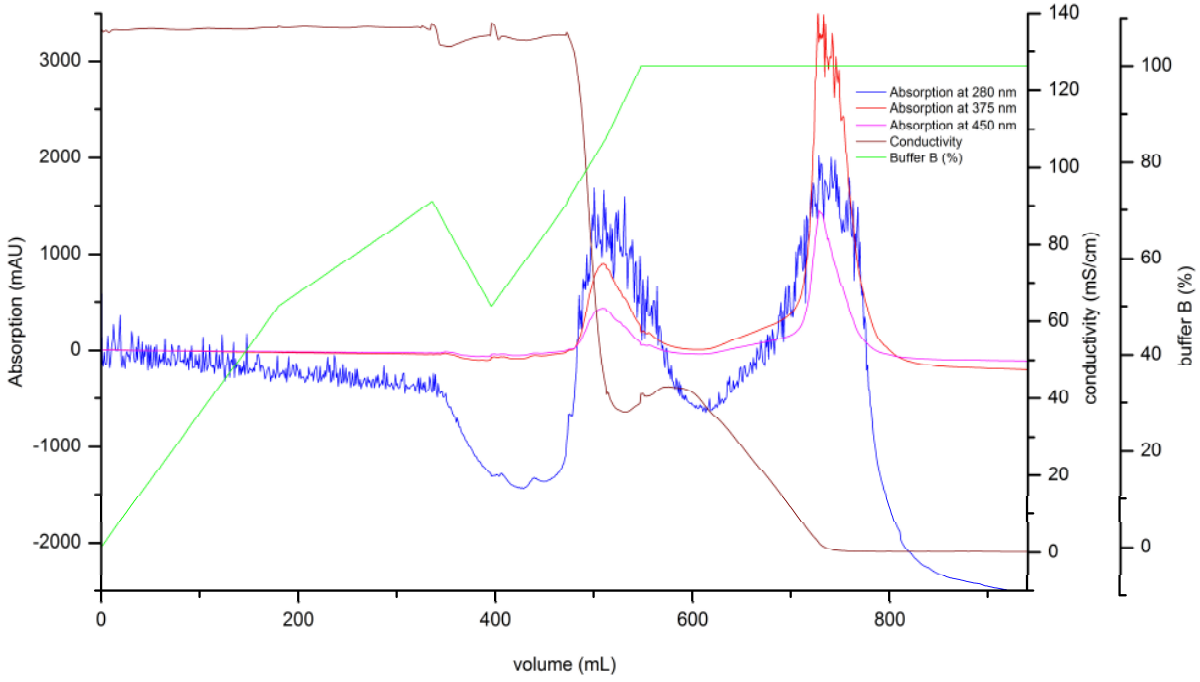

Figure 20: Trend of conductivity (brown), % buffer B (green), absorption at 280 nm (blue), 375 nm (red) and 450 nm (pink), detected during purification with a Ph-sepharose column.

For BBE it is known that the protein elutes at concentrations between 60% and 80% of buffer B (buffer B: 20% EtOH) giving a hint that EasE could also come off the column at similar EtOH concentrations. To verify that assumption, the fractions at the corresponding buffer concentrations were analyzed on an SDS-PAGE (Figure 21).

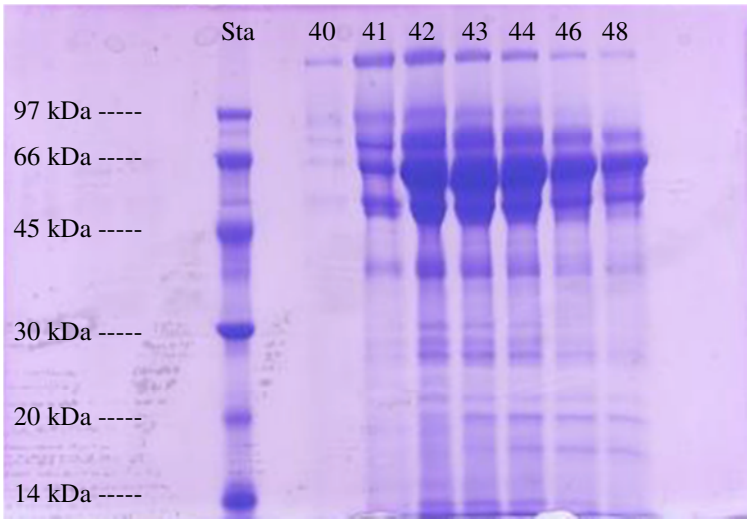

Figure 21: SDS-PAGE analysis of different fractions collected upon purification of $EasE_{30}$ via HIC (hydrophobic interaction chromatography): Sta (LMW-standard), 40-48 (elution fractions)

As fractions 40 to 48 seemed to contain a protein of about 64 kDa in size, which was the size predicted for $EasE_{30}$, the latter were merged and concentrated to allow further purification via gel filtration.

4.2.6 Gel filtration (SEC)

As the protein obtained from the HIC-purification was rather impure, it was decided to use SEC (Superdex 200 prep grade) for further purification. The corresponding chromatogram can be seen in Figure 22.

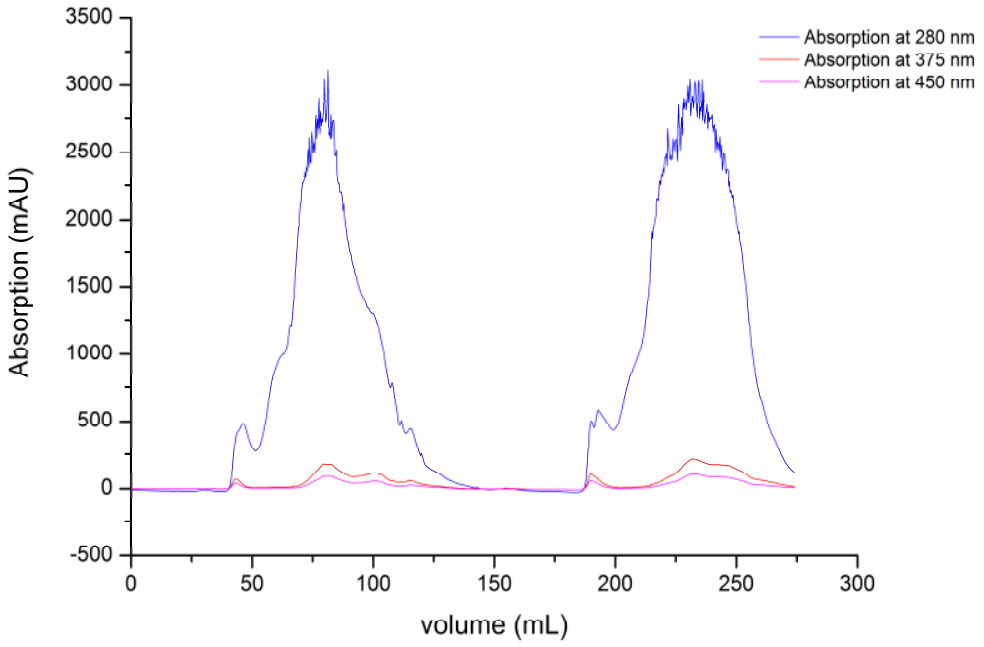

Figure 22: Trend of absorption at 280 nm (blue), at 375 nm (red) and 450 nm (pink), detected during gel filtration.

All fractions showing absorption at 280, 375 and 450 nm were analyzed with an SDS-PAGE.

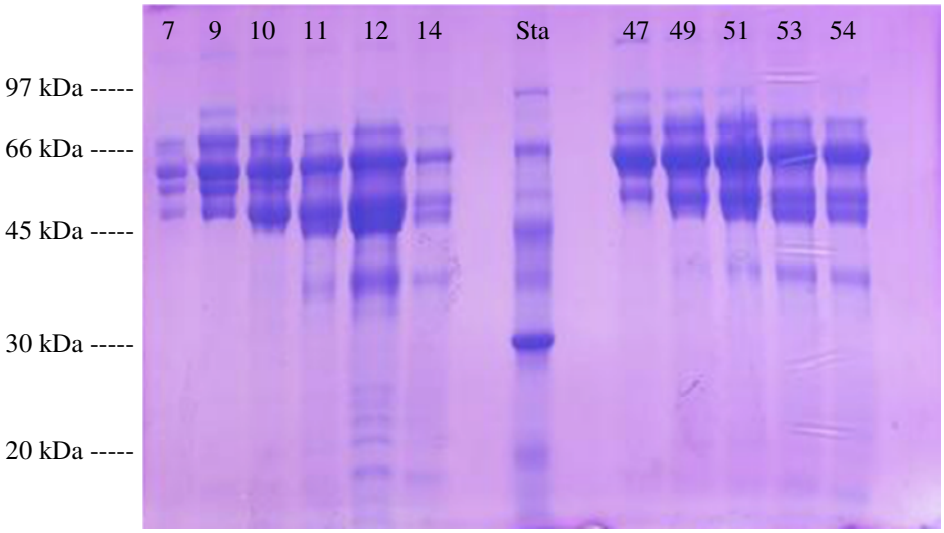

Figure 23: SDS-PAGE analysis of various elution fractions collected during gel filtration: Sta (LMW-standard), 7-14 (elution fractions purification 1), 47-54 (elution fractions purification 2)

4.2.7 Homology model of EasE

A homology model using EncM as a template was generated in order to be able to have a closer look at the active site of EasE, allowing the identification of amino acids that could possibly be involved in the enzymatic reaction. The figure below (Figure 24) shows the proposed overall topology (A), as well as a close-up view of the active site (B).

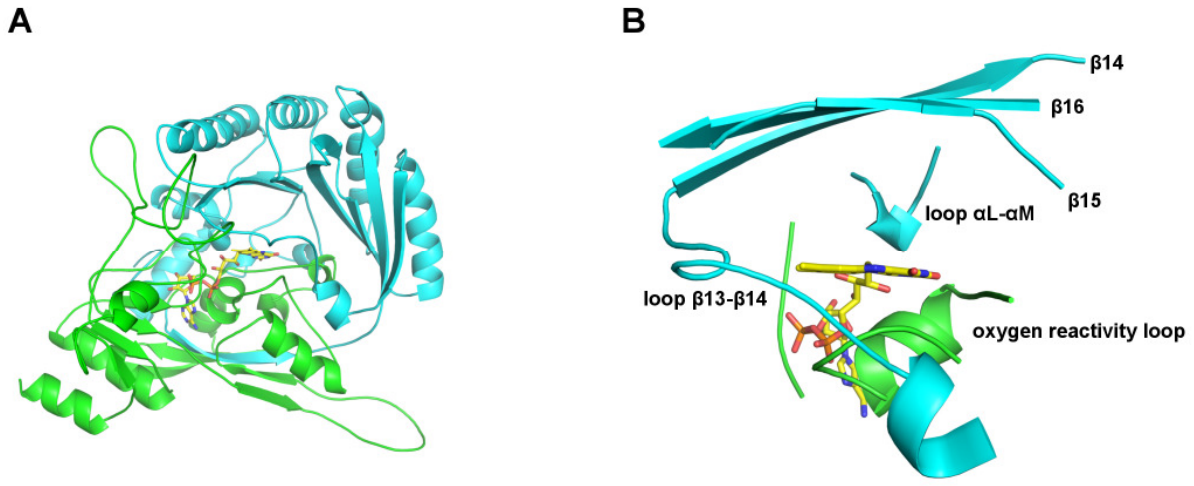

Figure 24: Analysis of the homology model of EasE, which was generated using EncM as a template: A: overall topology of EasE. Like proteins adopting the VAO topology, EasE seems to consist of an FAD-binding domain (green) and a substrate binding domain (blue). The FAD cofactor is depicted in yellow. **B**: close-up view of the active site. While loop αL-αM, loop β13-β14 and β14 to β16 (labelling as for THCA-synthase) are most likely part of the substrate binding, the oxygen reactivity loop seems to be embedded in the FAD binding domain. The cofactor is again shown as yellow sticks.

Analysis of the amino acids in the active site suggests that either Tyr427 or Glu239 could serve as catalytic base thereby triggering "diene"-formation. Figure 25 provides a deeper insight into the active site, with panel A and B focusing on the overall organization of the active site. Panel C and D just show the FAD-cofactor as well as two tyrosines (Tyr378 and Tyr427) and a glutamate (Glu239), which are all likely to be involved in catalysis.

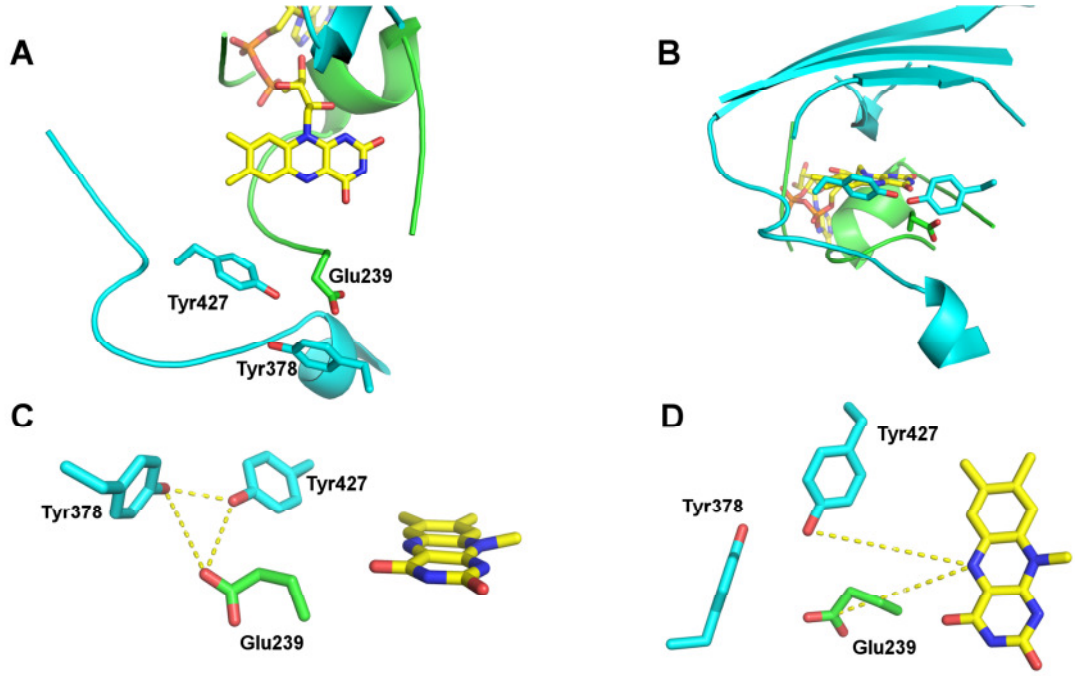

Figure 25: A and B: Different close-up views of the active site: **B** shows the overall organization of the active site, while **A** allows a closer look on the cofactor and the polar amino acids of the active site. **C:** close-up view of the polar amino acids found in close vicinity to the FAD, with the dashed lines (yellow) indicating possible interaction partners. **D:** close-up view of the polar amino acids found in close vicinity to the FAD. The dashed lines (yellow) are used to estimate the distance between the cofactor and possible catalytic bases.

4.2.8 Activity assays

Small-scale biotransformations were used to analyze the activity of EasE as well as to exclude unspecific modifications of DMAT by enzymes found in the fermentation supernatant. The different assays showed that EasE is likely to induce the formation of a "diene", as proposed by the reaction mechanism provided by Schardl *et al.* (2006). While knockout experiments performed by Ryan *et al.* (2013) suggested that EasC and not EasE was responsible for that substrate modification, incubation of EasC with DMAT did not lead to the alteration of the substrate (see also Nielsen *et al.*, 2014).

Interestingly, it could also be shown that "diene" formation is a very fast process as analysis of the samples, which were incubated for three hours, showed a high degree of DMAT conversion to that particular intermediate. At the same time this highly conjugated molecule seemed to be rather unstable as analysis of the samples, which were incubated for 72 h, revealed a high amount of L-Trp and a far smaller peak of the reaction intermediate.

To make sure that EasE and no other protein contained in the fermentation supernatant was able to modify DMAT, the biotransformation was also performed with a fermentation supernatant, which was known not to contain the protein of interest. Since HPLC analysis (performed by Jörg Schrittwieser) of that sample did just show the characteristic substrate peak, it is very likely that EasE is the main enzyme involved in "diene" formation.

Figure 26 to Figure 28 below show the chromatograms as well as the UV-VIS spectra (provided by Jörg Schrittwieser) obtained from various biotransformations:

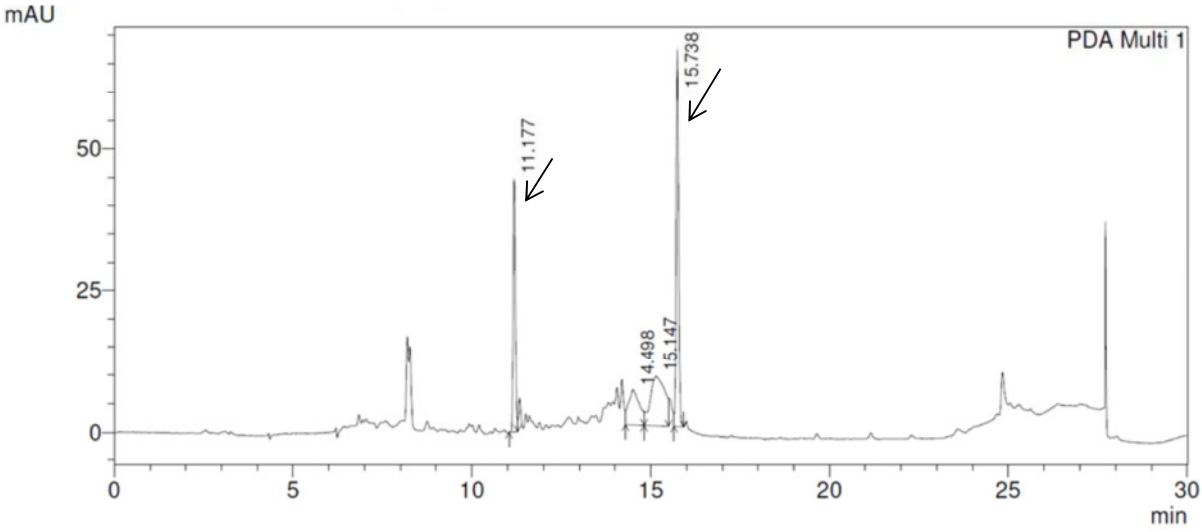

Figure 26: HPLC-chromatogram of the biotransformation sample containing $EasE_{30}$ and DMAT: the reaction batches were incubated at 37 °C for 3 h. The arrows are used to indicate the peaks of interest**.**

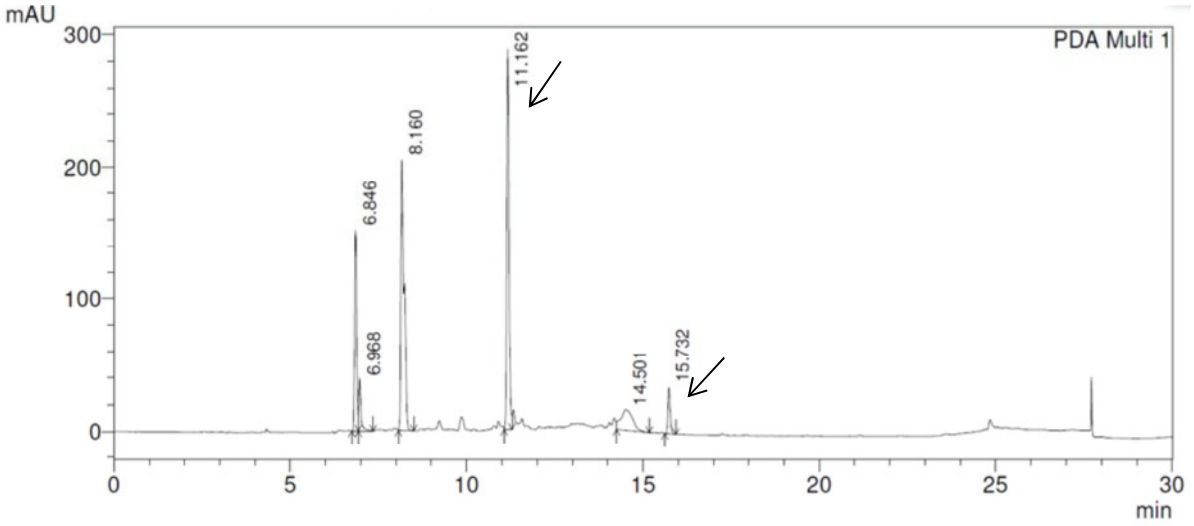

Figure 27: HPLC-chromatogram of the biotransformation sample containing $EasE_{30}$ and DMAT: the reaction batches were incubated at 37°C for 72 h. The arrows are used to indicate the peaks of interest**.**

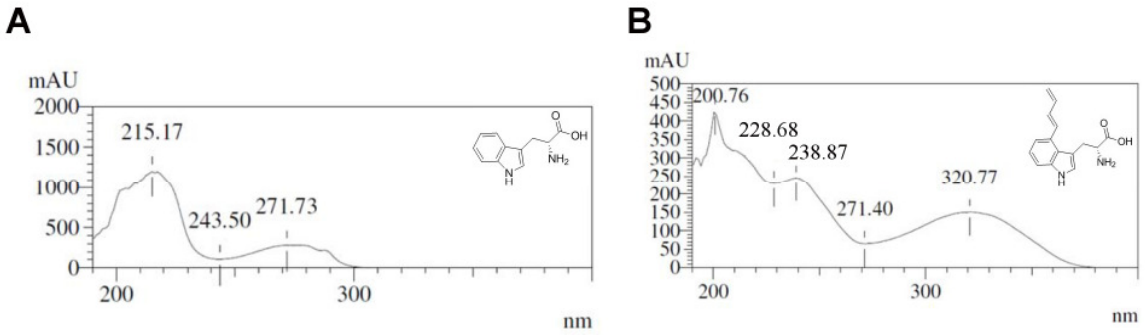

Figure 28: UV-VIS spectra corresponding to the most prominent peaks of the chromatograms: panel A: UV-VIS spectrum and structure of the molecule corresponding to the peak at 11.162 min, **panel B**: UV-VIS spectrum and structure of the molecule corresponding to the peak at 15.738 min

To get further information on the compound found, HPLC-MS analysis was performed (by Jörg Schrittwieser). These measurements revealed that the peak at 11.162 min corresponds to L-Trp, while the peak at 15.738 min corresponds to the "diene" (see Figure 28 and also reaction intermediate Figure 8).

Chapter 5: Discussion

5 Discussion

Ergot alkaloids are a group of interesting metabolites, as they are suggested to be substances that could be used in the treatment of severe neurological diseases (Schiff 2006). Their complex structure, though, does not really allow total organic synthesis of those metabolites. So, researchers are trying to use the naturally required enzymes in biocatalytic applications, to be able to generate at least intermediates of the complex pathway (Minami *et al.* 2008).
To deepen the understanding of reaction mechanisms used by enzymes involved in ergot alkaloid biosynthesis, their expression and biochemical characterization is important. Since ring closing reactions are chemically very challenging, especially chanoclavine I synthesis is interesting to many scientists. In previous studies it was tried to use the well-understood expression system *S. cerevisiae* to overexpress EasE in a soluble way, but the amounts of protein obtained did not allow the characterization of the protein (Nielsen *et al.* 2014).

Expression studies with EasE in *E. coli* showed that the gene can be overexpressed, but isolation of soluble protein was not possible because all EasE was found as insoluble inclusion bodies. Co-expression with various solubility tags, all fused to the C-terminus of EasE, showed the same results that EasE is not folded properly, when expressing it in *E. coli*. It is also possible that the protein is highly glycosylated and can therefore not be obtained in soluble form. Further, interaction with EasC could be required in order to stabilize EasE, but to find out more about the interaction between EasE and EasC, co-expression of both proteins has to be performed.

Since it is known that EasE exhibits high sequence similarity to BBE, which cannot be expressed in *E. coli* but in *K. pastoris*, expression studies with EasE were performed in *Komagataella pastoris*, too. Small-scale expression in deep-well plates suggested, that the secretory expression of the protein was possible, as dot blots analysis allowed the detection of his-tagged protein in the reaction supernatant. As *in vivo* observations of Nielsen *et al.* (2014) suggest co-expression of PDI with EasE seemed to enhance the amount of solubly expressed protein.
Expression experiments in shake flasks, which were used to optimize the expression conditions, showed that expression of EasE is likely to work better at a pH-value of 7 than at the for BBE commonly used pH 6 (Schrittwieser *et al.*, 2011). This is particularly interesting, as it is known that *K. patoris* prefers expression conditions in the pH-range between 5 and 6 or pH 3 if proteases are supposed to be inhibited (Invitrogen manual). Further, expression of

$EasE_{30}$ seemed to work better than the expression of $EasE_{85}$ indicating that the *N*-terminus might be important for the stability of EasE. This is very likely as five cysteines are part of the amino acid sequence between Val30 and Val86, which might strongly influence protein folding and therefore protein stability by forming intramolecular disulfide bonds.

Using all the information obtained from the screening experiments, a large-scale expression in a 7 L bioreactor was performed. Subsequent affinity purification of EasE from the fermentation supernatant led to surprising results. EasE could not be purified using Ni-sepharose columns, which meant that either no protein was formed or that the his-tag was cleaved off during fermentation. SDS-PAGE analysis of the fermentation supernatant showed a thick 64 kDa protein band indicating that EasE was most likely formed, but was lacking the C-terminal his-tag. It is possible that already in the small-scale expressions the his-tag of EasE was cleaved off, since dot blot analysis just allows the detection of all proteins and peptides in the fermentation supernatant (also the his-tag epitopes) but does not enable the identification of the detected fragment. This means if the his-tag was present in the supernatant it was detected no matter whether it was (still) attached to a protein or not.
As affinity chromatography could no longer be used to purify EasE, less specific strategies had to be chosen. With hydrophobic interaction chromatography and gel filtration the amount of impurities could be reduced, but no pure yellow protein fraction, clearly showing that EasE was expressed, could be obtained. MALDI-TOF analysis of the 64 kDa band again found on the SDS-PAGE further indicates that EasE was produced during the large-scale fermentation, but as not many masses corresponding to the flavoprotein could be found further studies were required to identify EasE.
E.g. biotransformations with the protein and DMAT should help to get more information on the mysterious protein (see later).

In addition, it could be shown that EasC can easily be expressed in *E. coli*, as previously described by Nielsen *et al.* (2014) Interestingly the absorption spectrum of the protein did just show a very small peak at 410 nm, indicating that the enzyme was not fully loaded with cofactor. So, different expression methods were used to increase cofactor saturation, but neither addition of hemine nor of δ-aminolevulinic acid to the expression medium affected the 280 nm to 410 nm absorption ratio. This suggests that *E. coli* is either not able to produce enough heme to fully saturate all EasC proteins or that the interaction of EasC with its heme-

cofactor is so weak that the enzyme is not able to always bind the cofactor. Further, it is also possible that incorrect or improper folding of the cofactor binding domain of EasC is responsible for the low cofactor saturation.
However, the catalase assays performed with the enzyme showed that EasC was active, allowing us to use it in different biotransformation assays.

The biotransformations showed that DMAT can neither be modified by EasC nor by any protein found in the fermentation supernatant, if EasE is not present. Incubation of EasE together with the substrate led to the formation of a reaction intermediate ("diene"), which was formed very quickly as very high amounts of that molecule were found in samples with a reaction time of 3 h. At the same time, HPLC-analysis of the samples with 72 h incubation time revealed that the "diene" is likely to be very unstable. This is assumed as the amount of intermediate found in those samples was a lot lower than in the "3 h samples", while the peak corresponding to L-Trp seemed to be far higher.
In addition, it could be shown that EasC is not able to introduce the hydroxyl group, as no new intermediate could be found, adding the catalase to the biotransformations with EasE and DMAT. This makes sense as the hydroxylation is thought to be an oxidative process, which is not likely to be mediated by a "normal" catalase. Recent studies suggest that catalases might be involved in oxidative processes if NADPH was available as second cofactor (Zamocky *et al.* 2008). Therefore it is possible the activity of EasC could be changed upon addition of NADPH in the reaction batches allowing it to catalyze the hydroxylation.
Biotransformations with EasE, EasC, NADPH and DMAT still have to be performed, but incubation of the substrate with the catalase and NADPH did not lead to the modification of DMAT at all.

Analysis of the homology models generated further strengthens the theory that EasE is involved in "diene" formation, as possible catalytic bases could be found in the active site. Comparison of our model with known active site types suggests that Tyr427 serves as the important base deprotonating *N*-Me-DMAT thereby triggering the formation of the second double bond and the hydride transfer to the N(5) of the isoalloxazine ring. Glu239 is expected to be responsible for the deprotonation of Tyr427, while Tyr378 is interacting with Tyr427 thereby stabilizing the latter (Daniel *et al.*, 2015).

The model of EasC also allowed analysis of its active site. Taking a closer look at the composition and the organization of the active site, many hydrophobic amino acids could be found (Val54, Leu109, Leu128, Phe133, Phe141) all together forming a hydrophobic pocket. His55 seems to interact with the heme-cofactor using π-π-stacking, while Ser94 and Thr96 are almost the only polar amino acid residues found in the active site. The high similarity of EasC's active site to *Saccharomyces cerevisiae*'s catalase A's active site (see Fig. 3 Zamocky *et al.*, 2008) strengthens our theory that EasC belongs to clade 3 of "normal" catalases. This means that EasC is possibly able to catalyze the epoxide formation thereby triggering the subsequent ring closing reaction, which would make sense as Vetrano *et al.* (2005) suggest that clade 3 catalases are able to modify indole derivatives by catalyzing oxidation reactions.

All in all, lots of new information on EasE and EasC could be obtained, but still further studies are required to prove the role of those two enzymes in chanoclavine I synthesis.

5.1 Conclusion

The aim of this master project was the expression and characterization of the two key enzymes of chanoclavine I biosynthesis. EasC could easily be expressed and purified, while EasE could not be obtained in soluble form for a long time. Expression of the enzyme in *E. coli* just led to the formation of inclusion bodies, resulting in the decision that another expression host had to be tested. Having transferred the gene into the *K. pastoris* vector pPICZα, small-scale screening experiments in deep-well plates and shake flasks allowed the investigation of the ideal expression conditions for EasE. As those studies suggested that the enzyme could successfully be expressed in soluble form when setting the pH of the expression medium to 7, a large-scale expression in a 7 L bioreactor was conducted. The isolated protein was further used in biotransformation assays, which suggested that EasE triggers the formation of the "diene" reaction intermediate. Addition of EasC to the biotransformation did not lead to the alteration of the intermediate as incubation of DMAT with EasC did not lead to the formation of any other product.

To get a deeper understanding of the reaction mechanism further biotransformations will be required: e.g. adding NADPH to a reaction batch containing EasE, EasC, as well as DMAT to find out whether EasC is able to mediate the epoxidation reaction if it has the chance to act as an oxidase. Further, the co-expression of EasE and EasC in *K. pastoris* is planned, to find out more about possible interactions between the two enzymes.

All in all, lots of new information on EasE and EasC could be obtained, but still the role of both enzymes in chanoclavine I synthesis remains a mystery.

5.2 References

Aebi, H., Catalase in Vitro, Meth. Enz., 1984, 105, 121-126.

Aniszewski, T., Alkaloids – Secrets of Life, Elsvier 2007, p. 13.

Bacher, A., Eberhardt, S., Fischer, M., Kis, K., Richter, G., Biosynthesis of vitamin B_2 (riboflavin), Annu. Rev. Nutr. 2000, 20, 153–167.

Coyle, Ch. M., Panaccione, D. G., An Ergot Alkaloid Biosynthesis Gene and Clustered Hypothetical Genes from Aspergillus fumigatus, Appl. Environ. Microbiol. 2005, Vol. 71, No. 6, 3112-2118.

Daniel, B., Pavkov-Keller, T., Steiner, B., Dordic, A., Gutmann, A., Nidetzky, B., Sensen, Ch. W., van der Graaf, E., Wallner, S., Gruber, K., Macheroux, P., Oxidation of Monolignols by Members of the Berberine Bride Enzyme Family Suggests a Role in Plant Cell Wall Metabolism, J. Biol. Chem. 2015, 290, 30:18770-18781.

Facchini, P. J., St-Pierre, B., Synthesis and trafficking of alkaloid biosynthetic enzymes, Curr. Opin. Plant Biol 2005, 8, 657-666.

Fischer, M., Bacher, A., Biosynthesis of vitamin B_2: structure and mechanism of riboflavin synthase, Arch. Biochem. Biophys. 2008, 474, 252–265.

Gerhards, N., Neubauer, L., Tudzynski, P., Li, Sh.-M., Biosynthetic Pathways of Ergot Alkaloids, Toxins 2014, 6, 3281-3295.

Goetz, K. E., Coyle, Ch. M., Cheng, J. Z., Ergot cluster-encoded catalase is required for synthesis of chanoclavine I in Aspergillus fumigatus, Curr. Genet. 2011, 57:201-211.

Haarmann, T. Y., Rolke, Y., Giebert, S., Tudzynski, P., Ergot: from witchcraft to biotechnology, Mol. Plant Pathol. 2009, 10:563-577.

Hesse, M., Alkaloids: Nature's Curse or Blessing?, Wiley-VCH 2002, pp. 283-291.

Heuts D. P. H. M., Scrutton N. S., McIntire, W. S., Fraaje, M. W, What's a covalent bond?: On the role and formation of covalently bound flavin cofactors, FEBS J. 2009, 276:3405-3427.

Huang, C. H., Lai, W. L., Lee, M. H., *et al.*, Crystal structure of glucooligosaccharide oxidase from Acremonium strictum: A novel flavinylation of 6-S-cysteinyl, 8α-N1-histidyl FAD, J. Biol. Chem. 2005, 280:38831-38838.

Invitrogen, Life Technologies, Pichia Fermentation Process Guidelines, Version B, 053002.

Kearney, E. B., Salach, J. I., Walker, W. H., *et al.*, The covalently bound flavin of hepatic monoamine oxidase. 1. Isolation and sequence of flavin peptide and evidence for binding the 8alpha position, Eur. J. Biochem. 1971, 24:312-327.

Kutchan, T. M., Molecular genetics of plant alkaloid biosynthesis, In: Cordell GA, ed. The alkaloids: Chemistry and biology, San Diego, CA, USA: Academic press 1998, 50:257-316.

Lorenz, N., Olsovska, J., Sulc, M., Tudzynski, P., Alkaloid Cluster Gene ccsA of the Ergot Fungus Claviceps purpurea Encodes Chanoclavine I Synthase, a Flavin Adenine Dinucleotide-containing Oxidoreductase Mediating the Transformation of N-Methyl-Dimethylallyltryptophan to Chanoclavine I, Appl. Environ. Microbiol. 2010, 76(6):1822.

Macheroux, P., Kappes, B., Ealick, S. E., Flavogenomics – a genomic and structural view of flavin dependent proteins, FEBS J. 2011, 278:2625-2634.

Masuoka, N., Sugiyama, H., Ishibashi, N., Wang, D. H., Masuoka, T., Kodama, H., Nakano, T., Characzerization of acalasemic erythrocytes treated with low and high dose hydrogen peroxide: hemolysis and aggregation, J. Biol. Chem. 2006, 281:21728-21734.

McIntire, W., Edmondson, D. E., Hopper, D. J., Singer, T. P., 8α-(O-tyrosyl)flavin adenine dinucleotide, the prosthetic group of bacterial *p*-cresol methylhydroxylase, Biochemistry 1981, 20:3068-3075.

Metzger, U., Schall, Ch., Zocher, G., Unsöld, I., Stec, E., Li, S.-M., Lutz, H., Stehle, T., The structure of dimethylallyl tryptophan reveals a common architecture of aromatic prenyltransferases in fungi and bacteria, PNAS 2009, 106, 34:14309-14314.

Minami, H., Kim, J. s., Ikezawa, N., Takemura, T., Katayama, T., Kumagai, H., Sato, F., Microbial production of plant benzylisoquinoline alkaloids, Proc. Natl. Acad. Sci. USA 2008, 105, 7393-7398.

Nielsen *et al.*, The important ergot alkaloid intermediate chanoclavine-I produced in the yeast *Saccharomyces cerevisiae* by combined action of EasC and EasE from Aspergillus japonicus, Microb. Cell Fact. 2014, 13:95.

Petrova, V. Y., Drescher, D., Kujumdzieva A. V., Schmitt M. J., Dual targeting of yeast catalase A to peroxisomes and mitochondria, Biochem. J. 2004, 380:393-400.

Rigbers, O., Li, S. M., Ergot alkaloid biosynthesis in Aspergillus fumigatus. Overproduction and biochemical characterization of a 4-dimethylallyltryptophan N-methyltransferase, J. Biol Chem. 2008, 283:26859–26868.

Ryan, K. L., Moore Ch. T., Panaccione, D. G., Partial Reconstruction of the Ergot Alkaloid Pathway by Heterologous Gene Expression in Aspergillus nidulans, Toxins 2013, 5, 445-455.

Salach, J. I., Walker, W. H., Singer, T. P., *et al.*, Studies on succinate dehydrogenase. Site of attachment of the covalently-bound flavin to the peptide chain, Eur. J. Biochem. 1972, 26:267-278.

Schardl, C. L., Panaccione, D. G., Tudzynski, P., Ergot alkaloids – biology and molecular biology, Alkaloids Chem. Biol. 2006, 63:45-86.

Schiff, P. L., Ergots and Its Alkaloids, American Journal of Pharmaceutical Education 2006; 70 (5) Article 98.

Schrittwieser, J. H., Resch, V., Wallner, S., Lienhart, W.-D., Sattler, J. H., Resch, J., Macheroux, P., Kroutil, W., Biocatalytic Organic Synthesis of Optically Pure (S)-Scoulerine and Berbine and Benzylisoquinoline Alkaloids, J. Org. Chem. 2011, 76, 6703-6714.

Singer, T. P., Kearney, E. B., Biochem. Biophys. Acta 1956, 15:151.

Terlecky, S. R., Koepke, J. I., Walton, P. A., Peroxisomes and aging, Biochem. Biophys. Acta. 2006, 1763:1749-1754.

Trudzynski, P., Correia, T., Keller, U., Biotechnology and genetics of ergot alkaloids, Appl. Microbiol. Biotechnol. 2001, 57:593-605.

Vetrano, A. M., Heck, D. E., Mariano, T. M., Mishin, V., Laskin, D. L., Laskin, J. D., Characterization of the oxidase activity in mammalian catalase, J. Bio.l Chem. 2005, 280:35372-35381.

Wallner, S., Dully, C., Daniel, B., Macheroux, P., Berberine bridge enzyme and the family of bicovalent flavoenzymes, Chapter 1 of the Handbook of Flavoproteins, Volume 1 by Hille, R., Miller, S. M., Palfey, B., De Gruyter, Berlin, 2013.

Wallwey, Ch., Matuschek, M., Li, S.-M., Ergot alkaloid biosynthesis in Aspergillus fumigatus: conversion of chanoclavine-I to chanoclavine-I aldehyde catalyzed by a short-chain alcohol dehydrogenase FgaDH, Arch. Microbiol. 2010, 192:127-134.

Wallwey, C., Li, S.-M., Ergot alkaloids: structure diversity, biosynthetic gene clusters and functional proof of biosynthetic genes, Nat. Prod. Rep. 2011, 28:496-510.

Winkler, A., Lyskowski, A., Riedl, S., *et al.*, A concerted mechanism for berberine bridge enzyme, Nat. Chem. Biol. 2008, 4:739-741.

Winkler, A., Motz, K., Riedl, S., Puhl, M., Macheroux, P., Gruber, K., Structural and mechanistic studies reveal the functional role of bicovalent flavinylation in berberine bridge enzyme, J. Biol. Chem. 2009, 284:19993-20001

Young, C. A., Schardl, Ch. L., Panaccione, D. G., Florea, S., Takach, J., Charlton, N. D., Moore, N., Webb, J. S., Jaromczyk, J., Genetics, Genomics and Evolution of Ergot Alkaloid Diversity, Toxins 2015, /, 1273-1302.

Zymocky, M., Furtmüller, P. G., Obinger, Ch., Evolution of Catalases from Bacteria to Humans, Antioxid. Redox Signal. 2008, 10(9): 1527-1548.

Chapter 6: Appendix

6.1 List of figures

6.2 List of tables