Graz University of Technology

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Expression of Ergot Alkaloid degrading Enzymes in *Pichia pastoris*

Comparison with the Expression in different Strains of *Escherichia coli*

Master Thesis

by

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"If you want to build a ship, don't drum up people to collect wood and don't assign them tasks and work, but rather teach them to long for the endless immensity of the sea."
(Antoine de Saint-Éxupery)

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Abstract

Mycotoxins are secondary metabolites produced by moulds. One major group of mycotoxins are the ergot alkaloids that are mainly produced by the genus *Claviceps* infecting cereals such as wheat, rye and barley. The infected host plant develops a so called ergot body, sclerotium that contains the ergot alkaloids. Other producers of the toxins are *Neotyphodium*, *Balansia* and *Epichloë*. These fungi are endophytes and infect forage grasses such as perennial rye grass and tall fescue.

In history ergot alkaloids caused major diseases in humans, called ergotism. Nowadays the alkaloids are used in treatment of certain diseases because of their pharmacological effects. The risk of a human intoxication is low. However, ergotism in animals is still a big medicinal and economic problem around the world, especially in the USA, where tall fescue is widely used as feed for grazing animals.

In this work the main ergot alkaloids were ergotamine and ergine. Ergine is one of the major alkaloids found in endophyte infected tall fescue. Two enzymes, a hydrolase and an amidase, have been isolated from an ergot alkaloid-degrading strain, *Rhodococcus erythropolis* MTHt3, and detected as being able to degrade the toxins. The main focus was to produce a proper amount of both enzymes to be able to purify and characterize them. For production of both enzymes the expression system *Pichia pastoris* CBS7435 was selected because of its several advantages concerning expression of foreign proteins such as extracellular secretion and availability etc. Additionally, comparisons with different expression strains of *Escherichia coli* were made.

Although transformation results into *P. pastoris* were examined via real time PCR and the conditions during expression were varied hydrolase but no amidase were obtained. Also different expression strains of *E. coli* did not lead to a positive result concerning the expression of the amidase. Overall, it was found out that neither *P. pastoris* nor *E. coli* were the right expression systems for the production of the hydrolase and the amidase.

Kurzzusammenfassung

Mykotoxine sind sekundäre Metabolite, die von Schimmelpilzen produziert werden. Eine Gruppe von Mykotoxinen sind die Ergotalkaloide, die von der Spezies *Claviceps* produziert werden und Getreide, wie Weizen, Roggen und Hafer befallen. Die infizierten Pflanzen entwickeln das sogenannte Sclerotium, in dem die Ergotalkaloide zu finden sind. Des weiteren produzieren auch die Endophyten *Neotyphodium*, *Balansia* und *Epichloëe* die Toxine und infizieren Gräser, wie das deutsche Weidelgras und den Rohrschwingel.

In früheren Zeiten waren Ergotalkaloide die Ursache vieler Krankheiten. Heutzutage werden die Alkaloide, aufgrund ihrer pharmakologischen Aktivität, eher in der Medizin zur Behandlung verwendet. Obwohl das Risiko einer menschlichen Erkrankung relativ gering ist, sind die Vergiftungen von Weidetieren noch immer ein großes medizinisches und wirtschaftliches Problem. Vor allem in den USA kommt es meist zu krankhaften Erscheinungen, da dort Rohrschwingel (tall fescue) großflächig als Weidegras genutzt wird.

Die verwendeten Alkaloide in dieser Arbeit sind Ergotamin und Ergin. Ergin ist eines des häufigsten Ergotalkaloide im infizierten Rohrschwingel. Für einen potenziellen Toxinabbau konnten zwei Enzyme, eine Hydrolase und eine Amidase, aus *Rhodococcus erythropolis*, MTHt3, isoliert werden. Das Hauptaugenmerk meiner Arbeit lag auf der rekombinanten Produktion beider Enzyme, um diese dann reinigen und charakterisieren zu können. Für die Produktion wurde *Pichia pastoris* CBS7435 herangezogen, da dieses Expressionssystem zahlreiche Vorteile für die Expression von fremden Proteinen hat. Zusätzlich wurden auch Vergleiche mit unterschiedlichen Expressionsstämmen von *Escherichia coli* gemacht.

Obwohl die Transformation der Expressionskassette in *P. pastoris* mittels real time PCR überprüft und die Bedingungen während der Expression variiert wurden, konnte nur wenig von der Hydrolase und keine Amidase gewonnen werden. Ebenso kam es bei der Expression der Amidase in *E. coli* zu keinem positiven Ergebnis. Im Allgemeinen konnte erkannt werden, dass weder *P. pastoris* noch *E. coli* das geeignete Expressionssystem für die Produktion der Hydrolase und der Amidase sind.

1. Introduction

1.1. Mycotoxins

Mycotoxins are produced by moulds such as *Aspergillus*, *Penicillium*, *Claviceps*, *Fusarium* and *Alternaria*. Moulds producing mycotoxins can infest cereal grains, oil seeds, tree nuts and dehydrated fruits depending on the conditions during growth of the plant and storage such as substrate availability, pH-value, humidity and temperature (Steyn and Stander, 2005, Hof, 2008). Fungi producing mycotoxins can be classified into field fungi (growth before harvest) and storage fungi (growth after harvest) (Osweiler, 2001). On the one hand one single species of fungi can produce one specific or several mycotoxins. However, specific mycotoxins can be produced by different species' of fungi (Hussein and Brasel, 2001).

Mycotoxins are stable low-molecular secondary metabolites and do not play a significant biochemical role for fungal growth or development. Over 300 different mycotoxins have already been isolated (Hussein and Brasel, 2001). They have carcinogenic, mutagenic, teratogenic, estrogenic, immunotoxic, nephrotoxic and neurotoxic effects (Steyn and Stander, 2005). Because of their size they cannot be detected by the immune system. Therefore, a recurrent infection will again cause symptoms. A few mycotoxins are even able to accumulate in the body and stay there for quite a long time. Mycotoxins can act in an additional manner but also in a synergistical manner, meaning that they might have positive influences on each other concerning their toxic effects (Hof, 2008). Major groups of mycotoxins are aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, tremorgenic toxins and ergot alkaloids (Hussein and Brasel, 2001). Throughout the history until now mycotoxins have always been associated with diseases. Mycotoxins might have been the reasons for one of the "Ten Plagues of Egypt" as well as for the "Witchcraft Trials" in Salem, Massachusetts, when people were obsessed with "evil spirits". A more recent example of a disease outbreak with mycotoxins as a cause is St. Anthony's Fire (Richard, 2007). In the 12th century there was an outbreak of a mycotoxicosis named *ignis sacer*. People consumed contaminated food and suffered of this disease. It became quite popular to go to the Hospital of the Brothers of St. Anthony where they thought to be healed by Saint Anthony himself, giving the disease a name. Actually people were not really healed by a saint but more because of the fact that the infected people got food of better quality in the hospital and recovered (Gensthaler, 2011, Meyer, 2011). Nowadays hardly any toxicoses in humans are known because humans usually avoid consuming contaminated food (Steyn and Stander, 2005). The

European Unit also set strict limits for mycotoxin concentrations in food (Hof, 2008). Additionally, certain food processes such as sorting, milling, cleaning, trimming, cooking, brewing, baking, frying, roasting, canning etc. have positive influences on the reduction of mycotoxins in food (Bullerman and Bianchini, 2007). Nonetheless contaminated food and feed is a worldwide serious problem (Hussein and Brasel, 2001). Especially for animals the toxins are still a major risk. Serious diseases and disorders in farm animals can be the result of consumption of contaminated feed (Kolosova and Stroka, 2011). Depending on the type of animal (also ruminants or non-ruminants), type of toxin, intake level, gender, age, diet, physiological state and duration of exposure different symptoms and disease patterns can manifest. Symptoms include decreased performance, feed refusal, poor feed conversion, diminished body weight gain, immune suppression and reproductive disorders (Kolosova and Stroka, 2011). Furthermore, infected animals can also produce milk and meat containing toxic residues or biotransformation products (Bennett and Klich, 2003). Toxicoses can be divided into acute toxicity with a quick onset of response and symptoms and chronic toxicity meaning an exposure over a long time span (Bennett and Klich, 2003).

Detoxification can be chemically or physically. Chemical methods are treatment with gases, e.g. ammonia, chlorine, hydrogen chloride, ozone and sulphur dioxide, treatment with solutions like bleach, diluted acids, alkali and hydrogen peroxide. Physical methods include heat treatment or UV-light as well as separation methods based on different sizes and densities of the toxin and the product (Young et al., 1983). These days certain substances are explored and used to reduce mycotoxins in food and feed. These substances are called mycotoxin binders (MB). They are added to the diet to bind mycotoxins to their surface via adsorption. For biodegradation and biotransformation steps living microorganisms as well as enzymes and genes responsible for these processes act in the intestinal tract of animals before mycotoxins can be absorbed by the animal (Kolosova and Stroka, 2011, Hartinger et al., 2011, Heinl et al., 2010).

An overview of mycotoxins can be seen in Table 1.

Table 1 Short overview of major mycotoxins, their sources and certain features (Bennett and Klich, 2003, Osweiler, 2001).

Overview of major mycotoxins		
Toxin	**Sources**	**Symptoms and other features**
Aflatoxin	*Aspergillus flavus*, *Aspergillus parasiticus* in cereals, oil seeds, figs, nuts and tobacco	toxic and carcinogenic; death, cancer, immune suppression; probably plays a role in kwashiorkor, Reye's syndrome and neoplasms in extra hepatic tissue
Ochratoxin	*Aspergillus ochraceus*, *Penicillium viridicatum* in corn, wheat and peanuts	nephrotoxic, toxic to the liver, immune suppressant, teratogenic, carcinogenic; disturbs cellular physiology
Zearalenone	certain *Fusarium* species in corn, wheat and barley	low toxicity; estrogenic effects: hyperestrogenism, abortions etc.
Ergot alkaloids	*Claviceps* species in cereals; *Neotyphodium coenophialum* in grasses	Gangrene, abortions, convulsions, low milk production, hypersensitivity, ataxia, smooth muscle contractions etc.
Trichothecenes	*Fusarium*, *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichoderma*, *Trichothecium* and others in grains	potent inhibitor of eukaryotic protein synthesis; hypothesized to play a role in the human disease: alimentary toxic aleukia
Tremorgenic toxins	certain *Penicillium* species in corn, peanuts, pecans etc.	Tremors, incoordination and weakness; Usually it is considered to be not lethal;
Fumonisins	certain *Fusarium* species in corn	dependent on the species hepatotoxic and carcinogenic effects, oedema etc.

1.2. Ergot alkaloids

A few members of mycotoxins are substances of the group of ergot alkaloids. They can be divided into four groups: clavine alkaloids, simple lysergic acid derivatives, peptide alkaloids or ergopeptines and lactam ergot alkaloids (also called ergopeptams) (Table 2) (Schardl et al., 2006, Uhlig and Petersen, 2008). Clavine alkaloids are substituted 6, 8-dimethylergolines, except of a few members. 35 alkaloids of this group have already been isolated but none of them is used in pharmacology. Lysergic acid derivatives have an amidation of the C-8 carboxy-group. Therefore, two types of compounds can be formed (Schiff, 2006). These two epimers can be either in the α-form or the β-form. The two forms can be distinguished by the prefix *iso*- or by the suffix *–inine* (Flieger et al., 1997). The simple nonpeptidic acids carry quite short carbon chains, whereas the peptidic amides mostly are tripeptides. Peptide alkaloids or ergopeptines are tetrapeptides containing lysergic acid as the first member. The three other members consist of classical amino acids. This also explains the great diversity of ergopeptines. Common amino acids are L-alanine, L-phenylalanine, L-valine, L-leucine and L-isoleucine. Ergotamine is the only natural ergopeptine that is used in medicine. However, a

lot of other semisynthetic ergopeptines play a role in pharmacy (Schiff, 2006). Lactam ergot alkaloids are non-cyclol tripeptidic ergot alkaloids. The differences to ergopeptines are that L-proline is replaced by a D-proline and that the tripeptides chain is a non-cyclol lactam. They are quite instable. As a consequence, only three naturally occurring ergopeptams are known (Uhlig and Petersen, 2008).

Table 2 Summary of major groups of ergot alkaloids and their representatives (Scott, 2009, Schiff, 2006, Uhlig and Petersen, 2008).

Main groups of ergot alkaloids	
Group	**Toxins**
Clavine alkaloids	Agroclavine
Simple lysergic acid derivatives	Ergometrine, Ergine
Peptide alkaloids or ergopeptines	Ergovaline, Ergotamine, α-Ergosine, Ergocornine, Ergocryptine, Ergocristine
Lactam ergot alkaloids	Ergocristam, Ergocornam, α-Ergocryptam

Basically all ergot alkaloids have one structural characteristic in common, a tetracyclic ergoline ring structure. This feature is also one of the main reasons for the major pharmaceutical influence of ergot alkaloids as well as a lot of the symptoms of ergotism (disease resulting of the consumption of ergot alkaloids). The tetracyclic ergoline ring gives the alkaloid a certain similarity to common neurotransmitters, which usually bind to various receptors of the central nervous system (Tudzynski et al., 2001) (Figure 1). These receptors include receptors for uterotonic activity, regulation of blood pressure, secretion of pituitary hormones, migraine prevention and dopaminergic and neuroleptic activities meaning that the alkaloids are similar to the neurotransmitters noradrenaline, dopamine and serotonin. They can act as agonists as well as antagonists (Wallwey and Li, 2010). It is also thought that they might act in a dual role as partial agonists and antagonists. Furthermore, the side chains of the tetracyclic ring play an important role concerning the effects of ergot alkaloids interacting with CNS receptors (Haarmann et al., 2009).

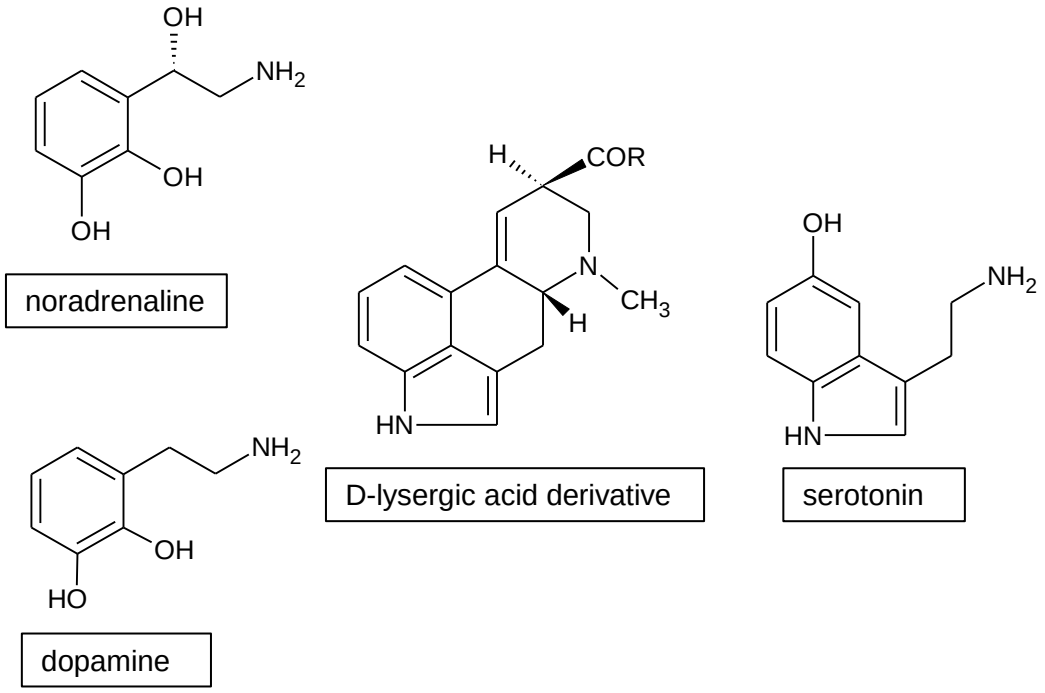


Figure 1 Similarity of tetracyclic ergoline ring and three neurotransmitters (Tudzynski et al., 2001, Haarmann et al., 2009).

Ergot alkaloids are produced by a wide range of different fungi, especially by the fungi of the grass parasitizing genus *Claviceps* (Tudzynski et al., 2001). This genus consists of a group of phytopathogenic ascomycetes with approximately 36 members. They can parasitize over 600 plants such as forage grasses, corn, wheat, barley, oats, millet, sorghum, rice and rye (Schiff, 2006). The most notable fungus of this genus is *Claviceps purpurea* which mainly infects cereals and replaces the growing seed with a seed like wintering body called sclerotium or ergot. Ergot alkaloids are found in the sclerotium (Krska and Crews, 2008). The sclerotia are dark grey, purple or black with variable sizes (Eadie, 2003) (Figure 2). Fungi of the genus *Claviceps* are called aposematic. Aposematism is a defensive phenomenon where warning coloration (seen in the dark coloured sclerotia) is associated with chemical defence (Lev-Yadun and Halpern, 2007).

Figure 2 a) Sclerotia and normal corn; b) Different sizes of sclerotia compared to a normal corn (on the right);

Sclerotia vary in their total ergot alkaloid content as well as the pattern of produced alkaloids. This depends on the fungal strain, the host plant and the geographical region. Ergot is ubiquitous but it seems to appear more often in favourable climatic conditions which are seasons with heavy rainfall and wet soils (Krska and Crews, 2008, Pedrosa and Grießler, 2010). Ergot alkaloids are also produced by some plants, most of them members of the morning glory family (Krska and Crews, 2008). Other sources are grasses, such as tall fescue or perennial ryegrass that are infected with endophytic fungi e.g. *Neotyphodium, Epichloëe* and *Balansia* species (Krska and Crews, 2008, EFSA, 2005, Strickland et al., 2011). These fungi colonize the intercellular spaces of the grasses. They infect only the parts above ground, including the reproductive tissues, but they do not infect the roots (Schardl, 1996). *Epichloëe* and *Balansia* are termed as sexual endophytes whereas *Neotyphodium* is the asexual counterpart. Most but not all fungi produce ergot alkaloids when they infect the grasses (Schardl and Phillips, 1997).

These fungi can grow within a plant without sporulation (Strickland et al., 2011). The major ergot alkaloid found in endophyte infected tall fescue is ergovaline. Additionally, lysergic acid amide (ergine) and the clavine alkaloids can be found (Porter, 1995, De Lorme et al., 2007). Intoxication of livestock by infected tall fescue pose a major problem nowadays. Tall fescue is a very resistant, highly adaptive, cool season turf grass species and is widely used in the USA. When the grass is infected there is no seed that forms, such as the sclerotia. Therefore, an infection cannot be seen with the eye. Livestock grazing on infected tall fescue can develop certain diseases such as fescue foot and fescue toxicosis (Bacon, 1995, Powell and Petroski, 1992, Lyons et al., 1986). The symptoms are described later in this chapter.

Host plants and parasites live in symbiosis leading to a better protection of the plant against environmental stress and certain factors for example herbivores and insects (Strickland et al., 2011, Lev-Yadun and Halpern, 2007, Wallwey and Li, 2010, Bacon, 1995).

The parasitic lifecycle of ergot producing fungi starts in spring. The acospores of the fungus are distributed by wind. They land on susceptible host plants and invade forming hyphae. The ovaries are colonized which leads to the production of masses of spores that exude the so called honeydew, a syrupy fluid. This honeydew is then transferred to other plants by insects, rain or head to head contact of the plants which allows the ergot fungus to spread in a field. Sclerotia grow and mature in 5 weeks and honeydew production is stopped (Schiff, 2006). Sclerotium is formed out of the primary ergot mycelium, which darkens (EFSA, 2005). The number and size of produced sclerotia vary between different types of cereals (Schiff, 2006). The ripe and pigmented sclerotia either are eaten by grazing animals or harvested together with the cereals or they fall to the ground where they form sexual spores. The spores can stay in the soil where they are able to survive over the winter or they become airborne and infect susceptible host plants of the next season (Eadie, 2003) (Figure 3).

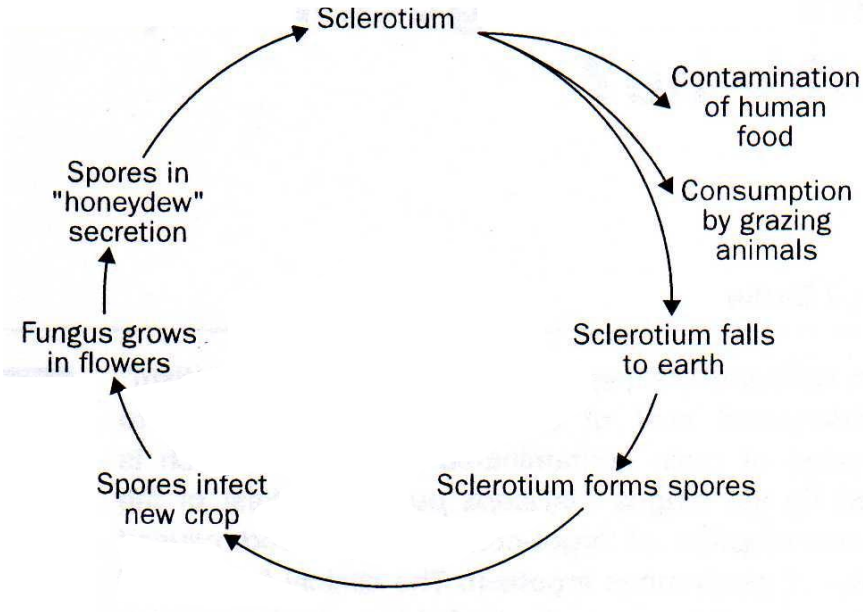


Figure 3 Life cycle of fungi of the genus Claviceps, infecting cereals and producing ergot alkaloids (Eadie, 2003).

When sclerotia are harvested together with the cereals, the toxin can also get into food and feed. Ingestion of these products can lead to so called ergotism in animals and humans (Krska and Crews, 2008). Ergotism has been known for centuries. It already occurred in ancient

times but had its greatest outbreak in Europe in the Middle ages as the so called St. Anthony's fire (Schiff, 2006, De Costa, 2002, Meyer, 2011). On the other hand midwives used ergot alkaloids as an aid in childbirth as well as for abortions in earlier days, giving them also the name "Mothercorn" (Haarmann et al., 2009, Tudzynski et al., 2001). It is also thought that the name comes from a spirit called "Kornmutter" that was believed to live in corn and rye (Lee, 2009a). The "Mothercorn" is also described in a German folklore telling about the mother of corn and rye that passes through the fields and produces ergot (Koehler and Isler, 2002). The toxic effects of these treatments probably have killed a lot of women. As a consequence, the powder the midwives used was actually called "powder of birth" (Pulvis ad partum) but very often it also got the name "powder of death" (Pulvis ad mortem) (Gensthaler, 2011, Lee, 2009b). The symptoms of ergot poisoning vary based on the particular pattern of ergot alkaloids in a certain food product. However, there are two different forms of ergotism registered: the convulsive (*Ergotismus convulsivus*) and the gangrenous ergotism (*Ergotismus gangraenosus*) (Haarmann et al., 2009). Symptoms for the convulsive form are involuntary muscle contractions, flexion or extension of the fingers, wrists and ankles, involuntary twisting, paraesthesia, tinnitus, headaches, double vision, sweating, fever, hallucinations, mania, melancholy and delirium (Schardl et al., 2006). The gangrenous form is characterized by loss of peripheral sensation, jaundice, diarrhoea, swollen and inflamed hands and feet, oedema and vasoconstriction leading to dry gangrene in the extremities and finally their loss and even death (van Dongen and de Groot, 1995, Haarmann et al., 2009, Eadie, 2003, Lee, 2009a).

Due to their biological and pharmacological effects ergot alkaloids are widely used in medicine and pharmacy as prolactin inhibition, treatment of migraine and cluster headaches (Tfelt-Hansen et al., 2000, Rostoff et al., 2010), treatment of Parkinson's disease as well as uterine stimulation (Krska and Crews, 2008). Since the 19th century ergot alkaloids have also been used to stop excessive bleeding during or after child birth (Krska and Crews, 2008, Haarmann et al., 2009). Another semi-synthetic ergot alkaloid made its way through history to our modern days: lysergic acid diethylamide (LSD). It was accidentally synthesized by Albert Hofmann in 1938 (Hofmann, 1978). It was thought to help as an antidepressant drug as well as against schizophrenia and alcoholism. The drug was widely used and abused as it still is in these days (Haarmann et al., 2009, Lee, 2010). In 1966 the drug was declared illegal (Flieger et al., 1997).

Nowadays certain techniques allow effective removal of ergot alkaloids. There are a lot of improvements in crop genetics and management as well as in screenings, regulations of grain

quantities in food and in grain, cleaning techniques and removal of sclerotia (Strickland et al., 2011, Burk et al., 2006). Up to 82 % of ergots can be removed with cleaning at mills. Problems can occur when ergot sclerotia break into smaller fragments or the sclerotia has a similar size to the actual grain, which can happen under certain dry climatic conditions (Krska and Crews, 2008). Additionally, there are strict regulations in many countries only allowing a certain amount of ergots in food products (Scott and Lawrence, 1980, Scott, 2009, Burk et al., 2006). In the European Union a maximum of 0.05% of ergots in grains used for human food is allowed (Haarmann et al., 2009, EFSA, 2005).

Ergotism has basically been eliminated as a human condition. Nowadays ergotism in humans mostly occurs as a result of an overdose of a pharmacologically used ergot drug e.g. ergotamine tartrate. Unfortunately there are still a few recent major outbreaks in humans registered, e.g. in France (Krska and Crews, 2008), India (Bhat et al., 1976) and Ethiopia (Strickland et al., 2011, Krska and Crews, 2008). Additionally, ergotism still poses a major problem in veterinary medicine, particularly in cattle, horses, sheep, pigs and chicken (Krska and Crews, 2008). Especially fescue toxicosis which is caused by ingestion of endophyte infected tall fescue is still very common in grazing animals, predominantly in the USA, Australia and New Zealand. Animals grazing on endophyte infected tall fescue develop symptoms like loss of appetite, poor weight gain or even loss of weight, dry gangrene which can lead to loss of extremities e.g. hooves (this condition is called fescue foot (Garner et al., 1993, Porter and Thompson, 1992), low or no milk production, reduced fertility, long gestations and even still births. Additionally, convulsions, abortions and death can occur. Again symptoms depend on the set of ingested ergot alkaloids as well as the animal species. Mainly the ergopeptine ergovaline plays a major role in poisoning of animals (Schardl et al., 2006, Scott, 2009, Porter and Thompson, 1992, EFSA, 2005). Not only that the animals show similar symptoms that also occur in ergotism but also economic losses put a major pressure on the grazing livestock industry (Strickland et al., 2011, Schardl et al., 2006).

1.3. Enzymes and the degradation reaction

In this work the main focus is on the biotransformation of ergotamine (ergopeptine) and ergine (lysergic acid amide). Two enzymes of the *Rhodococcus erythropolis* strain MTHt3 were isolated by Michaela Thamhesl (paper in preparation). One of the enzymes is a hydrolase termed ErgA, the other one is an amidase called ErgB.

The metabolisation of ergotamine takes place in two steps. The simplified reaction mechanism can be seen in figure 4. In the first step ergotamine is transformed into ergine and other yet unknown metabolites. This step is catalysed by ErgA. In the second step ErgB removes the NH_2-group of ergine which is replaced by a hydroxyl-group giving the final product of lysergic acid.

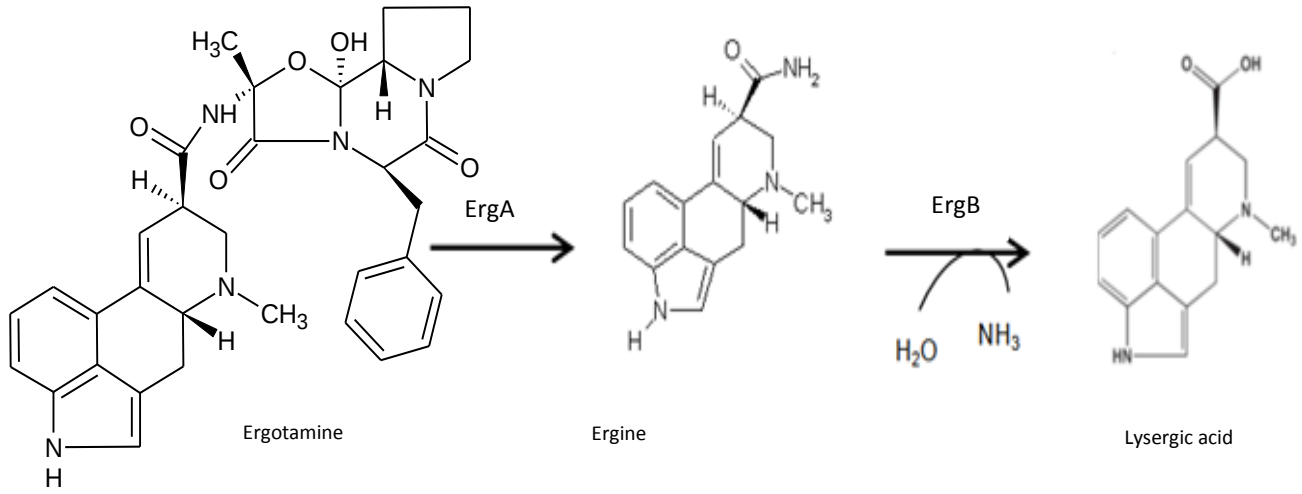


Figure 4 Simplified reaction mechanism of the degradation of ergotamine and ergine. (Metabolites are not mentioned in this scheme.)

Ergotamine and ergine cause vasoconstriction in animals, which is also one of the symptoms of fescue toxicosis and fescue foot (Abney et al., 1993, Oliver et al., 1993). There are different opinions about the vasoconstrictive effects of lysergic acid, which is an ergot alkaloid as well. Researchers in this field reported that lysergic acid is a weak vasoconstrictor and that it is unlikely that it has a lot of influence on the vasoconstrictive effects in an animal suffering of fescue toxicosis (Klotz et al., 2006, Foote et al., 2011).

1.4. Expression in *Pichia pastoris*

The two enzymes described above, ErgA and ErgB, were expressed in the methylotrophic yeast *Pichia pastoris* CBS7435, also termed NRRL Y-11430 whose genome was already sequenced (Kuberl et al., 2011). This strain has been classified as *Komagataella phaffii* (Kurtzman, 2009, Kurtzman, 2005).

P. pastoris is often chosen as an expression system because only simple techniques for molecular and genetic manipulation are needed. Additionally, *P. pastoris* can produce high

amounts of foreign proteins either intracellularly or extracellularly. An extracellular secretion is an efficient way of secretion. Furthermore, *P. pastoris* secrets only low levels of its own proteins (Werten et al., 1999). The organism is also able to carry out a lot of eukaryotic posttranslational modifications e.g. glycosylation, disulphide bond formation and proteolytic processes. One possible disadvantage using this expression system can be a degradation of the expressed protein by extracellular proteases, cell-bound proteases or intracellular proteases of lysed cells. This proteolysis can lead to a lower product yield, loss of biological activity and contamination of products with intermediates (Macauley-Patrick et al., 2005, Yin et al., 2007, Joshi and Sahni, 2010).

P. pastoris can grow using methanol as a carbon source. For the methanol metabolism certain enzymes such as alcohol oxidase, catalase and dehydroxyacetone synthase are needed. When the cells grow on media based on carbon sources some of the enzymes needed for methanol metabolism are not detectable (Cereghino and Cregg, 2000).

To express foreign genes in *P. pastoris* certain steps are necessary, namely the insertion of the chosen gene into a *Pichia* expression vector, the integration of the vector in the *Pichia* genome and finally the analysis of the potential of expression (Cereghino and Cregg, 2000). Alternatively, transformation processes exist maintaining the transformed fragment as an extra-chromosomal element (Cregg et al., 1985, Lee et al., 2005). All expression vectors are so called *Escherichia coli*/*P. pastoris* shuttle vectors, which allow transformation of the vector into *E. coli* and their maintenance in the cell. Most expression vectors have an expression cassette consisting of a promoter and a terminator sequence. In between those two exists the multiple cloning site for insertion of the foreign gene. The AOX1 promoter is a very strong promoter and has been widely and successfully used with a methanol induction. Methanol is not always appropriate for protein expression for example for large scale fermentation. Therefore, other promoters do exist, that are not induced with methanol e.g. *P. pastoris* GAP promoter. The GAP promoter (glyceraldehyde 3-phosphate dehydrogenase) allows expression on glucose with a potential that can be compared to the one with AOX1 promoter. Working with the GAP promoter has the advantage that methanol is not needed and that the cultures do not have to be shifted from one carbon source to the other. A disadvantage might be that the promoter is constitutively expressed and expression of proteins that are toxic to the yeast would not work. In the following experiments the *P. pastoris* expression vector pGAPZα C was used. The plasmid map and the features of the vector can be seen in Figure 5 and Table 3.

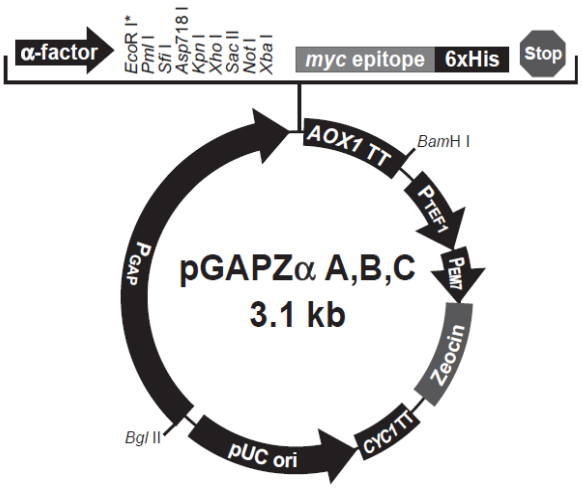


Figure 5 *Pichia pastoris* expression vector pGAPZα C that is used in the following experiments.

Table 3 Summary of all features of the expression vector pGAPZα C (User manual by (invitrogen, 2010).

Vector Site	Feature
GAP promoter	constitutive high level expression (Waterham et al., 1997); targets plasmids integration;
α-factor	codes for the α-factor secretion signal allowing extracellular secretion
Multiple cloning site	site where foreign gene sequence is inserted
C-terminal *myc* epitope	detection of fusion proteins with the help of Anti-*myc* antibody (Evan et al., 1985)
C-terminal polyhistidine-tag	codes for 6 histidine residues, enabling easy purification of expressed protein via affinity chromatography
AOX1 transcription termination (TT)	termination signal including 3' mRNA processing
TEF1 promoter	transcription elongation factor 1 gene promoter for *Sh ble* gene expression in *Pichia*, giving zeocin resistance
EM7 (synthetic prokaryotic promoter)	promoter for *Sh ble* gene expression in *E. coli*, giving zeocin resistance
Sh ble gene	gene for zeocin resistance (Calmels et al., 1991)
CYC1 transcription termination region	allows 3' mRNA processing of the *Sh ble* gene
pUC origin	important for replication and maintenance of the plasmid in *E. coli*

Transformation of a certain gene into *P. pastoris* can happen via electroporation, spheroblast method or whole cell methods namely those involving lithium chloride and polyethylene $glycol_{1000}$. The *P. pastoris* vector has to be cleaved in a sequence that is shared by the host genome so that homologous recombination events can take place and the target vector is integrated into the genomic locus. After integration of the foreign coding sequence proteins can be produced either intracellularly or extracellularly. Mostly extracellular secretion is

preferred to avoid the steps of purification, cell lysis and removal of cell debris. In most expression vectors the so called *Saccharomyces cerevisiae* α-factor prepro-peptide is the secretion signal. In some cases this standard signal has not worked. As a consequence, synthetic signals have been created (Cereghino and Cregg, 2000, Macauley-Patrick et al., 2005).

P. pastoris is ideal for large scale fermentation. All components of the media used are easily available and inexpensive. Furthermore, the media has a relatively acidic pH which lowers the risk of contamination with other microorganisms (Cereghino and Cregg, 2000, Macauley-Patrick et al., 2005, Sasagawa et al., 2011, Sreekrishna et al., 1997).

1.5. Expression in *Escherichia coli*

It is quite common to use bacterial expression systems to produce heterologous proteins. A very popular and widely used bacterial expression system is *E. coli* (Terpe, 2006). The advantages of this system are low costs, easy techniques, availability of multiple vectors and hosts with different features and rapid evaluation because of low doubling times. Unfortunately there are also a few limitations, especially for the expression of eukaryotic proteins. Accessory proteins such as chaperones, posttranslational modification proteins and maturation proteins are not available. As a consequence, other expression systems often have to be in consideration. However, *E. coli* is the most commonly used expression system for heterologous protein production (Jana and Deb, 2005, Yin et al., 2007, Daly and Hearn, 2005).

Protein expression of foreign proteins in *E. coli* can happen in three forms. They can be expressed as fusion proteins being fused to another sequence that enables easy purification, monitoring and other further steps (Klein et al., 2005, Yin et al., 2007). Proteins can also be secreted into the periplasm or the extracellular medium, where the newly expressed proteins are protected against intracellular proteases (Pugsley, 1993, Yin et al., 2007). Expressed proteins can also form inclusion bodies. In this form the proteins are protected against proteases by host cell enzymes. However, these inclusion bodies are usually insoluble and misfolded. For further working the proteins need to be refolded and solved (Clark, 1998, Yin et al., 2007).

Most of the vectors used for this system are for cytoplasmic expression. The vectors consist of various selectable markers, bacterial promoters, plasmid replication origins, localization signals and optional fusion tags. Expression is usually induced with IPTG. A disadvantage of

this step is that different growth rates will be observed during expression and that perfect induction conditions cannot always be reached. After expression the validation usually happens via denaturing gel electrophoresis and is based on the amount of expression and solubility of a protein. Expression rates in *E. coli* can reach 80 % but solubility can sometimes be a limiting factor. Expression levels of foreign proteins can be characterized compared to the expressed *E. coli* proteins. If the target bands on the SDS gel are less intensive than the *E. coli* proteins the expression level is 1. If the bands are stained more intensively they are scored as level 2 or even level 3 (Zerbs et al., 2009).

In the following experiments the vector pET28a(+) containing the coding sequence for the foreign protein was used. Vectors of the pET series have a T7 promoter and need to be induced with IPTG (Zerbs et al., 2009).

Table 4 gives a summary of the features of all *E. coli* expression systems that were used. The designation DE3 points out that the strain is a lysogen of λDE3, which means that it carries a chromosomal copy of the T7 RNA polymerase gene under control of the *lacUV*5 promoter. These strains are used for the production of proteins from the target genes cloned into pET-vectors (Novagen EMD Biosciences, Merck KGaA, Darmstadt, Germany; User protocol TB009 Rev. F0104; Competent cells).

Table 4 Summary of all *E. coli* expression systems used in this work (Novagen EMD Biosciences, Merck KGaA, Darmstadt, Germany; User protocol TB009 Rev. F0104; Competent cells).

Strain	Features
BL21(DE3)	This strain is deficient in the *lon* and *opmT* proteases. This should enable stable expression.(Studier and Moffatt, 1986)
BL21(DE3)pLysS	Additionally, to the features of the BL21(DE3), this strain carries a plasmid that codes for a T7 lysozyme. This lysozyme is a natural inhibitor of T7 RNA polymerase leading to a repression of the expression of the target gene before induction. The strain is resistant to chloramphenicol.
HMS174(DE3)	This strain has a *recA* mutation which should provide a stabilization of certain target genes whose products might lead to the loss of the DE3 prophage. It is resistant to rifamycin.
Origami 2(DE3)	This strain has mutations in the thioredoxin reductase and the glutathione reductase genes, enhancing the disulfide bond formation in the cytoplasm. It is resistant to tetracycline and streptomycin.(Derman et al., 1993)
Rosetta 2 (DE3)	This strain is a derivative of BL21 and it should enhance the expression of proteins with codons that are hardly used in *E. coli*. The seven rare codons are AUA, AGG, AGA, CUA, CCC, GGA and CGG. The strain is resistant to chloramphenicol.
Tuner(DE3)	This strain is a *lacZY* deletion mutant of BL21. The lac permease mutation allows the ubiquitous entrance of IPTG into all cells. This allows an induction with IPTG that is concentration dependent. With different amounts of IPTG expression of foreign proteins can vary between low levels up to fully induced expression levels. Low levels might even guarantee a better solubility of expressed proteins. As BL21 the strain is also deficient in *lon* and *opmT* proteases.
ArcticExpress(DE3)	This strain derived from the *E. coli* strain BL21-Gold. Its benefit is that it should overcome problems of insolubility of the target protein without fusion tags or in-vitro refolding methods. (information from the product description of competent *E. coli* ArcticExpress(DE3) cells of Agilent Technologies)

2. Material and Methods

2.1. Material

All materials were from Sigma-Aldrich; St. Louis, MO, USA, if not stated otherwise.

Media: Luria-Bertani (LB) medium, Low-Salt-LB medium, S.O.C-medium, YPD-medium, YPDS-medium; Agar Bacteriological (Agar No. 1) (Oxoid; Cambridge, UK); $NZY^{\pm}$-medium; Compositions of all media can be found in the book Molecular Cloning, A Laboratory manual (Sambrook and Russell, 2001).

DNA: synthesis of codon optimised *erg*A in pMA-T and the codon optimised *erg*B in pGAPZα C (Invitrogen, GeneArt; Regensburg, Germany); expression vector pGAPZα C and vector pUC19 (Invitrogen; Carlsbad, CA, USA); pUC18 (0.1 ng/µl) (Stratagene; Santa Clara; CA, USA);

Oligonucleotides: Amit3_int_for, pGAPZ_for and pGAPZ_rev (VBC Biotech; Vienna, Austria); ErgA1_fw, ErgA1_rev, ErgA2_fw, ErgA2_rev, ErgA3_fw, ErgA3_rev, ARG4_fw, ARG4_rev, ErgB1_fw, ErgB1_rev, ErgB2_fw, ErgB2_rev, ErgB3_fw, ErgB3_rev

Strains: *E. coli* DH10B (Invitrogen; Carlsbad, CA, USA); *E. coli* XL10-Gold (Stratagene Agilent Technologies; Santa Clara, CA, USA); *E. coli* strains Origami 2(DE3), HMS174(DE3), *E. coli* BL21(DE3) pLysS, Tuner(DE3), ArcticExpress(DE3) and Rosetta 2(DE3) (Novagen EMD Biosciences, Merck KGaA; Darmstadt, Germany); *P. pastoris* CBS7435 (NRRLY-11430);

Plasmids and hosts (all prepared by employees of Biomin, Tulln; Austria): Amidase T3-his_Klon #4 *E. coli* DH10B; pET28a(+) *E. coli* XL1-Blue; Amidase T3-his-Klon #1 *E. coli* BL21(DE3); pET28-a-BL21 *E. coli* BL21(DE3); Amidase T3-his_Klon #4-1 *E. coli* ArcticExpress; Amidase T3-his_Klon #4-1 *E. coli* ArcticExpress; Amidase T3-his_Klon #4-2 *E. coli* ArcticExpress; Amidase T3-his_Klon #4-3 *E. coli* ArcticExpress; pET28a_ArcticExpress *E. coli* ArcticExpress;

Enzymes: all restriction enzymes and appropriate buffers (Fermentas; St. Leon-Rot, Germany) except *Apa*I, *Dra*I and *Sfi*I (New England BioLabs; Ipswich, MA, USA); T4 DNA Ligase (EL0014), associated ligase buffer and RNaseA (Fermentas; St. Leon-Rot, Germany); ErgA, positive control; (with 25% glycerine; concentration: 0.71 mg/ml; stored at -20 °C since 22.12.2010; purified via His-Tag;); Calf Intestinal Alkaline Phosphatase (Invitrogen; Carlsbad, CA, USA);

Antibiotics: Zeocin (Invivogen, Eubio; Vienna, Austria); Ampicillin (Oxoid; Cambridge, UK); Kanamycin sulphate and Gentamicin sulphate

Chemicals: Glycerine; Isopropanol (VWR; Radnor, PA, USA); Ethanol; Ergotamine D-tatrate; Acetonitrile (VWR; Radnor, PA, USA); Phenol:Chloroform:Isoamylalcohol (PCI, 25:24:1); Chloroform:Isoamylalcohol (CI, 24:1); 1 M sorbitol solution; $MgCl_2$-$CaCl_2$-solution (80 mM $MgCl_2$ Merck; Vienna, Austria; 20 mM $CaCl_2$); Beta-ME-mix consisting of 5% β-mercaptoethanol, 13% NaCl and distilled water; Ergine (667 ppm) (Alfarma; Cernosice, Czech Republic); IPTG; $MgSO_4$ (Merck; Darmstadt, Germany);

Buffer: TE-buffer pH 8.0 containing 100 mM Tris-HCl (pH 8.0) and 10 mM EDTA (pH 8.0); sodium-phosphate buffer pH 7.0 (2.52 g NaH_2PO_4 and 4.12 g Na_2HPO_4); Yeast Lysis Buffer: 2% Triton X-100; 1% SDS, 100 mM NaCl, 1 mM EDTA, 10 mM Tris pH 8.0; Resuspension buffer: 9.5 ml 10 mM Tris-HCl buffer (pH=8.2), 0.5 ml 500 mM EDTA, 25 µl beta-mercaptoethanol; 10 mg/ml lysozyme solution; 0.5 % Triton X-100 (Roth; Karlsruhe, Germany) in 10 mM Tris-HCl buffer (pH=8.2);

Kits: Wizard SV Gel and PCR Clean-Up System (Promega; Madison, WI, USA); QIAprep® Spin Miniprep Kit (QIAGEN; Hilden, Germany); QIAGEN Plasmid Midi Kit (QIAGEN; Hilden, Germany);

Devices: Spectrophotometer Nanodrop ND-1000 (PeqLab Biotechnologie GmbH; Erlangen, Germany); 0,2 cm cuvettes (BioRad Laboratories; Hercules, CA, USA); Gene Pulser™, Capacitance Extender and Pulse Controller (BioRad Laboratories; Hercules, CA, USA); Avanti™ Centrifuge J-25 (Beckman Coulter™; Indianapolis, IN, USA); Centrifuge 5417R (Eppendorf; Vienna, Austria); Brown glass HPLC vials (Roth; Karlsruhe, Germany); Precellys and Precellys tubes (PeqLab Biotechnologie GmbH; Erlangen, Germany); Centrifuge 5804R (Eppendorf, Vienna, Austria); Incubator Ecotron (Infors HT; Bottmingen, Switzerland); Incubator 37 °C (Binder GmbH; Tuttlingen, Germany); Spectrophotometer U-2001 (Hitachi; Tokyo, Japan); Light microscope Eclipse E400 (Nikon; Tokyo, Japan) + Camera Visi Cam 5.0 (VWR; Radnor, PA, USA); Thermocycler Mastercycle Gradient (Eppendorf; Vienna, Austria); PCR cycler: Eppendorf realplex² Mastercycler epgradient S (Eppendorf; Vienna, Austria); Run One™ Electrophoresis cell (Embi Tec; San Diego, CA, USA); Electrophoresis Power supply EV231 (Consort; Turnhout, Belgium); Dark room (PeqLab Biotechnologie GmbH; Erlangen, Germany); Electrophoresis Chamber (Hoefer; Holliston, MA, USA);

PCR: 10x reaction buffer S, dNTP-mix, peqGOLD Taq-DNA-Polymerase'all inclusive (all from PeqLab Biotechnologie GmbH; Erlangen, Germany); SYBR-supermix (KAPA) (PeqLab Biotechnologie GmbH; Erlangen, Germany); 0.2% - DEPC-H_2O (sb);

Agarose Gel Electrophoresis: Agarose (PeqLab Biotechnologie GmbH; Erlangen, Germany); TAE-Buffer containing 0,4 M Tris, 0,01 M EDTA-Na_2-Salt and 0,2 M acetic acid ; 5x loading dye (Fermentas; St. Leon-Rot, Germany); Gene Ruler ™ 1 kb DNA ladder and FastRuler™ Low Range DNA Ladder, ready-to-use (Fermentas; St. Leon-Rot, Germany); Gelred ™ (Biotium; Hayward, CA, USA);

SDS-PAGE (Laemmli, 1970): Separating gel (12%): 1.98 ml distilled water, 2.4 ml acrylamide 30%, 1.5 ml Separating Gel buffer (1.5 M Tris/HCl pH 8.8), 60 µl SDS 10%, 60 µl APS 10%, 2.4 µl TEMED; Loading gel (5%): 1.7 ml distilled water, 415 µl acrylamide 30%, 315 µl Loading Gel buffer (0.5 M Tris/HCl pH 6.8), 25 µl SDS 10%, 25 µl APS 10%, 2.5 µl TEMED; Isopropanol (VWR; Radnor, PA, USA), 2x sample buffer; Isopropanol (VWR; Radnor, PA, USA); Molecular weight ladder: BenchMark Protein Ladder (Invitrogen; Carlsbad, CA, USA); BSA-Standards (0.1 mg/ml and 0.5 mg/ml); GelCode™ Blue Stain Reagent (Thermo Scientific; Waltham, MA, USA); 5x running buffer;

2.2. Expression of ErgA

2.2.1. Molecular cloning

Preparation of *erg*A pMA-T

Vector pMA-T containing the gene encoding the enzyme ErgA (synthesis of codon optimised sequence and insertion were done by the company Invitrogen [Regensburg, Germany]) was solved in 50 µl TE-buffer (pH 8,0) giving a final concentration of 0,1 µg/µl. Transformation of the plasmid into *E. coli* DH10B was carried out via electroporation. 1 µl plasmid was added to 40 µl electro competent *E. coli* DH10B cells. (Settings at electroporation: 0.1 cm cuvettes; 2.0 kV; 200 Ω; 25 µF) After the electric pulse 1 ml S.O.C medium was quickly added to the cuvettes and the samples then transferred to 15 ml Falcon tubes. Incubation for 1 h at 37 °C and 300 rpm was carried out. The sample plus a negative and a positive (pUC 19) control were plated out onto LB-agar plates containing 100 µg/ml ampicillin and were incubated overnight at 37 °C. Four transformants were picked and each one was inoculated into LB-medium (incubation 37 °C, 200 rpm, overnight) to carry out a miniprep isolation gaining *erg*A pMA-T. The isolation procedure was done as suggested by the company QIAGEN. Additionally, the concentration of plasmid-DNA was spectrophotometrically measured with nanodrop.

As a control the plasmids were digested with the restriction enzyme *Sfi*I to obtain the DNA fragments with a size of 1025 bp and 2321 bp. (2 µl of each sample was used (colony 1:

260.4 ng DNA; colony 2: 359.8 ng; colony 3: 326.0 ng; colony 4: 302.2 ng). Gel electrophoresis was carried out (1.5% agarose gel; 5x loading dye; electrophoresis duration: 25 min; potential: 100 V; staining with Gelred™ for 30 min).

Preparation of pGAPZα C

Isolation of the *P. pastoris* vector pGAPZα C, which was in *E. coli* XL10-Gold, was also carried out with the QIAprep Spin Mini Prep Kit from QIAGEN. As a control pGAPZα C was digested with *Apa*LI and the plasmid parts were observed with the help of gel electrophoresis. Two approaches were made. 2 µl of each sample were used for each digestion: 269.4 ng and 229.2 ng.

Digestion of pGAPZα C and *erg*A pMA-T with *Xho*I and *Xba*I

pGAPZα C and *erg*A pMA-T were digested with the restriction enzymes *Xho*I and *Xba*I to gain the vector pGAPZα C and the insert ErgA ready for ligation. Each 20 µl sample (1254 ng insert and 1546 ng vector), 8 µl Tango Buffer 10x, 6 µl distilled water., 4 µl *Xba*I and 2 µl *Xho*I were used and incubated in a water bath (37 °C) for 4 h. Settings for each approach of restriction digest were calculated on the website http://www.fermentas.com/en/tools/doubledigest/?country_code=AT.

A preparative gel electrophoresis was carried out. 40 µl of each sample was mixed with 4 µl 5x loading dye and applied onto a 1.5 % agarose gel. Electrophoresis was carried out for 1 h at a potential of 90 V. The gel was stained with Gelred™ for approximately 1 h. Under the observation of UV-light the appropriate bands for the cut vector pGAPZα C (3060 bp) and the insert ErgA (960 bp) were cut out. DNA was isolated with Wizard SV Gel and PCR Clean up System according to the manufacturer´s instructions and its concentration determined with the spectrophotometer nanodrop as well as an agarose gel electrophoresis.

To prevent a religation of pGAPZα C the so called CIPing of the vector was carried out. An alkaline phosphatase cuts off the phosphate groups at the cut ends of the vector. This step was done with the Calf Intestinal Alkaline Phosphatase (CIAP). To find out how much enzyme is needed the amount of plasmid was calculated. As a result 1 µl of CIAP was added to 15 µl of the restriction mixture of pGAPZα C. Furthermore, the mix was then incubated for 30 min at 37 °C. For inactivation of the alkaline phosphatase 0.4 µl of 10mM EDTA (pH 8.0) were added and the mixture incubated for 15 min at 65 °C.

Production of *erg*A pGAPZα C

1 µl T4 DNA ligase, 1 µl digested vector pGAPZα C ($n=3.827*10^{-14}$ mol), 3 µl digested insert ErgA ($n=1.9138*10^{-13}$ mol), 1 µl 10x ligase buffer and 4 µl distilled water were mixed together. Three different negative controls were also prepared. Control 1 contained the enzyme and buffer but no DNA. Control 2 contained the enzyme, buffer and the insert coding for ErgA. Control 3 contained the enzyme, buffer and the digested vector pGAPZα C. All samples were incubated at 16 °C for 16 h. To observe the ligation process an agarose gel electrophoresis was carried out.

E. coli DH10B cells were made electro competent. After 16 h of incubation of the pre culture (*E. coli* DH10B inoculated in Low Salt media), 25 ml were transferred to 475 ml fresh media. (Incubation: 37 °C, 200 rpm, 1.5. h). After reaching an OD_{600} of approximately 0.6 the culture was divided into four parts and incubated on ice for 20 min. Cell were harvested via centrifugation (15 min; 1000 x g; 4 °C) and resuspended in 125 ml ice cold distilled water. The cells were harvested (20 min; 1000 x g; 4 °C) and resuspended in 30 ml ice cold distilled water. With the same settings as used before the cells were centrifuged and the pellets resuspended in 2.5 ml ice cold and sterile 10 % glycerine. After a final centrifugation (15 min; 1000 x g; 4 °C) the pellets were solved in 250 µl ice cold and sterile 10 % glycerine. Aliquots of 40 µl were stored at -80 °C. To determine efficiency of transformation a transformation with pUC19 (1 µl plasmid to 40 µl electro competent *E. coli* cells; 0.1 cm cuvettes; 2.0 kV; 200 Ω; 25 µF) was carried out.

Transformation of ErgA pGAPZα C plus the 3 control samples into *E. coli* DH10B was carried out via electroporation (1 µl sample of the ligation to 40 µl electro competent *E. coli* DH10B cells; 0.1 cm cuvettes; 2.0 kV; 200 Ω; 25 µF).

The cells were plated out on LB Low Salt agar plates containing 25 µg/ml zeocin and incubated at room temperature for 77.5 h.

Four transformants were picked to carry out miniprep isolation. The difference to the protocol of QIAGEN was that the final DNA pellet was solved in 30 µl elution buffer rather than in 50 µl.

Concentrations of the samples were determined via nanodrop measurements. After a restriction digest with *Xho*I and *Xba*I each sample was also observed on an agarose gel. 2 µl of each sample were used (colony 1: 119.8 ng plasmid DNA; colony 2: 135.6 ng; colony 3: 110.8 ng; colony 4: 110.6 ng) and the samples were incubated in a water bath at 37 °C for 2 h.

Additionally, digestions with different combinations of restriction enzymes (*Xho*I and *Xba*I; *Xba*I (linearization); *Dra*I and *Xho*I) and following gel electrophoresis were carried out as a control with colony 2.

To get a sequence analysis of the produced plasmid plus insert a forward and a reverse primer were diluted to gain a concentration of 100 pmol/µl. 50 µl per primer (Table 5) and 15 µl of isolated DNA were sent to the company Sequiserve (Vaterstetten, Germany) to perform sequencing.

Table 5 List of primers used for sequencing.

Name	Sequence	Length	GC-content	Annealing Temperature[°C]
pGAPZ_for	5'- TTC CAA CAG CAC AAA TAA CGG G -3'	22-mer	45.50%	56.4
pGAPZ_rev	5'- ATG GTC GAC GGC GCT ATT C -3'	19-mer	57.90%	54.9

2.2.2. Transformation

Production of electro competent *P. pastoris* CBS 7435-cells

This procedure was carried out as described in the manual pGAPZ A, B, and C pGAPZα A, B, and C *Pichia* expression vectors for constitutive expression and purification of recombinant proteins of Invitrogen by life technologies (Carlsbad, CA, USA). One colony of *P. pastoris* CBS 7435 was inoculated in 5 ml YPD-medium and incubated at 30 °C overnight to gain a pre culture. 250 µl of the pre culture was transferred into 2 flasks containing 250 ml YPD-medium. These cultures were incubated at 30 °C, 210 rpm for 23 h. Cells were harvested with centrifugation (4 °C;1500 x g; 5 min). After each pellet was resuspended in 400 ml ice cold and sterile water another centrifugation step, with the same settings as described before, was carried out. Each pellet was washed with 250 ml ice cold and sterile water. After the water had been removed via centrifugation (4 °C; 1500 x g; 5 min) each pellet was resuspended in 15 ml ice cold and sterile 1 M sorbitol-solution. The solution was removed with another centrifugation (4 °C; 1500 x g; 5 min) and each pellet dissolved in 1 ml ice cold and sterile 1 M sorbitol solution. The electro competent cells were used straightaway.

Transformation of *erg*A pGAPZα C into electro competent *P. pastoris* cells

For this step the sequenced plasmid *erg*A pGAPZα C was used. Isolation was carried out as described in the manual for high copy plasmids of the QIAprep® Spin Miniprep Kit from QIAGEN with the difference that the DNA pellets were tried overnight to ensure that all

ethanol was vaporized. Each pellet was resolved in 50 µl TE-buffer. Concentrations of DNA were measured with the spectrophotometer nanodrop.

To be able to integrate the plasmid into the *Pichia* genome 80 µl of isolated plasmid were linearized with the restriction enzyme *Avr*II (*Xma*JI, *Bln*I). The sample was incubated in a water bath at 37 °C for approximately 24 h. To ensure a complete restriction digestion an agarose gel electrophoresis was carried out. (100 V, electrophoresis duration: 30 min) With the help of Wizard Gel and PCR Clean-Up System from Promega the DNA was purified and the concentration measured with nanodrop.

Transformation was carried out as described in the user manual "pGAPZ A, B, and C pGAPZα A, B, and C *Pichia* expression vectors for constitutive expression and purification of recombinant proteins" of Invitrogen by life technologies (Carlsbad, CA, USA). 80 µl electro competent *P. pastoris*-cells were mixed with 30 µl of the linearized and purified sample DNA (approximately 3 ng). The sample and a negative control (instead of DNA sterile water was used) were incubated on ice for 5 min. Settings at electroporation were 0.2 cm cuvettes; 1.5 kV; 400 Ω and 25 µF. 1 ml of ice cold and sterile 1 M sorbitol-solution was added and the sample plus negative control incubated at 37 °C, 210 rpm for 1.5 h. Aliquots of the cells were plated out on YPDS-agar plates containing 100 µg/ml zeocin and incubated at 30 °C for 4 d.

Colony PCR

To find out which colonies contained the plasmid with the insert after transformation a colony PCR was carried out. All colonies that formed on the plates after transformation were chosen and transferred to a master plate. 10 of these colonies were picked and used for the screening experiment. Furthermore, one negative control (without template DNA) and one positive control (containing DNA of *erg*A pGAPZα C) were used.

The PCR-mixture per sample contained 5 µl 10x reaction buffer S, 1 µl dNTP-mix, 0.5 µl forward primer pGAPZ_for (n=0,05 nmol), 0.5 µl reverse primer pGAPZ_rev (n=0,05 nmol) (Table 6), 42.75 µl distilled water and 0.25 µl Taq-polymerase. Having used these primers an amplification product with a length of approx. 1200 bp were expected. The PCR-program can be seen in Table 7.

Table 6 Primers used for colony PCR.

Name	Sequence	Length	GC-content	Annealing Temperature [°C]
pGAPZ_for	5'- TTC CAA CAG CAC AAA TAA CGG G -3'	22-mer	45.50%	56.4
pGAPZ_rev	5'- ATG GTC GAC GGC GCT ATT C -3'	19-mer	57.90%	54.9

Table 7 Time frame and temperature settings at colony PCR.

PCR - program			
	Timeframe [min]	Temperature [°C]	
	10	96	
	4	40	Taq Polymerase was added
Denaturation	1	94	These three steps were repeated 30 times
Annealing	0.5	50	
Elongation	2	72	
	1	72	
	Hold	10	

For the actual screening process an agarose gel electrophoresis was carried out.

2.2.3. Expression

Seven colonies of *P. pastoris* CBS7435 were chosen. Colony PCR proved that these cells contained the plasmid *erg*A pGAPZα C. The colonies were picked and each one of them was streaked on one YPDS plate and incubated at 30 °C for 4 d. A single colony of each of these plates was inoculated into 10 ml YPD-medium. As a negative control one colony of *P. pastoris* without the vector was used. These pre cultures were incubated at 30 °C, 250 rpm, overnight. 0.1 ml of each of the pre cultures was transferred on 50 ml YPD medium. These cultures were incubated at 30 °C and 150 rpm.

1 ml samples of each of these cultures were taken after 24 h, 48 h, 72 h and 96 h. The samples were centrifuged (room temperature; 25000 x g; 5 min), the supernatant transferred to a fresh tube and both supernatant and pellet stored at -20 °C. Pellets and supernatants were used for SDS-PAGE analysis. The pellets of all 7 colonies taken after 96 h of expression were resolved in 1 ml YPD medium. 10 µl of the dissolved pellets and 10 µl of the supernatants (5 colonies, 4 time points) were each mixed with 10 µl 2x sample buffer and derivatized on a heat block at 95 °C for 10 min. 13 µl of each mixture were loaded on SDS-PAGE gels. Additionally, two BSA-standards (0.1 mg/ml and 0.5 mg/ml) were used for quantification of the protein. Electrophoresis was carried out at 200 V for approximately 1.5

h. After electrophoresis the gels were washed three times (each 5 min) with distilled water. Then they were stained with GelCode™ Blue for 1 h. Before the results could be assessed the gels were washed in distilled water over night.

2.2.4. Analysis

Determination of activity – Degradation of ergotamine

The samples that were used for this experiment were chosen from the expression process. The experiment was carried out three times on different days but with the same conditions. The first time colony 1 with ErgA after 96 h of expression, diluted 1:100, 1:1000 and 1:10000 was used. In the second approach again colony 1 was used with expressed ErgA after 24 h, 48 h, 72 h and 96 h expression, diluted 1:100. The third time colonies 1, 8 and 10 at 0 h, 24 h, 48 h, 72 h and 96 h of expression were used. These samples were diluted 1:100. For each approach a negative control (without the enzyme) and a positive control (ErgA expressed in *E. coli* ArcticExpress(DE3), cleaned up by affinity chromatography and diluted 1:10000) were added. All samples were diluted with sodium phosphate buffer to a final volume of 4.9 ml. To each sample 100 µl ergotamine were added and the mix incubated at 25 °C in a water bath. At 0 h; 0.5 h; 1 h; 2 h and 4 h 320 µl samples were taken and the enzyme inactivated with 80 µl of 100% acetonitrile (final concentration 20%). The samples were centrifuged (10 min; 15682 x g; room temperature) and 360 µl of each supernatant was transferred into a brown glass vial for further HPLC analysis. HPLC-Fluorescence measurement was carried out at IFA Tulln.

Determination of copy number via real time PCR

At the beginning a rough assessment of copy number determination was made. All colonies that contained the plasmid and insert, proven with colony PCR, were streaked out on YPD agar plates with increasing zeocin concentrations (250 µg/ml; 500 µg/ml; 1000 µg/ml). For real time PCR extraction of genomic DNA was necessary. 10 ml YPD were each inoculated with colonies 1, 2, 6, 8 and 10 from the previous transformation. Colony PCR proved that these colonies contained the vector pGAPZα C with the insert coding for ErgA. Additionally, colonies 3, 5 and 9 from the same transformation were also used to inoculate media, although colony PCR showed that these colonies did not contain the insert. Furthermore, 10 ml medium were also inoculated with empty *P. pastoris* CBS7435 cells to get a negative control. The cultures were incubated overnight at 30 °C and 180 rpm. 2 ml of each culture was

harvested via centrifugation (15000 x g; 10 min; RT). The pellets were resuspended in 20 µl yeast lysis buffer. The suspension was transferred into Precellys tubes VK05. To get rid of the RNA, RNaseA (20 µl) was added to each tube and the tubes put into the Precellys (1x20 s; 5500 rpm). The tubes were incubated in a water bath (37 °C) for 10 min. 200 µl PCI was added and the samples centrifuged (15682 x g; 3 min; RT). 200 µl water was added and the tubes again centrifuged (25000 x g; 10 min; 4 °C) to get two phases. To each water phase 350 µl CI was added. After centrifugation was carried out (15682 x g; 3 min; 4 °C) 210 µl 100% isopropanol was added to the water phase to precipitate DNA. DNA was pelleted with the help of centrifugation (15682 x g; 5 min; 4 °C). Pellets were washed with 500 µl 70% EtOH (15682 x g; 5 min; 4 °C). Pellets were tried on a heater 37 °C for 45 min. The DNA was dissolved in 100 µl 10 mM Tris-HCl pH 7.5 and put on the heater at 65 °C for 15 min and 700 rpm to enable complete solving. Concentrations were measured three times with nanodrop and were also applied onto an agarose gel for electrophoresis.

The volume of all approaches at PCR was 15 µl. Depending on the amount of samples the PCR-super mix was created. Each 15-µl-approach contained 7.5 µl 2x SYBR-super mix (KAPA), 0.3 µl primer forward, 0.3 µl primer reversed, 4.9 µl sb and 2.0 µl template DNA. The used primers are listed in Table 8.

The PCR was carried out in three steps. First step was the first optimization of annealing temperature. For this step colony 8 was diluted to get a final concentration of 1 ng/µl and used at the temperatures 54.6 °C, 56.2 °C, 59.7 °C, 62.9 °C and 65.1 °C. All pairs of primers were used. The PCR program used can be seen in Table 9 to 11.

Table 8 Primer used for real time PCR.

Primer Name	Sequence	Length	Annealing Temperature [C°]	GC content [%]
ErgA1_fw	5'-GCTGTTTACTTCTACGACCCAACT-3'	24	63.4	45.8
ErgA1_rev	5'-AAGCCATAACAACCTCAAAAGTTC-3'	24	63.2	37.5
ErgA2_fw	5'-GCTTCTTTGTTGTCCAGAACTTTT-3'	24	63.5	37.5
ErgA2_rev	5'-CAATGTCTGAACCAAATCAAAGTC-3'	24	63.4	37.5
ErgA3_fw	5'-TACAGAGACTGGGTTGCTACTTTG-3'	24	63.3	45.8
ErgA3_rev	5'-GGTGATGTTGTCTAATACCGTTTG-3'	24	63.1	41.6
Arg4_fw	5'-TCCATTGACTCCCGTTTTGAG-3'	21	66.1	47.6
Arg4_rew	5'-TCCTCCGGTGGCAGTTCTT-3'	19	67.1	57.8

Table 9 PCR program for the first optimization of annealing temperature.

PCR-program			Melting curve	
T [°C]	Time [sec]		T [°C]	Time [min]
95	60		95	0.3
95	20	45 cycles	60	0.3
52-65	20		slow heating	20
72	40		95	0.3

In the second step the final annealing temperature was determined. Colony 8 (1 ng/µl) was used with primer pairs ErgA1, ErgA3 and ErgA4 at the temperatures 60.8 °C, 61.9 °C, 62.4 °C, 62.9 °C and 64.0 °C.

Table 10 PCR program of second optimization of annealing temperature.

PCR-program			Melting curve	
T [°C]	Time [sec]		T [°C]	Time [min]
95	60		95	0.3
95	20	45 cycles	60	0.3
60-65	20		slow heating	20
72	40		95	0.3

Afterwards the PCR efficiency was determined. Colony 8 was diluted to get the concentrations 1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl and 0.1 pg/µl. Each stock was used with primer pairs ErgA1 and ARG4. Additionally, for each primer pair a negative control was done where the template DNA was replaced with water.

Table 11 PCR program of determination of PCR efficiency and for actual quantification.

PCR-program			Melting curve	
T [°C]	Time [sec]		T [°C]	Time [min]
95	60		95	0.3
95	20	40 cycles	60	0.3
63	60		slow heating	20
			95	0.3

Finally the actual quantification was carried out. The same PCR program and melting curve was used in this step as in step before that can be seen table 11. The DNA of colonies 1, 2, 6, 7, 3, 5, 9 and the DNA of native *P. pastoris* CBS7435 was diluted as colony 8 in the step before with the exception that no dilution of 0.1 pg/µl was made. Each colony was used for PCR with both primer pairs. Additionally, for each primer pair a negative control was done.

As a positive control 1 ng/µl DNA of colony 8 was used with the primer pair ErgA1 (Hoffman and Winston, 1987, Lee et al., 2006, Abad et al., 2010, Hartner et al., 2008).
After quantification an agarose gel electrophoresis was carried out to observe the amplified products on the basis of the length

2.3. Expression of ErgB

2.3.1. Transformation

The production of chemically competent *E. coli* XL10 Gold was carried out as described in the manual of (Sambrook and Russell, 2001). The lyophilized vector *erg*B pGAPZα C was dissolved in 50 µl TE-buffer gaining a concentration of 0.1 µg/µl. The heat shock was carried out as described in the manual XL10-Gold Ultracompetent Cells of Stratagene Agilent Technologies with the difference that the cells were plated out on Low Salt agar plates containing 25 µg/ml zeocin. Furthermore, a transformation with the test plasmid pUC18 was carried out the same way to analyse transformation efficiency. After incubation two colonies of transformation of *erg*B into *E. coli* were picked, streaked out on a master plate (LB Low Salt medium) and used for cultivation. Isolation was carried out as described in the manual for high copy plasmids of the QIAprep® Spin Miniprep Kit from QIAGEN with the difference that the DNA pellets were tried for 2.5 h to ensure that all ethanol was vaporized. Each pellet was resolved in 50 µl TE-buffer. Concentrations of DNA were measured with the spectrophotometer nanodrop. 200.4 ng of the isolated plasmid were each digested with a different set of restriction enzymes. (Linearization with *Xba*I; digestion with *Xho*I and *Xba*I; digestion with *Xba*I and *Nco*I;) To observe the results an agarose gel electrophoresis was carried out.
In order to be integrated into the *Pichia* genome 8.904 µg of the vector *erg*B pGAPZα C needed to be linearized with 10 µl *Avr*II (*Xma*JI, *Bln*I). The enzyme was added stepwise throughout incubation. Linearization was carried out the same way as for the transformation of *erg*A pGAPZα C into *P. pastoris* CBS7435 (chapter 2.2.2.).
Production of electro competent *P. pastoris* CBS7435 cells, transformation and colony PCR were carried out as described in the chapter 2.2.2, with the difference that 6.229 µg of linearized DNA was used.
Based on the results of the previously described transformation another experiment of integrating *erg*B pGAPZα C into *P. pastoris* CBS7435 was carried out using published articles as a basis (Wu and Letchworth, 2004, Lin-Cereghino et al., 2005, Cregg et al., 2009).

Linearization was done the same way as described above (13.4535 µg DNA). Additionally, the linearized vector was digested with *Xho*I to prove that linearization had actually taken place. Production of electro competent *Pichia* cells was similar to the production before but with a few optimizations. A 5 ml pre culture (YPD-medium) was incubated at 30 °C, 200 rpm, overnight. 3.5 ml of this culture were inoculated in 100 ml YPD-medium and incubated at 30 °C, 200 rpm, for 2.4 h to gain and OD_{600} of 0.8. 4 times each 20 ml ($8*10^8$ cells) of this culture were transferred into 50 ml Falcon tubes and harvested via centrifugation (500 x g; 5 min; room temperature). Each pellet was resuspended in 8 ml incubation solution and incubated at room temperature for 30 min. Centrifugation (500 x g; 5 min; 4 °C) was carried out and the pellets were washed 4 times with 1.5 ml of ice cold 1 M sorbitol-solution. Finally the pellets were resuspended in 80 µl ice cold 1 M sorbitol solution gaining $1*10^{10}$ cells/ml. Each suspension was transferred to an Eppendorf tube.

For electroporation 3 different amounts of linearized plasmid and a negative control were used. 10 ng, 1 µg and 4 µg of DNA were each mixed with 80 µl of electro competent cells. For the negative control no DNA was used. Each mix was transferred into a 0.2 cm electroporation cuvette (0.2 cm) and incubated on ice for 5 min. Settings at electroporation were: charging voltage: 1500 V; capacitance: 25 µF; resistance: 200 Ω; After the electric pulse 1 ml of YPD medium/1 M sorbitol solution (1:1) was added, the mixture transferred into 15 ml Falcon tubes and incubated at 30 °C, 100 rpm for 3.5 h. Manual for the production of electro competent *P. pastoris* CBS7435 cells and settings at electroporation were found in the following literature (Wu and Letchworth, 2004, Lin-Cereghino et al., 2005, Pipes et al., 2005, Cregg et al., 2009). The following steps of transformation and colony PCR were carried out the same way as described in the chapter for the production of ErgA with the difference that a different primer pair (Table 12) was used for the PCR expecting bands at a height of 480 bp.

Table 12 Primers used for colony PCR of *erg*B pGAPZα C.

Name	Sequence	Length	GC-content	Annealing Temperature [°C]
Amit3_int_for	5'- AGG ACT TGA AAG AAG GTT CCC C -3'	22-mer	50.00%	56.8
pGAPZ_rev	5'- ATG GTC GAC GGC GCT ATT C -3'	19-mer	57.90%	54.9

2.3.2. Expression

Expression and SDS-PAGE analysis was carried out the way as described in the same-named chapter for the production of ErgA (2.2.3.).

2.3.3. Analysis

Determination of activity – Degradation of ergine

To determine activity of the enzyme a degradation experiment with ergine was carried out. The supernatants and pellets of all colonies of SDS-PAGE after an expression of 96 h were used. All samples were diluted 1:1 with sodium phosphate buffer and ergine (667 ppm stock) was added to gain a final concentration of 5 ppm. The volume of each sample was 1.5 ml. At the time of 0 h, 4 h and 24 h a sample was taken and the enzyme inactivated with 100% ACN (80 µl, gaining a final concentration of 20%). After centrifugation (15682 x g; room temperature; 10 min) each sample was transferred to a brown glass vial and measured with HPLC and fluorescence detection.

Determination of copy number via real time PCR

To get a rough estimation concerning copy number colonies 26, 28, 29 and 36 were streaked out on agar plates with increasing zeocin concentrations ranging from 250 µg/ml to 1000 µg/ml zeocin.

DNA extraction was carried out the same way as described for the determination of copy number of ErgA (chapter 2.2.4.). In general, also real time PCR was carried out the same way.

For the first step colony 28 was diluted to get a final concentration of 1 ng/µl and used at the temperatures 54.5 °C, 56.8 °C, 60.3 °C, 62.2 °C, 65.7 °C and 67.8 °C. All pairs of primers were used (Table 13). The different PCR-programs can be seen in Tables 14 to 16.

Table 13 Primer used for real time PCR.

Primer Name	Sequence	Length	Annealing Temperature [C°]	GC content [%]
ErgB1_fw	5'-ACTGCTGGTGTTGCTTTGGCTTT-3'	23	60.0	47.8
ErgB1_rev	5'-AGCTGGTTGAAGAGCAGTCATTGGA-3'	25	60.2	48.0
ErgB2_fw	5'-CCAGGTTACAGAAAGGACTTGG-3'	22	60.0	50.0
ErgB2_rev	5'-GTAGCACCTCTAGTTCTCAACTCGT-3'	25	59.6	48.0
ErgB3_fw	5'-CTTTCGGTATGGTTGCTAACTTG-3'	23	60.1	43.5
ErgB3_rev	5'-CTCAACGGATCTAGTCATTGGAC-3'	23	60.0	47.8
Arg4_fw	5'-TCCATTGACTCCCGTTTTGAG-3'	21	66.1	47.6
Arg4_rew	5'-TCCTCCGGTGGCAGTTCTT-3'	19	67.1	57.8

Table 14 PCR program for the first optimization of annealing temperature.

PCR-program			Melting curve	
T [°C]	Time [sec]		T [°C]	Time [min]
95	60		95	0.3
95	20	45 cycles	60	0.3
54-68	20		slow heating	20
72	40		95	0.3

To determine the exact annealing temperature colony 28 (1 ng/µl) was used with all primer pairs at the temperatures 60.1 °C, 60.8 °C, 61.2 °C, 62.0 °C, 62.8 °C, 63.6 °C, 64.4 °C, 65.1 °C and 66.0 °C.

Table 15 PCR program for determination of exact annealing temperature.

PCR-program			Melting curve	
T [°C]	Time [sec]		T [°C]	Time [min]
95	60		95	0.3
95	20	45 cycles	60	0.3
60-66	20		slow heating	20
72	40		95	0.3

To define PCR efficiency colony 28 was diluted to get the concentrations 1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl and 0.1 pg/µl. Each stock was used with primer pairs ErgB3 and ARG4. Additionally, for each primer pair a negative control was used replacing the template DNA with water.

Table 16 PCR program for determination of transformation of PCR efficiency and actual quantification.

PCR-program			Melting curve	
T [°C]	Time [sec]		T [°C]	Time [min]
95	60		95	0.3
95	20	40 cycles	60	0.3
62	60		slow heating	20
			95	0.3

For the actual quantification the same program for PCR and melting curve was used as in the step before (table 16). Genomic DNA of colonies 26, 29, 36, 20, 25, 30 and the DNA of native *P. pastoris* CBS7435 was diluted as colony 28 in the step before with the exception that no dilution of 0.1 pg/µl was made. Each colony was used for PCR with both primer pairs. Additionally, for each primer pair a negative control was done. As a positive control 1 ng/µl DNA of colony 28 was used with the primer pair ErgB3.

Furthermore, as a control an agarose gel electrophoresis was carried out with the samples ErgB CFU26, ErgB CFU36, ErgB CFU20, PTC ErgB, ARG4 CFU26, ErgB *P. pastoris*, ARG4 *P. pastoris*.

2.4. Expression of ErgB in different *Escherichia coli* expression strains

Production of chemically competent *E. coli* cells

Chemically competent cells were produced as described in protocol 25 (Sambrook and Russell, 2001). The strains used were *E. coli* Rosetta 2(DE3), HMS174(DE3), Origami 2(DE3) and Tuner(DE3).

Before use the chemically competent cells were thawed on ice and 1 µl of pUC18 (0.1 ng) was added to each tube to determine transformation efficiency. 10 ng pUC18 were used for transformation into *E. coli* Rosetta 2(DE3) and *E. coli* HMS174(DE3). One additional tube of each strain of competent cells was used as a negative control. The samples were incubated on ice for 5 min and heat shocked in a 42 °C water bath for 30 s. Afterwards they were incubated on ice for 2 min. 80 µl S.O.C-medium was added to each tube. The samples were incubated at 37 °C, 160 rpm for 1 h. All samples that contained DNA were plated out on LB-plates containing 100 µg/ml ampicillin (10 µl; 20 µl; 50 µl aliquots). Each negative control (50 µl) was plated out on one LB-plate containing 100 µg/ml ampicillin. Plates were incubated at 37 °C overnight.

Transformation of *erg*B pET28a(+) in different *E. coli* expression strains

5 ml LB-medium was each inoculated with Amidase T3-his_Klon #4 *E. coli* DH10B and pET28a(+) *E. coli* XL1-Blue and incubated at 37 °C, 150 rpm, overnight. After isolation of DNA with the QIAprep® Spin Miniprep Kit concentration was measured with nanodrop.

Chemically competent *E. coli* Rosetta 2(DE3), *E. coli* Origami 2(DE3), *E. coli* Tuner(DE3), *E. coli* BL21(DE3)pLysS and *E. coli* HMS174(DE3) were thawed on ice. For each strain there was one negative control with no DNA, one control with isolated pET28a(+) DNA and one sample with *erg*B pET28a(+). For the transformation in Rosetta 2(DE3) 10 ng of *erg*B pET28a(+) and 10 ng of pET28a(+) as control were used. For all other strains 21.2 ng *erg*B pET28a(+) and 29.95 ng pET28a(+) were used. All samples were incubated on ice for 5 min, heat shocked in a water bath at 42 °C for 30 sec and again incubated on ice for 2 min. 80 µl S.O.C-medium was added and the samples incubated at 37 °C, 150 rpm for 1 h. 10 µl, 20 µl

and 50 µl aliquots of each sample were plated out on LB-plates containing 50 µg/ml kanamycin. The exception was the strain Origami 2(DE3). Of this strain 10 µl, 20 µl, 50 µl and 100 µl aliquots were plated out on LB agar plates with 30 µg/ml kanamycin. The plates were incubated at 37 °C overnight. After transformation a few colonies per strain, which should contain the plasmid *erg*B pET28a(+) and one with the empty vector were picked and the plasmids isolated with the QIAprep® Spin Miniprep Kit. 2 µl isolated plasmid, 6.5 µl distilled water, 1 µl 10x buffer blue and 0.5 µl restriction enzyme *Apa*I were mixed together and incubated in a water bath at 37 °C for 2 h. All samples plus the original isolated plasmids after miniprep isolation were applied onto an agarose gel. Electrophoresis was carried out at 200 V. If the colony contained the vector *erg*B pET28a(+) three bands (4321 bp, 2010 bp and 568 bp) would be visible, an empty vector would be cut once (5369 bp).

Expression in *E. coli* Rosetta 2(DE3), HMS174(DE3), BL21(DE3) and BL21(DE3)pLysS

5ml LB-medium containing 50 µg/ml kanamycin was each inoculated with colonies from the transformations of *erg*B pET28a(+) and pET28a(+) in the different *E. coli* strains. Furthermore, the inoculation of *erg*B in *E. coli* BL21(DE3) (transformation done by another assistant of Biomin) was carried out. These pre cultures were incubated at 37 °C, 160 rpm, overnight. 600 µl of the cultures were transferred to each 29.4 ml fresh LB-medium + 50 µg/ml kanamycin and incubated at 37 °C and 160 rpm until an OD_{600} between 0.6 and 1.0 was reached. Expression was induced with 600 µl IPTG-solution (50 mM) to gain a final concentration of 1 mM. The samples were incubated at 37 °C and 160 rpm for 3 h. 10 ml aliquots were harvested in 15-ml-Falcon tubes via centrifugation (20 min; 4500 x g; 4°C). 1 ml aliquots were centrifuged in 1.5 ml Eppendorf tubes (5 min; 16600 x g; 4 °C). Pellets were stored at -20 °C. All these steps were also done for the expression of *E. coli* BL21(DE3)pLysS except that the used media for this strain contained 50 µg/ml kanamycin and 34 µg/ml chloramphenicol.

Expression in *E. coli* Tuner(DE3)

Expression was carried out the same way as for the *E. coli* strains Rosetta 2(DE3), HMS174(DE3) and BL21(DE3) with the modification that different concentrations of IPTG were used for induction, namely 25 µM, 100 µM, 500 µM and 1 mM.

Expression in *E. coli* ArcticExpress(DE3)

Transformation was done by another assistant of Biomin. The actual expression experiment was carried out three times. 5 ml LB-medium containing 20 µg/ml gentamycin and 50 µg/ml kanamycin were inoculated twice with 4 colonies of *E. coli* ArcticExpress(DE3) containing *erg*B (Amidase T3-his_Klon #4-1, Amidase T3-his_Klon #4-2, Amidase T3-his_Klon #4-3, Amidase T3-his_Klon #4-4;) and also with one colony containing the empty vector pET28a(+) and incubated at 37 °C, 150 rpm, overnight. 600 µl of each pre culture were transferred to 29.4 ml fresh LB-medium without antibiotics. The cultures were incubated at 30 °C, 170 rpm, for 3 h. The cultures were transferred to a cooling incubator to be incubated at 14.5 °C and 170 rpm for 10 min. Expression of cultures of all 4 colonies were induced with 60 µl IPTG (50 mM) to get a final concentration of 0.1 mM. The samples were incubated at 14 °C and 170 rpm for 24 h. 10 ml aliquots were harvested via centrifugation, 10 min, 4500 x g, 4 °C. 1 ml aliquots were harvested with centrifugation (5 min, 16600 x g, 4 °C). Additionally, an OD_{600}-measurement and a microscopic analysis were carried out. The second replication of this experiment was carried out the same way as described before with the difference that expression was additionally induced with 600 µl IPTG (50 mM) gaining a final concentration of 1 mM.

Due to the small size of the harvested pellets after expression a few optimisations for a third repeat of this experiment were established. Throughout the experiment OD_{600} measurements and microscopic observations were carried out to have an overview and a control of the growth. Fresh LB-medium was inoculated with the pre cultures to gain an initial OD_{600} of approximately 0.3. Before induction all samples containing the gene coding for ErgB were grown up to an OD_{600} between 0.8 and 1.0. Each colony was used three times; for induction with 0.1 mM IPTG, for induction with 1 mM IPTG, and for a sample without induction. During all incubation periods the shaking was raised to approximately 200 rpm.

Lysis of harvested cells and SDS-PAGE

The harvested cells from 1 ml expression culture were resuspended in 200 ml resuspension buffer. 50 µl lysozyme solution (10 mg/ml) was added and the mix vortexed and incubated at 37 °C for 5 min. 750 µl 0.5% Triton X-100 was added and the samples again vortexed and incubated at 37 °C for 5 min. Lysis was checked by light microscopy. Each sample was divided into sample A, B and C.

Sample A: 50 µl lysate was mixed with 50 µl 2x sample buffer.

Sample B: 50 µl lysate was centrifuged (18188 x g; 4 °C; 20 min) and 50 µl of the supernatant was mixed with 50 µl 2x sample buffer.

Sample C: 50 µl lysate was centrifuged (18188 x g; 4 °C; 20 min) and the pellet resuspended in 50 µl 10 mM Tris-HCl (pH=8.2) and 50 µl 2x sample buffer was added. Afterwards all samples were heated up to 96 °C for 5 min, they were applied onto polyacrylamide gels and SDS-PAGE was carried out with 200 V.

3. Results

3.1. Expression of ErgA

3.1.1. Molecular cloning

Preparation of *erg*A pMA-T

Transformation of *erg*A pMA-T into *E. coli* DH10B worked with an efficiency of $1{,}629*10^9$ transformants per µg DNA. Figure 6 shows the result of the control restriction digest of ergA pMA-T with *Sfi*I after miniprep isolation.

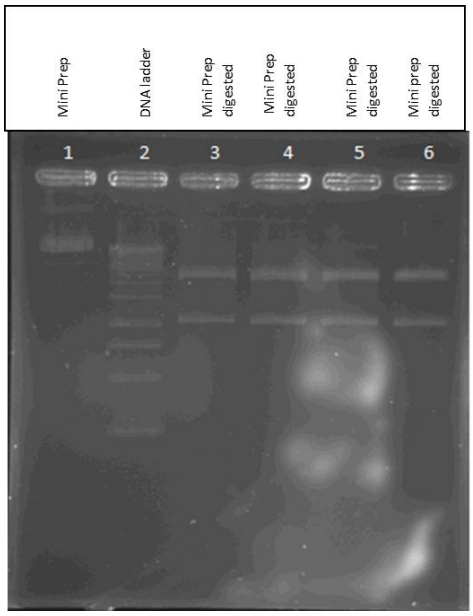


Figure 6 Restriction digest with *Sfi*I. Lane 1: undigested miniprep of *erg*A pMA-T; Lane 2: 1 kb DNA ladder; Lane 3 to 6: miniprep of *erg*A pMA-T after digestion with *Sfi*I with the fragment sizes of 1025 bp and 3020 bp.

Preparation of pGAPZα C

As expected control digest of isolated pGAPZα C with the restriction enzyme *Apa*LI resulted in two fragments with 2500 bp and 670 bp (Figure 7).

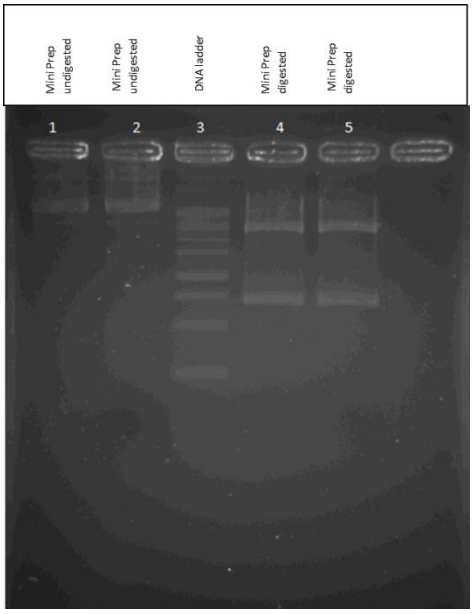


Figure 7 Control digest of isolated pGAPZα C. Lane 1 and 2: undigested miniprep pGAPZα C; Lane 3: 1 kb DNA ladder; Lane 4 and 5: pGAPZα C digested with *Apa*LI;

Digestion of pGAPZα C and *erg*A pMA-T with *Xho*I and *Xba*I

After restriction digest of *erg*A pMA-T and pGAPZα C with *Xho*I and *Xba*I the fragment of the 960 bp and the band in lane 4 at 3060 bp were cut out to gain the cut vector and insert ready for ligation (Figure 8).

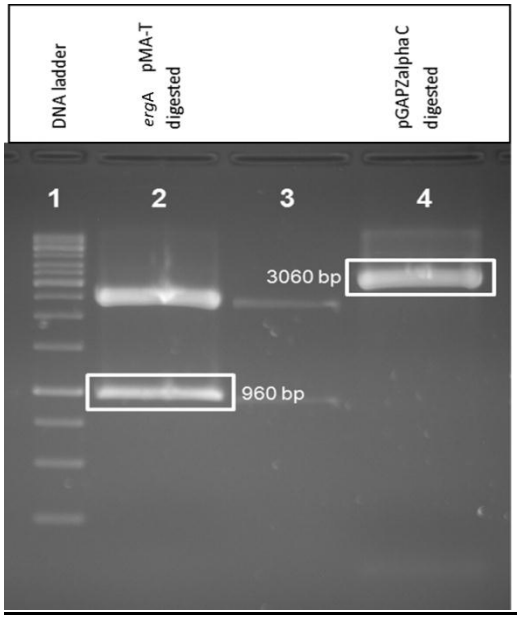


Figure 8 Preparative gel electrophoresis of digested *erg*A pMA-T and pGAPZα C. Lane 1: 1 kb DNA ladder; Lane 2: digested *erg*A pMA-T; Lane 4: digested pGAPZα C

After purification the concentrations were measured spectrophotometrically as well as with gel electrophoresis. The brightness and size of the appropriate band were compared to one of

the bands of the DNA ladder to estimate the concentration and allow further work e.g. CIPing and ligation. Measured concentrations and mean values can be seen in Table 17. (Although the differences between the determined concentration values between the two different measuring methods were quite big, the mean values were used for all further experiments and calculations.)

Table 17 Summary of concentration measurements of insert and vector.

Sample	Concentration [ng/µl] nanodrop	260/280 with nanodrop measurement	Concentration [ng/µl] gel electrophoresis	Mean value [ng/µl]
Insert ErgA	53.40	1.79	72.00	62.70
Vector pGAPZα C	28.60	1.50	126.00	77.30

Only at transformation of *erg*A pGAPZα C into *E. coli* DH10B it was found out that ligation had worked because only on the sample plates that contained antibiotics transformants were visible. Colonies were picked for cultures for further miniprep isolation and digested with *Xho*I and *Xba*I to figure out if the right plasmid plus insert has been transformed into the cell (Figure 9).

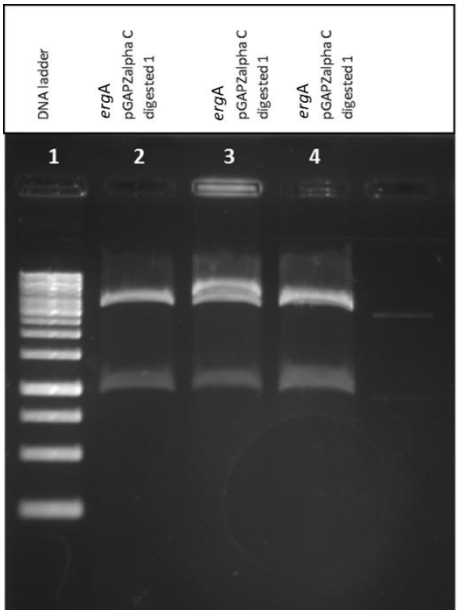


Figure 9 Restriction digest of *erg*A pGAPZα C miniprep samples. Lane 1: 1 kb DNA ladder; Lane 2 to 4: samples 1 to 3 digested with *Xho*I and *Xba*I showing fragments of the sizes 3060 bp and 970 bp.

Sample 2 has been chosen for further experiments. To eliminate all doubts that the plasmid of sample 2 could be contaminated with other plasmid parts, sample 2 was further observed with different restriction digests which proved that it is the right vector (pGAPZα C), the right

insert (*erg*A) and that the insert is ligated into the vector the right way as can be seen in figure 10.

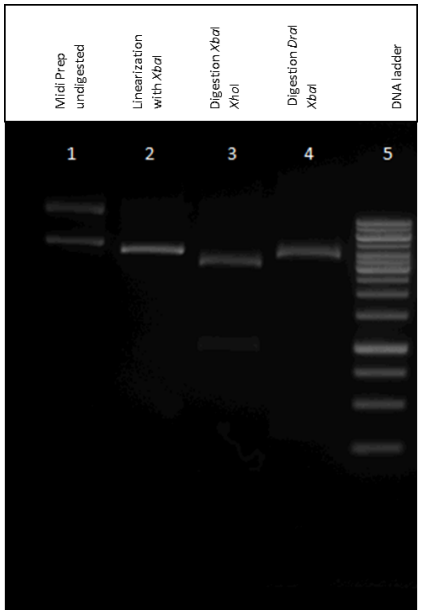


Figure 10 Restriction digest of *erg*A pGAPZα C as a control. Lane 1: Undigested *erg*A pGAPZα C Lane 2: Linearization getting a fragment of 4020 bp; Lane 3: Restriction digest gaining fragments of the sizes 3060 bp and 960 bp; Lane 4: Fragments of the sizes 3723 bp and 297 bp (this band was only visible after strong obscuration); Lane 5: Gene Ruler ™ 1 kb DNA ladder

The sequencing process of ergA pGAPZα C at Sequiserve proved that the sequence of the ligated insert and its reading frame was right (Figure 11). As a consequence, the plasmid containing *erg*A was used for further transformation into *P. pastoris*.

5'3' Frame 1
LLFINTTIASIAAKEEGVSLEKRMPLVVLSDGTRIHVETSGNGVPALVPCVGSSVPFERTFGEELKT DIQYNFVEVRGTSRSDGEPSEVASLDRISDDLEEVRQLLGLDKVIALGQSRNGMMAAHYAQKY PNSVLHLVTIGTPASLSMIKNEEYWNAFADDERKRLRAENDAAMEREGLLDLDNLNTAEKIVRL FDLEGAVYFYDPTTLMNDWWDASLLSRTFEVVMASNMGWADFDLVQTLQNSDVPAFVTFG KYDFMVSPLPKPGNPVDGKAGLFEDIPGVRVEVFEKSGHFPYWEQEQEFARRYRDWVATLPES AVRAAEAMTPNGIRQHHHHHH**Stop**SRTKTHLRRGS

Figure 11 Protein sequence of sequencing results of *erg*A pGAPZα C. The underlined sequence shows the enzyme and its histidine tag. Translation was done with ExPaSy (http://web.expasy.org/translate/).

3.1.2. Transformation

For transformation a great amount of DNA was needed. Therefore, midiprep isolation of ergA pGAPZα C was carried out. After isolation of ergA pGAPZα C out of *E. coli* DH10B the reliability of the isolation process with midiprep kit was observed via gel electrophoresis.

Gel electrophoresis of midiprep samples showed that isolation worked and that there were no losses of DNA during protein precipitation (Figure 12).

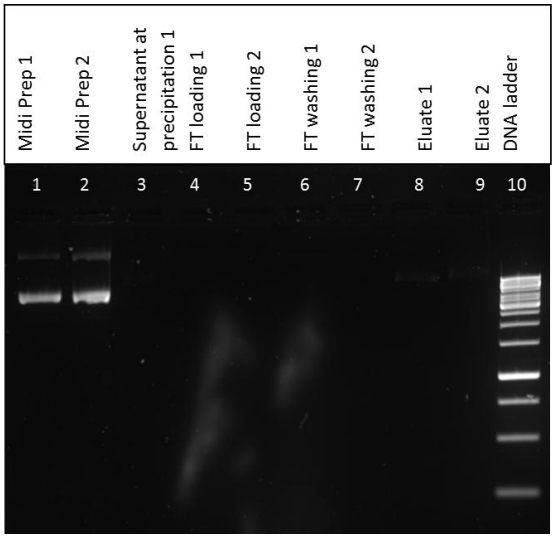


Figure 12 Midiprep Isolation. Lane 1 and 2: sample 1 and 2 of isolated *erg*A pGAPZα C; Lane 3: Supernatant after precipitation of protein; Lane 4 and 5: Flow Through after loading of the column; Lane 6 and 7: Flow through after washing of the column; Lane 8 and 9: Eluate of sample 1 and 2; Lane 10: 1 kb DNA ladder;

With the gel electrophoresis (Figure 13) that was carried out after linearization it could be seen that linearization was not complete. Linearization led to a band at a height of 3060 bp. Nevertheless the linearized DNA was purified. The measured concentration of linearized plasmid was 127.4 ng/µl (280/260: 1.85). Since the DNA was dissolved in 30 µl of buffer the resulting amount of plasmid was 3.822 ng.

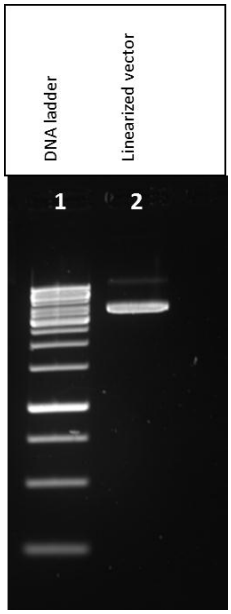


Figure 13 Linearized vector *erg*A pGAPZα C, control for complete linearization. Lane 1: 1 kb DNA ladder; Lane 2: linearized vector (3060 bp);

However, transformation of ErgA into *P. pastoris* worked. Evaluating the plates of transformation after incubation 57 big colonies could be counted on the sample plates as well as numerous small cells. The countless small cells grew on the sample as well as on the negative control plates. That phenomenon is called superposition, which means that even colonies that do not contain a certain plasmid with a certain resistance to an antibiotic can grow on the agar plate because a great volume was used at plating out the cells after transformation. This was also the reason why only the bigger colonies were chosen for further steps such as colony PCR because superposition could be excluded. 10 colonies were used for the colony PCR. 7 out of 10 colonies showed the appropriate band at 1200 bp and can therefore be used for expression experiments of ErgA (Figure 14).

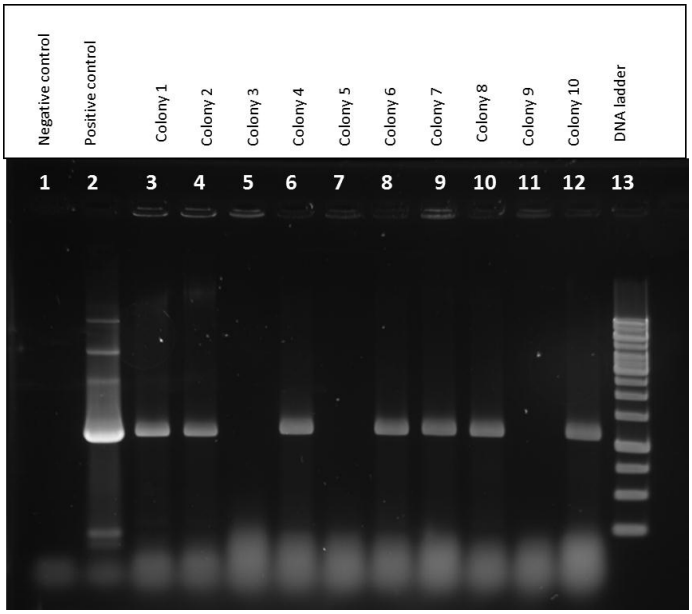


Figure 14 Screening after colony PCR. Lane 1: Negative control; Lane 2: Positive control *erg*A pGAPZα C; Lane 3 to Lane 12: amplified colonies 1 to 10 that were picked after transformation of *erg*A pGAPZα C into *P. pastoris*; Lane 13: 1 kb DNA ladder;

3.1.3. Expression

All positive colonies of colony PCR (Figure 14) were chosen for the expression experiment. The gels of SDS-PAGE of expression of ErgA showed that the enzyme (35 kDa) was expressed by every single colony, because a band at the appropriate height could be seen in all the supernatants (Figures 15 to 20). For each colony the supernatants from 24 h to 96 h plus the pellet after incubation of 96 h were applied onto the gels. Colony 6 and 7 seemed to have expressed the enzyme only at an incubation time of 96 h (Figure 17). Although the enzyme should be secreted there is some ErgA in each pellet. Best expression results were

gained with colony 1 and 2 but with amounts lower than 0.1 mg/ml. Based on the results of determination of activity of ErgA expressed with colony 1, this colony was chosen for a second expression experiment and determination of activity together with colonies 8 and 10 (Figures 18 to 20).

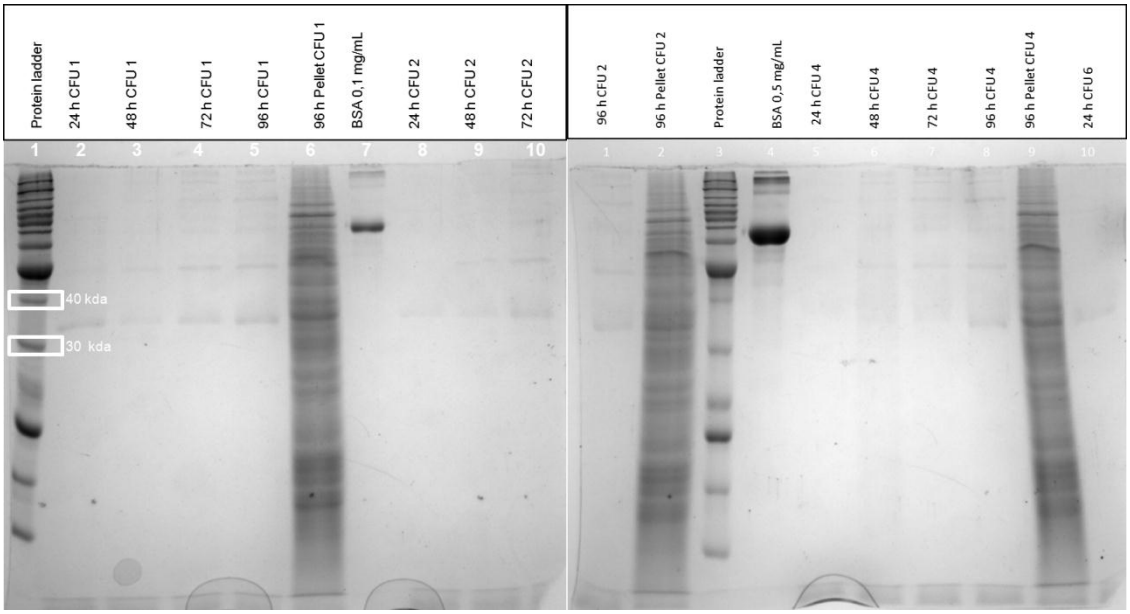


Figure 15(on the left) SDS-Gel of expressed ErgA. Lane 1: Bench Mark Protein Ladder; Lane 2 to 6: colony 1; Lane 7: BSA 0.1 mg/µl; Lane 8 to 10: colony 2;

Figure 16 (on the right) SDS-Gel of expressed ErgA. Lane 1 and 2: colony 2; Lane 3: BenchMark Protein Ladder; Lane 4: BSA 0.5 mg/ml; Lane 5 to 9: colony 4; Lane 10: Supernatant CFU 6 after 24 h;

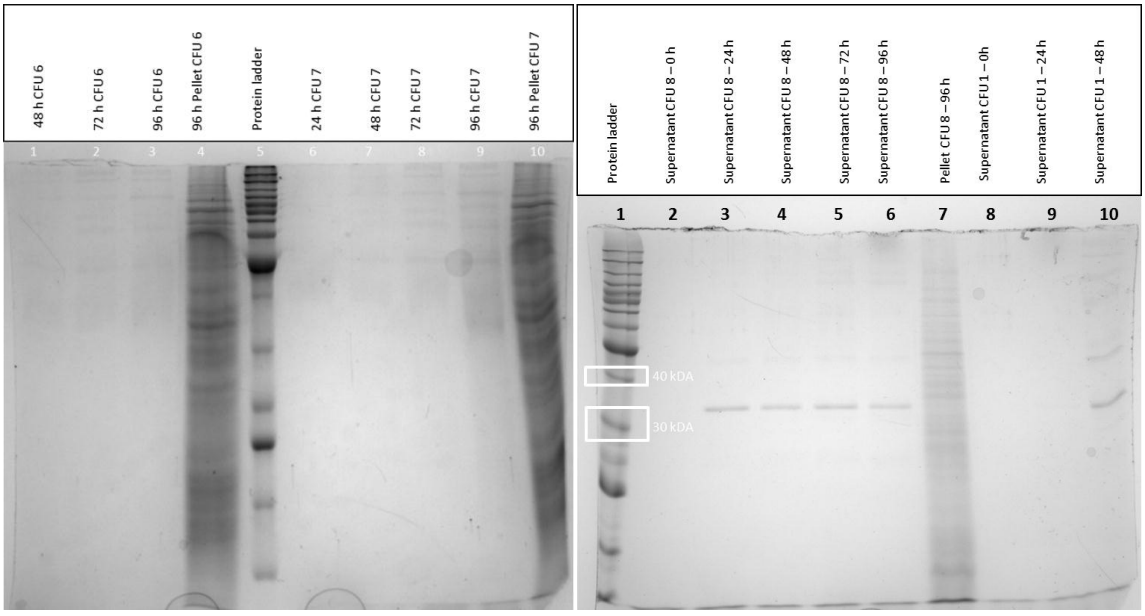


Figure 17 (on the left) SDS-Gel of expressed ErgA. Lane 1 to 4: colony 6; Lane 5: BenchMark Protein Ladder; Lane 6 to 10: colony 7;

Figure 18 (on the right) SDS-PAGE Gel of supernatants and pellets after expression. Lane 1: Protein ladder; Lane 2 to 7: colony 8 from 0h to 96 h plus pellet; Lane 8 to 10: Colony 1 from 0 h to 48 h;

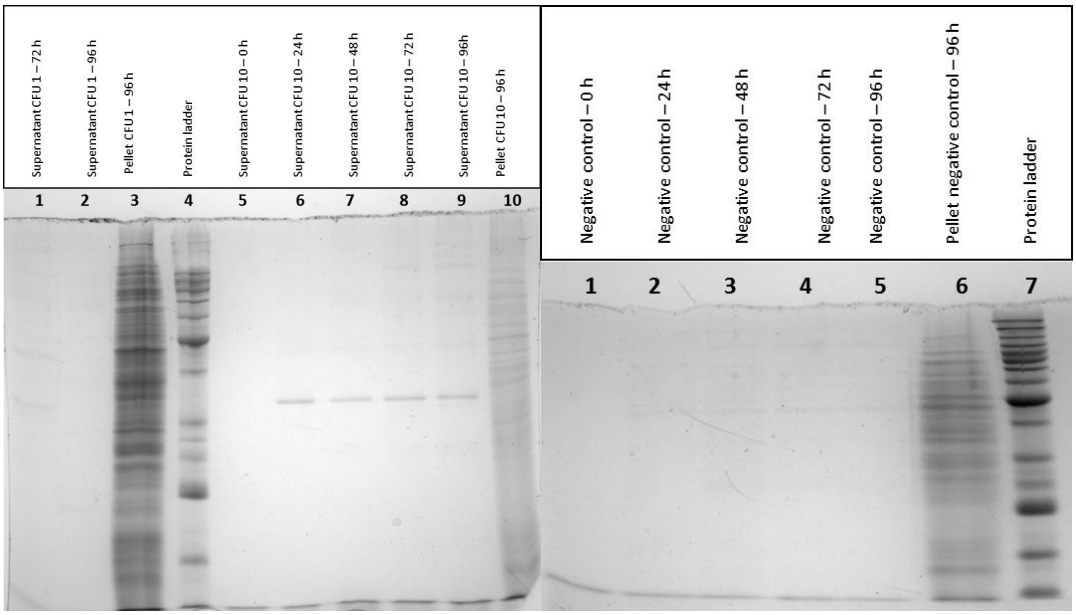


Figure 19 (on the left) SDS-PAGE Gel of supernatants and pellets of expression. Lane 1 to 3: colony 1 from 72 h to 96 h plus pellet; Lane 4: Protein ladder; Lane 5 to 10: colony 10 from 0 h to 96 h plus pellet;

Figure 20 (on the right) SDS-PAGE Gel 3 of supernatants and pellets of negative control at expression. (Lane 1 to 6). Lane 7: Protein ladder;

3.1.4. Analysis

Determination of activity

Approach 1

All results concerning ergotamine and ergine show the sum of both epimers (ergotamine + ergotaminine; ergine + erginine). The determined activity of ErgA expressed by colony 1 after 96 h of expression was not as high as expected, degradation only worked at a dilution of 1:100 (Figure 21).

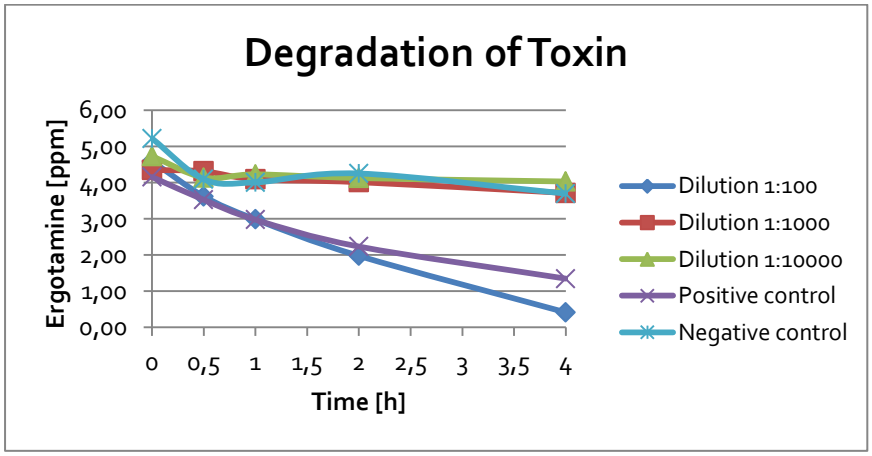


Figure 21 Summary of degradation of ergotamine with expressed ErgA of colony 1.

Figures 22 and 23 show the conversion of ergotamine into ergine and unknown intermediate by the expressed ErgA diluted 1:100 and the positive control (ErgA, purified after expression in *E. coli* ArcticExpress(DE3); diluted 1:10000).

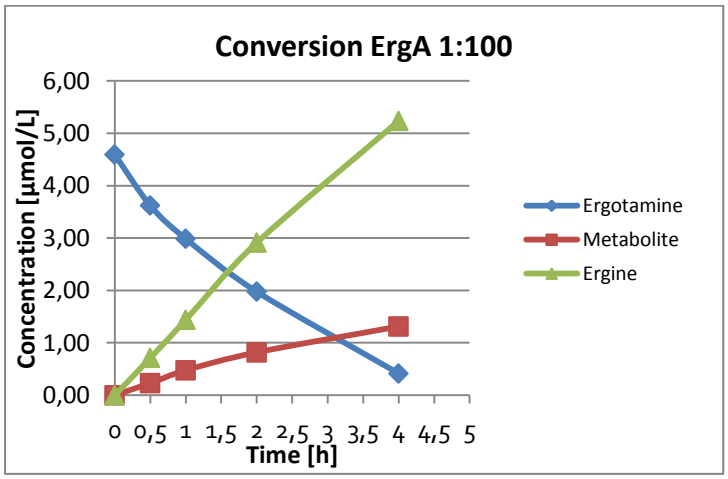


Figure 22 Degradation of ergotamine with expressed ErgA (colony 1) diluted 1:100. Concentration of products, educts and metabolites throughout the process.

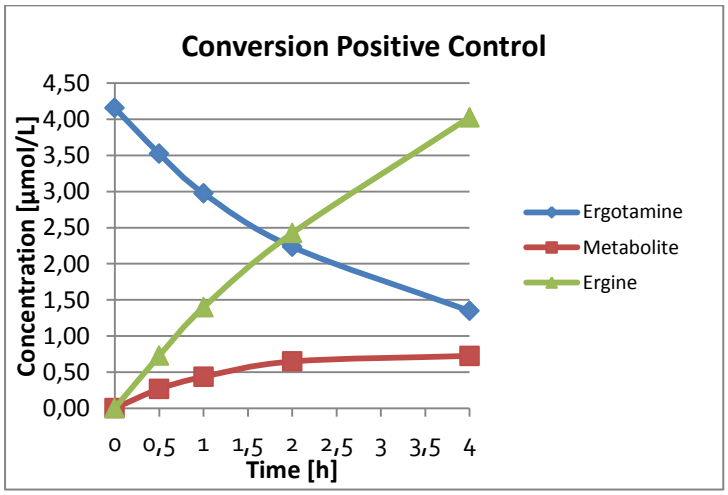


Figure 23 Degradation of ergotamine with purified ErgA (1:10000) as a positive control.

Approach 2

The second approach was done to find out if the expressed enzyme of colony 1 is active throughout the incubation process of expression or if there are changes in activity. The results of the degradation experiment showed that the enzyme was active throughout the expression process. However, after expression for 48 h no degradation of ergotamine could be seen. Furthermore, no ergotamine, ergotaminine or their degradation products could be detected for the negative control. First it was thought that a mistake concerning addition of toxin or enzyme has happened but following experiments with other colonies also showed that activity after an expression of 48 h has less or even no activity. Overall, the enzyme showed

activity with little deviations throughout the expression procedure. Hence the conditions during incubation did not change or if they changed they have not had any influence on the enzymes activity. Yet the enzyme after an expression for 96 h showed best activity (Figure 24). This also means that the expressed enzyme was stable up to an incubation duration of 96 h. The results for the negative control were not evaluable.

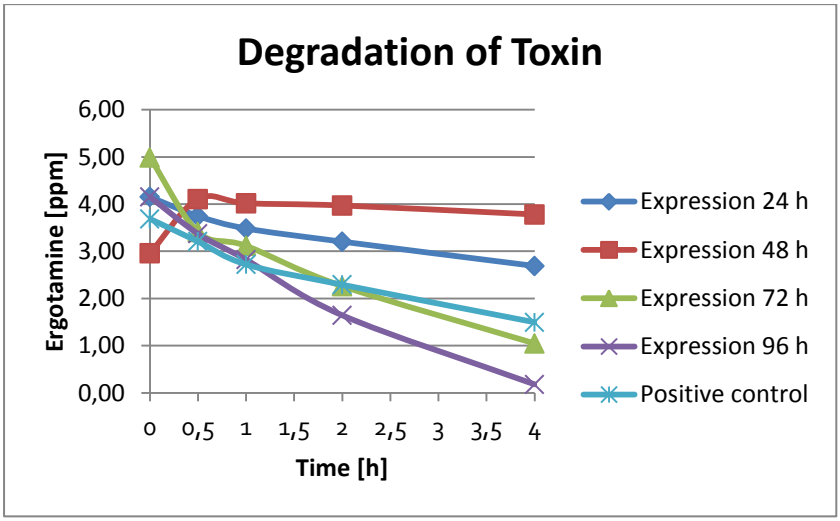


Figure 24 Degradation of ergotamine with expressed ErgA colony 1 after different expression periods.

Figure 25 (A to E) shows the conversion of ergotamine into ergine and unknown intermediate for the expressed ErgA after all time points of incubation and the positive control. Comparison of all figures with each other also suggests that the highest activity of expressed ErgA was gained after an incubation of 96 h.

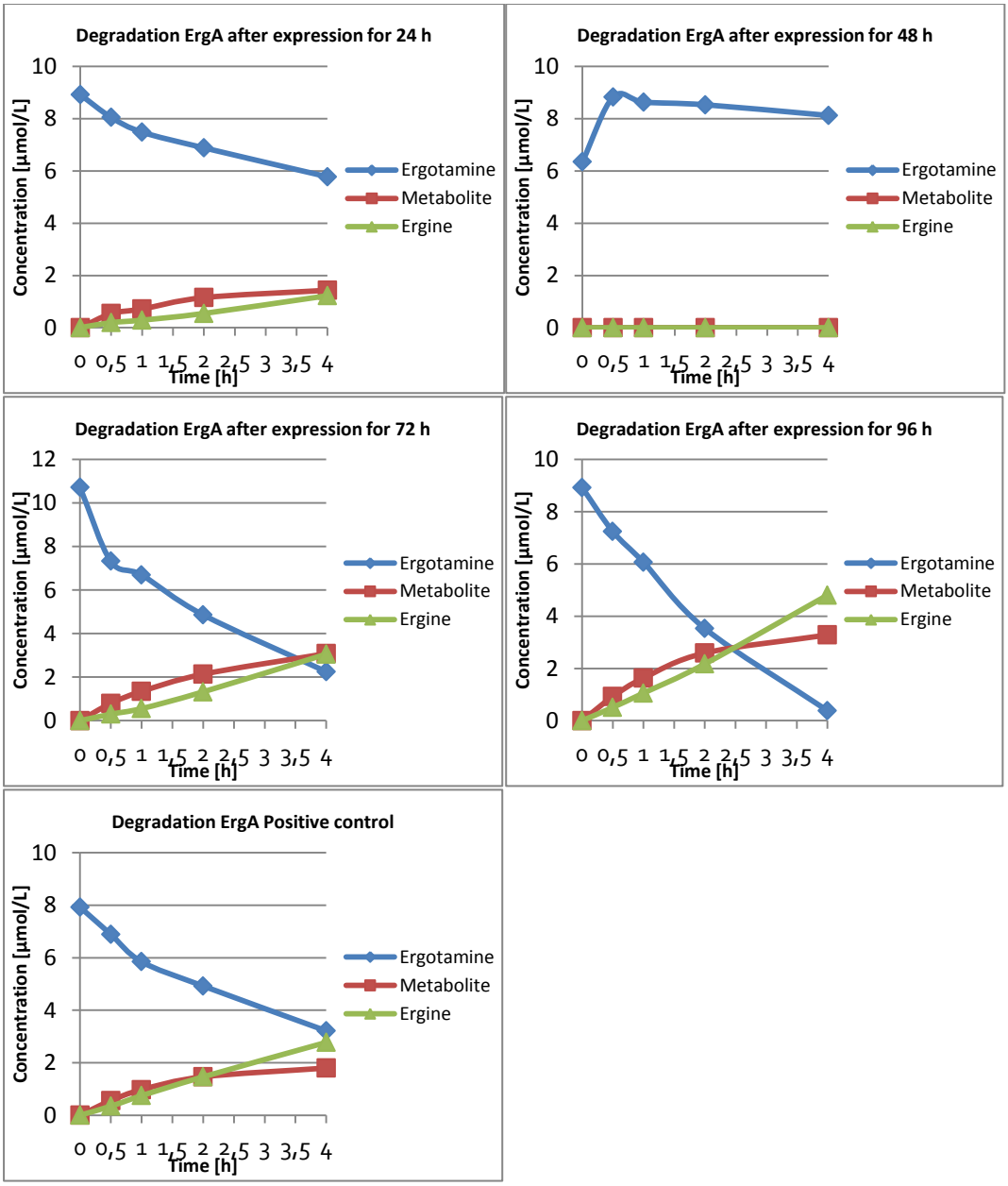


Figure 25 A, B, C, D and E Degradation of ergotamine with ErgA (colony 1) after different incubation periods at expression plus one positive control; Concentration of educts, products and metabolites of all samples.

Approach 3

The expression and degradation experiment was carried out with colonies 1, 8 and 10. SDS-PAGE (Figure 18 and 19) showed that colonies 8 and 10 expressed more ErgA (35 kDa) than colony 1. Concerning the amount of expressed enzyme of colonies 8 and 10, there is no increase from 24 h expression to 96 h expression visible. To have a better idea of the amount of expressed ErgA a degradation experiment was carried out. The contaminated culture of colony 1 showed no activity concerning the degradation of the toxin ergotamine for all samples taken at different time points of expression. This contamination maybe has an

influence on the expression of the enzyme and the activity itself. Colonies 8 and 10 both expressed enzymes that show good activity. However, the expressed enzymes by *P. pastoris* (diluted 1:100) are less active than the positive control that has been diluted 1:10000. Only at an expression of 48 h both colonies had a lack of activity. This might have something to do with the conditions during expression. Already on the gels of SDS-PAGE (Figure 18 and Figure 19) the bands for colonies 8 and 10 after an expression of 48 h look slightly lighter than the bands at an expression of 24 h and all other time points. Since the results of colony 1 in this experiment were not reliable the results from the previous described experiment (Figure 24 and Figure 25) were used to make slight comparisons between all three colonies. The degradation experiment showed that ErgA after 24 h expression of colony 8 and 10 degrades nearly all ergotamine. Throughout the expression (apart from 48 h) the enzyme is active and degrades nearly all the toxin. Having a look at colony 1 it shows that the enzyme after an expression of 48 h is also inactive. Best activity for colony 1 is gained after an expression of 96 h. From 24 h expression up to a 96 h expression the activity rises. Overall, ErgA is more active when expressed with colony 8 or 10 (Figure 26 and Figure 27).

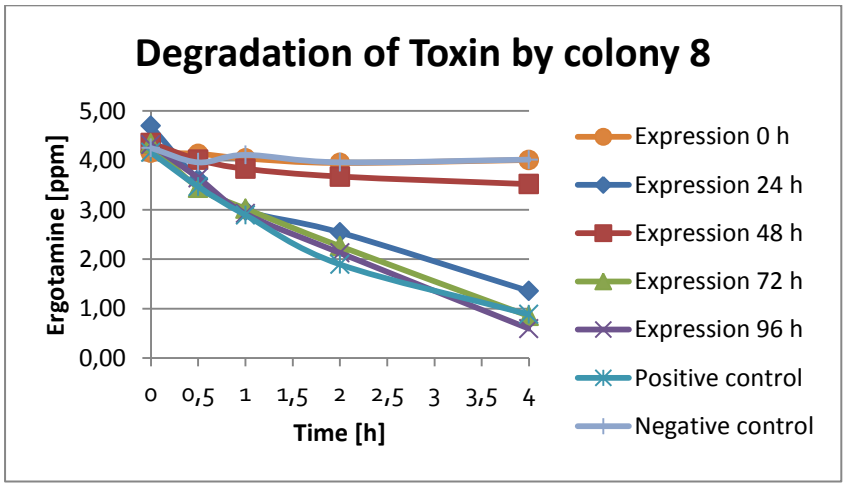


Figure 26 Degradation of ergotamine with expressed ErgA of colony 8 at certain time points of expression.

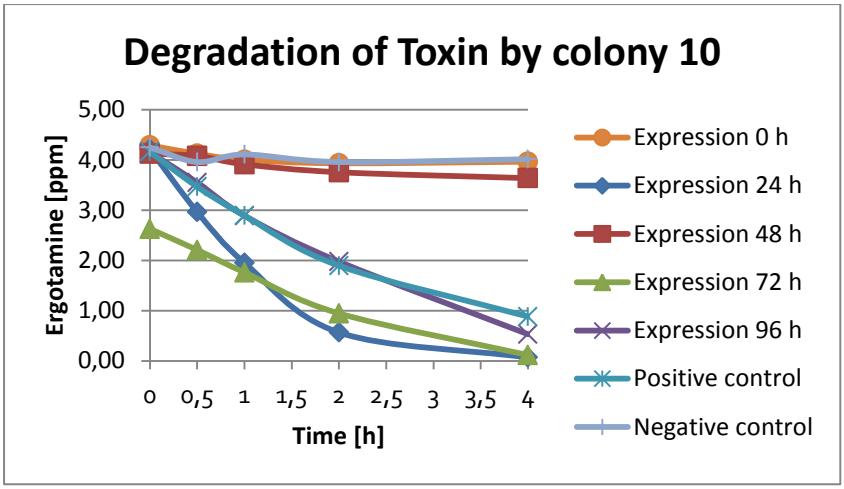


Figure 27 Degradation of ergotamine with expressed ErgA of colony 10 at certain time points of expression.

Determination of copy number via Real Time PCR

Table 20 displays the growth of all colonies used for the expression of ErgA on agar plates with increasing zeocin concentrations. Colonies 8 and 10 were the only two colonies growing on a zeocin concentration of 1000 µg/ml putting in question if these two colonies contained a higher copy number of integrated plasmids then the other colonies.

Table 18 Growth of colonies on increasing zeocin concentrations.

Growth of *P. pastoris* on different concentrations of Zeocin			
Sample	250 µg/ml Zeocin	500 µg/ml Zeocin	1000 µg/ml Zeocin
ErgA 1	x*	x*	
ErgA 2	x	x*	
ErgA 4	x	x*	
ErgA 6	x	x*	
ErgA 7	x	x*	
ErgA 8	x	x	x*
ErgA 10	x	x	x

* growth of cells was visible but not good

To determine the number of integrated plasmids real time PCR was carried out. Prior to this quantification the genomic DNA of all samples was extracted. The concentration measurements after DNA extraction showed the amounts of DNA that were gained. It seemed not a lot but considering the fact that a cell also contains lots of different RNAs and that for extraction RNaseA was used, degrading the RNA, the gained amount of extracted DNA seems plausible. DNA amounts seemed little and for a few samples the values 260/280 were far off 1.8 – 1.9. The gel electrophoresis (Figure 28 and Figure 29) proofed that all samples

contained extracted DNA. Some bands on the gel are less bright or longer than the others. This effect is caused by shearing, which naturally happens during the extraction process. However, all samples were chosen for copy number determination via real time PCR. In figure 28 bands were only visible after a strong obscuration, which cannot be seen here.

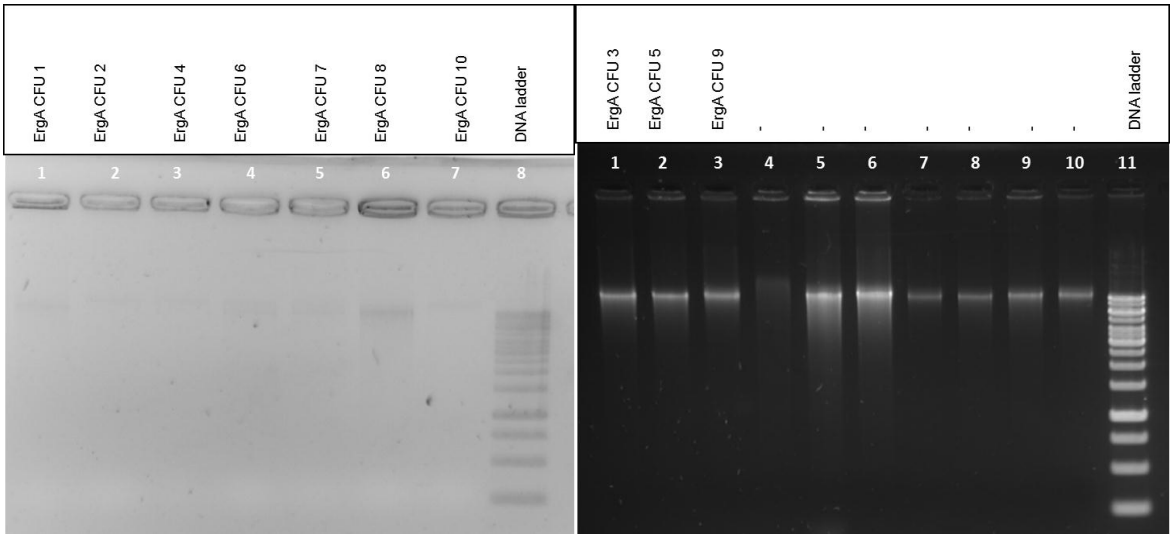
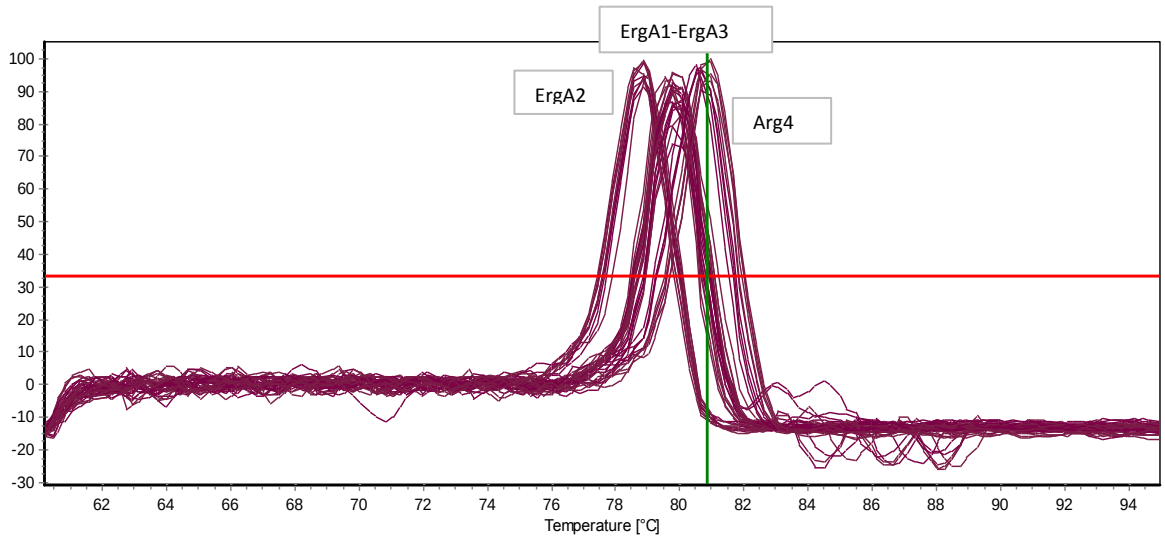


Figure 28 (on the left) gel electrophoresis after DNA extraction. Bands for all samples were only visible after a strong obscuration. Lane 1: CFU 1; Lane 2: CFU 2; Lane 3: CFU 4; Lane 4: CFU 6; Lane 5: CFU 7: Lane 6: CFU 8; Lane 7: CFU 10; Lane 8: DNA ladder;

Figure 29 (on the right) gel electrophoresis after DNA extraction of ergA pGAPZα C in *P. pastoris*. Lane 1: CFU 3, Lane 2: CFU 5: Lane 3: CFU 9; Lane 4 - 10: not relevant for this experiment; Lane 11: DNA ladder;

To develop an assay for gaining the results of copy number a few steps had to be carried out. First of all the optimization of annealing temperature was carried out in two steps. Based on the lowest Ct-value and the highest fluorescence (data not shown) the determined annealing temperature for primer pair ARG4 and the chosen primer pair ErgA1 was 63 °C. Another consideration for this result was the melting curves of the products of all primer pairs (Figure 30). Melting temperature of the amplified products should be similar but still a difference of at least 1 °C should be visible. For the third step, determination of PCR-efficiency, the chosen primers were used on one colony of which a standard with different concentrations was made. PCR-efficiency was determined to figure out if the used set of primers was working properly with the result that the efficiency of the chosen primers was close to or even higher than 100% (Table 19). This step was the last part of establishing the assay. Therefore, the actual quantification was carried out. The amplified products of ErgA1 were measured referring to the product of ARG4 primers amplifying parts of the *Pichia* gene ARG4. ARG4 is only present once in the whole *Pichia* genome and could therefore be used

as a reference for this experiment. The curves for the ARG4 products (1 ng/µl DNA) always appeared at a Ct-value (cycle threshold) around 19. The curves for the products of ErgA1 also appeared around that value ±1 (Figure 31). Usually a difference of one Ct means the double amount of DNA. This would mean if ARG4 comes at a Ct of 19 and ErgA1 at a Ct of 20 the copy number of ErgA would be 2 because the reference gene ARG4 is only a single copy. In some measured samples though ErgA1 products appeared one Ct value later than ARG4. Since there must be at least one copy of ErgA in the genome it is considered that it cannot be distinguished between one or two copy numbers. It is thought that when higher copy numbers of ErgA are reached that a difference of more Cts can be seen and therefore, the result of a higher copy number is stated. Overall, it can be said that all colonies used for real time PCR contained at least one copy of the integrated plasmid. A higher copy number was not detected.



Threshold: 33%

Figure 30 Melting curves of amplified products of all primer pairs.

Table 19 PCR efficiencies for determination of copy number of ErgA.

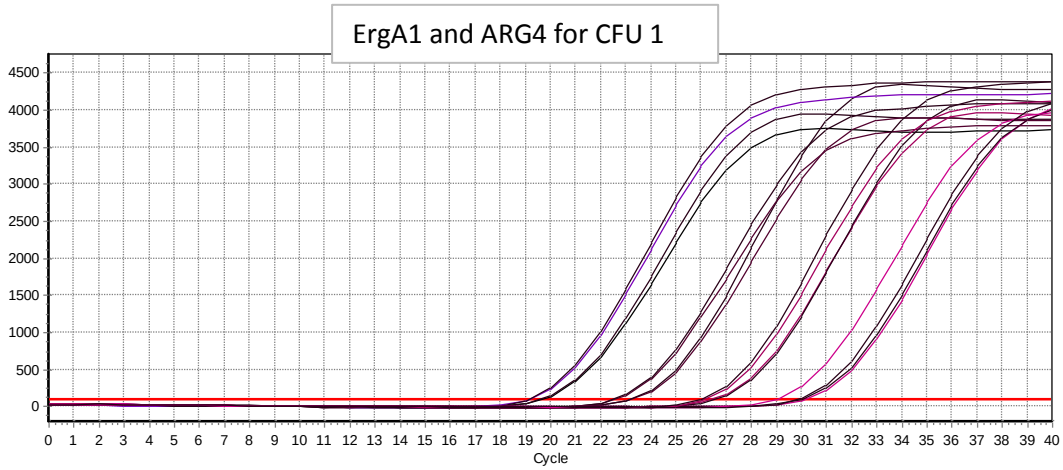
Sample	Efficiency	R^2
ErgA1	1.05	0.992
ARG4	0.95	0.997

At the actual quantification the PCR efficiencies of each sample with both primer pairs were determined as well and can be seen in Table 20.

Table 20 PCR efficiencies of all samples at quantification.

Sample	Efficiency	R^2
ErgA1 CFU1	0.95	1.000
ARG4 CFU1	0.95	0.996
ErgA1 CFU2	0.96	0.995
ARG4 CFU2	1.01	0.995
ErgA1 CFU6	0.88	0.994
ARG4 CFU6	0.98	1.000
ErgA1 CFU7	0.89	0.999
ARG4 CFU7	0.87	0.992
ErgA1 CFU10	0.94	0.983
ARG4 CFU10	1.02	0.997
ErgA1 CFU3	0.79	0.995
ARG4 CFU3	0.89	0.997
ErgA1 CFU5	0.87	0.997
ARG4 CFU5	0.86	0.998
ErgA1 CFU9	0.82	1.000
ARG4 CFU9	1.24	0.609

Regarding the final curves of all samples at quantification the results were defined to be the same for all colonies used. Representing these results the curves of colony 1 and the negative control (native *P. pastoris*) can be seen in Figure 31 and 32.

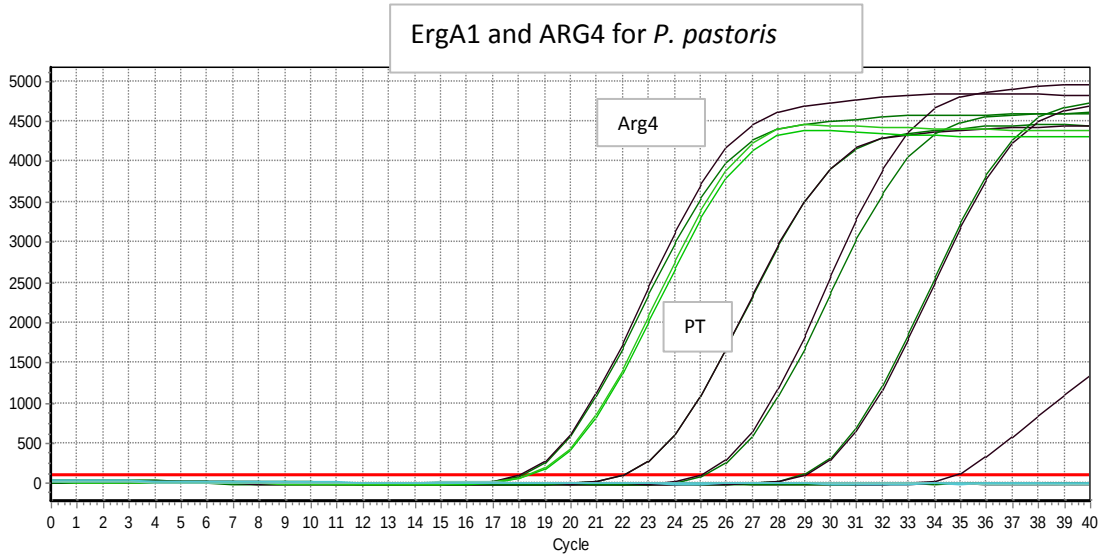


Threshold: 87 (Noiseband)
Baseline settings: automatic, Drift correction OFF

Figure 31 Quantification of colony 1 to determine copy number. Curves for ARG4 and ErgA1 appear at a Ct around 19.

As a negative control the genomic DNA of *P. pastoris* was also used which showed that no other products were amplified with the primer pair ErgA1 and that the gained results described above can be taken for real (Figure 32). The negative control of real time PCR

proofed that these colonies actually contained the insert and that the results were not gained because of unspecific primer binding.



Threshold: 93 (Noiseband)
Baseline settings: automatic, Drift correction OFF

Figure 32 Negative control for determination of copy number of ErgA.

To observe the amplified products based on their length a gel electrophoresis was carried out. In Figure 33, gel electrophoresis showed that the bands of all samples were approximately at a height between 80 and 100 bp. As expected a difference between amplified products of ARG4 and ErgA1 could not be made, because the products were all around the same length. However, the negative control showed no band and the positive control was at the same height as all other samples.

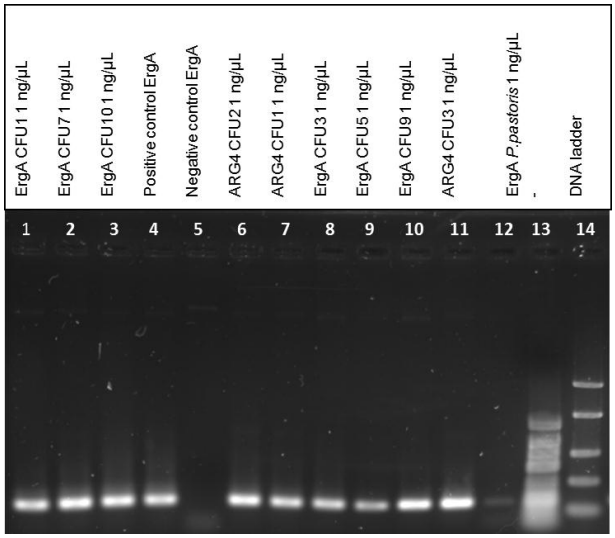


Figure 33 Gel electrophoresis of quantification at real time PCR. Lane 1: ErgA CFU 1 1 ng/µl; Lane 2: ErgA CFU 7 1 ng/µl; Lane 3. ErgA CFU 10 1 ng/µl; Lane 4: Positive control ErgA; Lane 5: Positive control ErgA; Lane 6: ARG4 CFU 2 1 ng/µl; Lane 7: ARG4 CFU 1 1 ng/µl; Lane 8: ErgA CFU 3 1 ng/µl; Lane 9: ErgA CFU 5 1 ng/µl; Lane 10: ErgA CFU 9 1 ng/µl; Lane 11: ARG4 CFU 3 1 ng/µl; Lane 12: ErgA *P. pastoris* 1 ng/µl; Lane 13: empty; Lane 14: FastRuler™ Low Range DNA ladder (50 bp);

3.2. Expression of ErgB

3.2.1. Transformation

Determined transformation efficiency of *E. coli* XL10 Gold was $4.523*10^5$ CFU/µg DNA. On the negative control plates no transformants were visible. Restriction digest of the two chosen colonies proved that all colonies contained the right insert. Linearization gained bands at a height of 4722 bp. Restriction digest with *Xho*I and *Xba*I showed the insert (1662 bp) and the vector (3060 bp). Bands of a length of 3834 bp and 888 bp were visible after a restriction digest with the enzymes *Xba*I and *Nco*I.

Since the undigested plasmid, isolated by midiprep Kit, could be seen on the same height as the linearized vector, a second restriction digest with *Xho*I was carried out (Figure 34). This gel electrophoresis showed that linearization of isolated *erg*B pGAPZα C wasn't complete prior to purification. However, a sufficient amount of DNA was gained to perform transformation into *P. pastoris*. In total 21 really small colonies grew on all sample plates after transformation of *erg*B pGAPZα C into *P. pastoris*. 6 colonies grew on the plates for the negative control. The colonies on the negative control might have been able to grow because of the phenomenon called superposition. The control of zeocin resistance via transferring the colonies on fresh zeocin containing plates showed that a few colonies of the sample plates

grew. These cells were then used for colony PCR with the result that none of the colonies contained the actual insert.

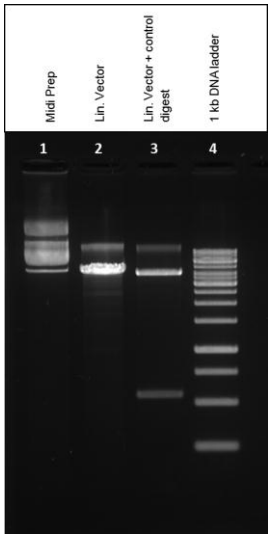


Figure 34 Linearization and control digest of linearized *erg*B pGAPZα C. Lane 1: midiprep; Lane 2: linearized *erg*B pGAPZα C; Lane 3: linearized and further digested *erg*B pGAPZα C with *Xho*I giving fragments of the size of 546 bp and 4176 bp; Lane 4: 1 kb DNA ladder;

For the second attempt of transformation with a slightly different protocol, as can be seen in the section 2.3.1., 16 colonies could be counted for the approach with 10 µg DNA, 13 for 4 µg DNA and 6 for 1 µg DNA. Unfortunately 26 colonies also grew on the plates for the negative control. As a consequence, all colonies (samples and negative control) were transferred to a fresh agar plate containing zeocin to observe the resistance of the colonies. 10 of the samples grew as broad colonies. The others were really small and grew into the air rather than having a lot of contact with the agar. The 10 broad colonies were picked and used for PCR. After PCR the positive control and a few other colonies showed two bands. As expected, one band was at a height of 480 bp. The other band was lower than 250 bp (Figure 35).

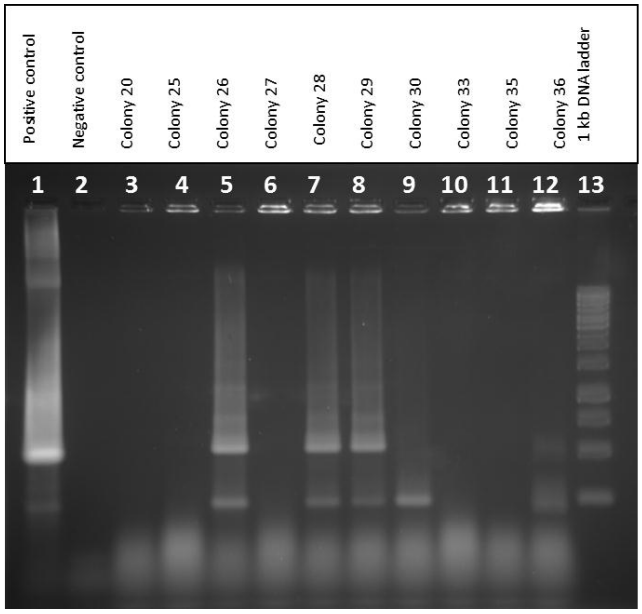
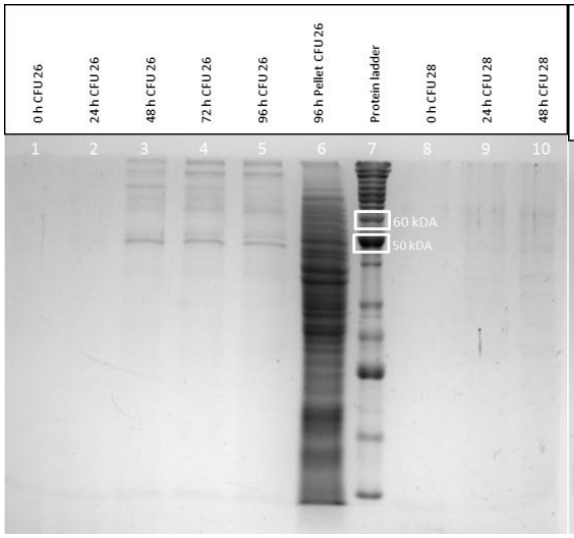


Figure 35 Colony PCR screening after transformation of *erg*B pGAPZα C in *P. pastoris*. Lane 1: positive control; Lane 2: negative control; Lane 3: colony 20; Lane 4: colony 25; Lane 5: colony 26; Lane 6: colony 27; Lane 7: colony 28; Lane 8: colony 29; Lane 9: colony 30; Lane 10: colony 33; Lane 11: colony 35; Lane 12: colony 36; Lane 13: Gene Ruler™ 1 kb DNA ladder;

3.2.2. Expression

For the enzyme ErgB a band at 58 kDA was expected at SDS PAGE. In all samples, neither supernatants nor pellets, no bands at this height were clearly visible (Figures 36 to 38).



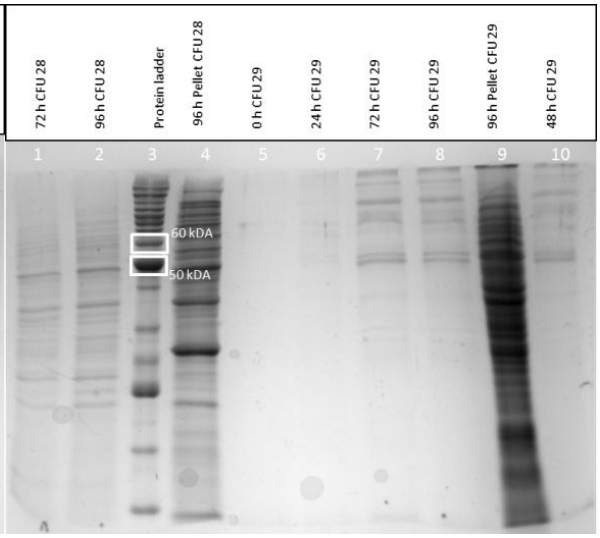


Figure 36 (left) SDS PAGE of expressed ErgB. Lane 1 to 6: colony 26; Lane 7: Bench Mark Protein Ladder; Lane 8 to 10: Colony 28;

Figure 37 (right) SDS PAGE of expressed ErgB. Lane 1 and 2: colony 28; Lane 3: Bench Mark Protein Ladder; Lane 4: Pellet colony 28; Lane 5 to 10: colony 29;

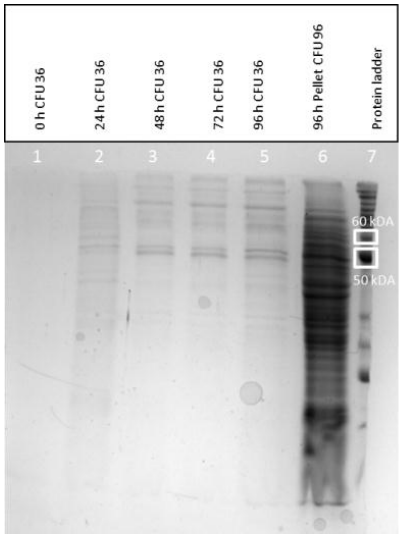


Figure 38 SDS PAGE of expressed ErgB. Lane 1 to 6: colony 36; Lane 7: Bench Mark Protein Ladder;

3.2.3. Analysis

Determination of activity

All results concerning ergine are concerning the sum of both epimers (ergine + erginine). Although SDS-PAGE showed no positive results concerning a positive expression of ErgB a degradation experiment of ergine to determine activity of the enzyme was carried out because even if the protein was not visible on the gel of SDS-PAGE there still might have been enough enzyme produced to observe activity. The degradation experiment of ergine with the supernatants and pellets of expression showed that the educts ergine and erginine could not be degraded as can be seen in figure 39. This means that the enzyme, if expressed, was not active. No appropriate bands were visible.

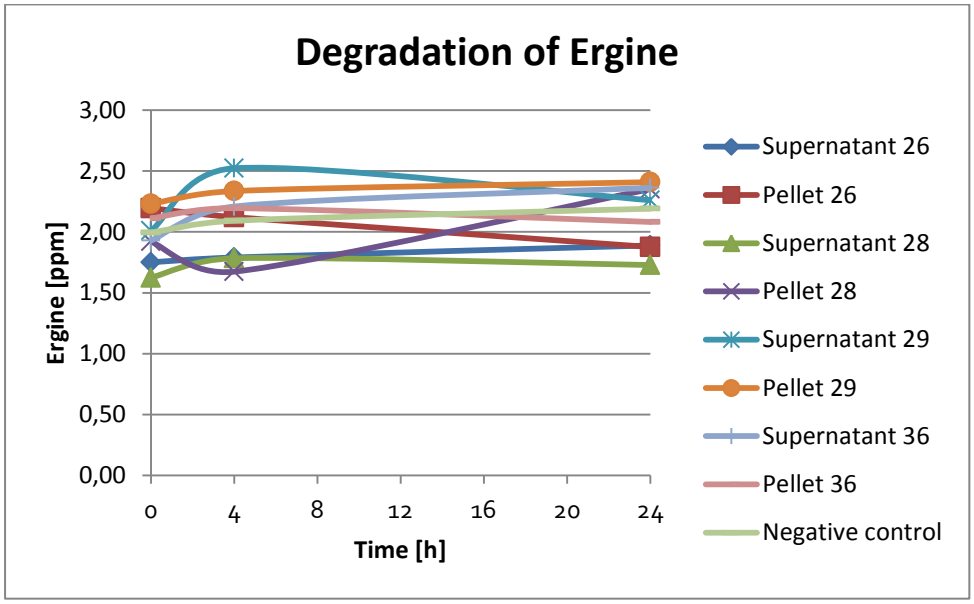


Figure 39 Degradation of ergine with expressed ErgB of different colonies after 96 h of expression.

Determination of copy number via Real Time PCR

Table 21 shows a summary of growth of different colonies used for expression of ErgB on agar plates with increasing zeocin concentrations. All four colonies were able to grow on the highest zeocin concentration of 1000 µg/ml.

Table 21 Growth of colonies on increasing zeocin concentrations.

Growth of *P. pastoris* on different concentrations of Zeocin			
Sample	250 µg/ml Zeocin	500 µg/ml Zeocin	1000 µg/ml Zeocin
ErgB 26	x	x	x*
ErgB 28	x	x	x*
ErgB 29	x	x	x
ErgB 36	x	x	x

* growth of cells was visible but not good

To determine if the insert has actually been transformed into *P. pastoris* and how often it was inserted a real time PCR was carried out to determine the copy number. Gel electrophoresis also proofed that all samples contained extracted DNA, although some DNA had undergone shearing effects (Figure 40).

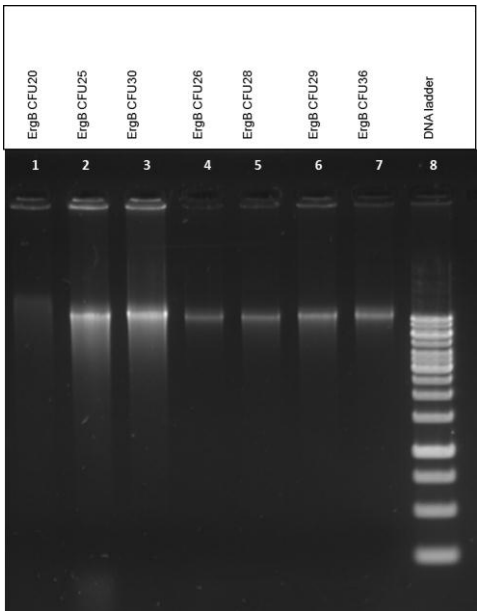
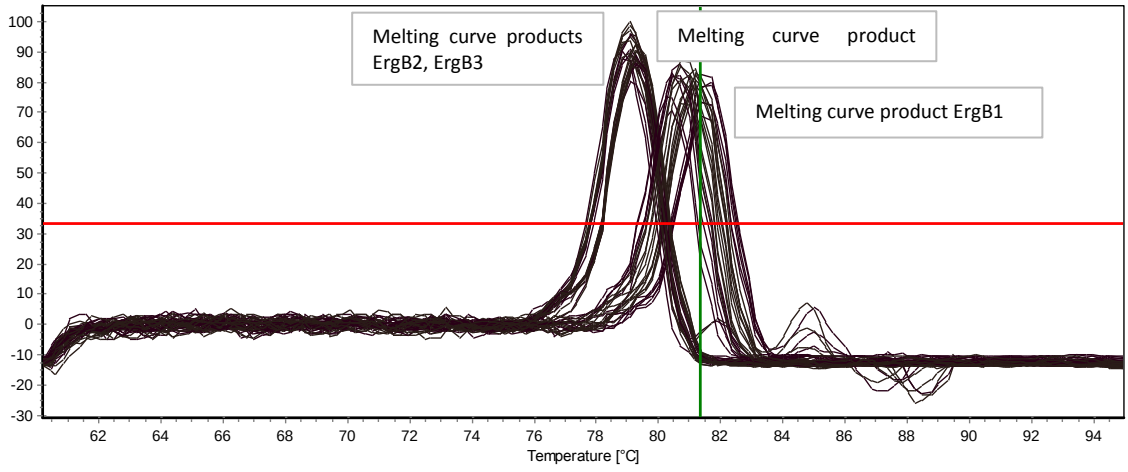


Figure 40 Agarose Gel of DNA extraction of *erg*B pGAPZα C in *P. pastoris.* Lane 1: CFU 20; Lane 2: CFU 25; Lane 3: CFU 30; Lane 4: CFU 26: Lane 5: CFU 28; Lane 6: CFU 29; Lane 7: CFU 36; Lane 8: 1 kb DNA ladder

The same steps for development of a real time PCR assay had to be carried out as described for the determination of copy number of ErgA see section 2.2.4. and 3.1.4. After the two steps of optimization of annealing temperature were carried out primer pairs ARG4 and ErgB3 and an annealing temperature of 62 °C were chosen for the following quantification based on low Ct-values (cycle threshold), regularity of fluorescence signals and a small difference of the melting temperature of the different products (Figure 41). For the third step, determination of PCR-efficiency, the chosen primers were used on one colony, colony 28, of which a standard with different concentrations was made. PCR-efficiency was determined to figure out if the used set of primers was working properly with the result that the efficiency of the chosen primers was nearly 100% (Table 22). This step was the last part of establishing the assay. Quantification was carried out in the same manner as described for ErgA. Again a difference between one or two copy numbers of the integrated insert was not able to determine. The results that could be seen were that colonies 26, 28 and 29 contained the insert (Figure 42). The copy number must be around 1 or 2. This result has also been proven by colony PCR after transformation. Additionally, real time PCR showed that colonies 20, 25, 30 and 36 did not contain the insert (Figure 43). When no insert is in the cell, the primers should not bind, no products should be amplified and no curves should be seen. However, for these colonies irregular curves appeared after a Ct of 30.



Threshold: 33%

Figure 41 Melting curves of amplified products of all primer pairs.

Table 22 PCR efficiency for the determination of copy number of ErgB with colony 28.

Sample	Efficiency	R^2
ErgB3	1.18	0.977
ARG4	0.96	0.990

At quantification efficiencies of all samples were also measured (Table 23). Those colonies that contained the insert had efficiencies around 100 %, whereas the others had either low efficiencies or the values was not even able to be measured.

Table 23 PCR efficiencies for all samples used for quantification.

Sample	Efficiency	R^2
CFU26 ErgB3	1.02	0.999
CFU26 ARG4	0.92	1.000
CFU29 ErgB3	0.93	1.000
CFU29 ARG4	0.95	1.000
CFU36 ErgB3	-1.00	0.009
CFU36 ARG4	0.90	0.999
CFU20 ErgB3	-1.00	0.227
CFU20 ARG4	0.99	0.991
CFU25 ErgB3	X	0.335
CFU25 ARG4	0.94	0.999
ErgB3 CFU30	X	0.433
ARG4 CFU30	0.96	1.000
ErgB3 *P. pastoris*	x	0.911
ARG4 *P. pastoris*	0.97	1.000

For colonies 26, 28 and 29 the same results at quantification could be gained. Representing them the curves of colony 29 can be seen in figure 42.

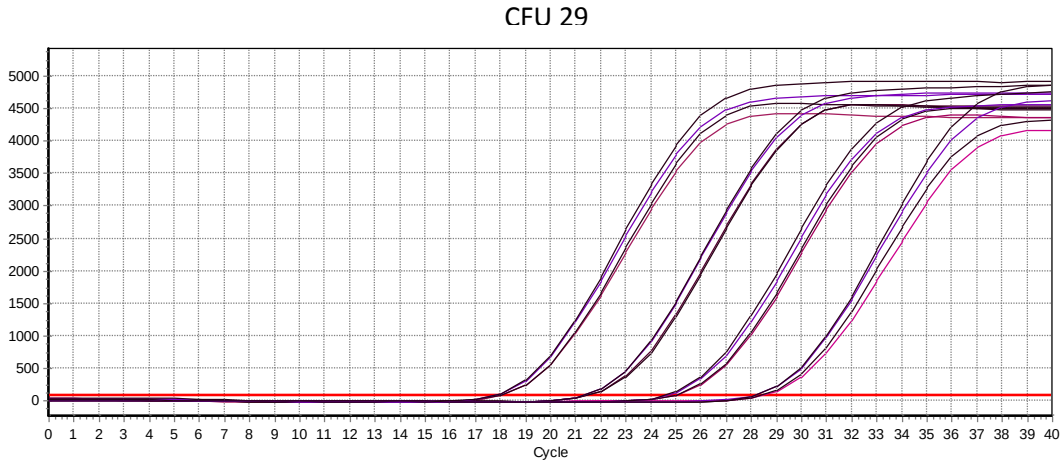
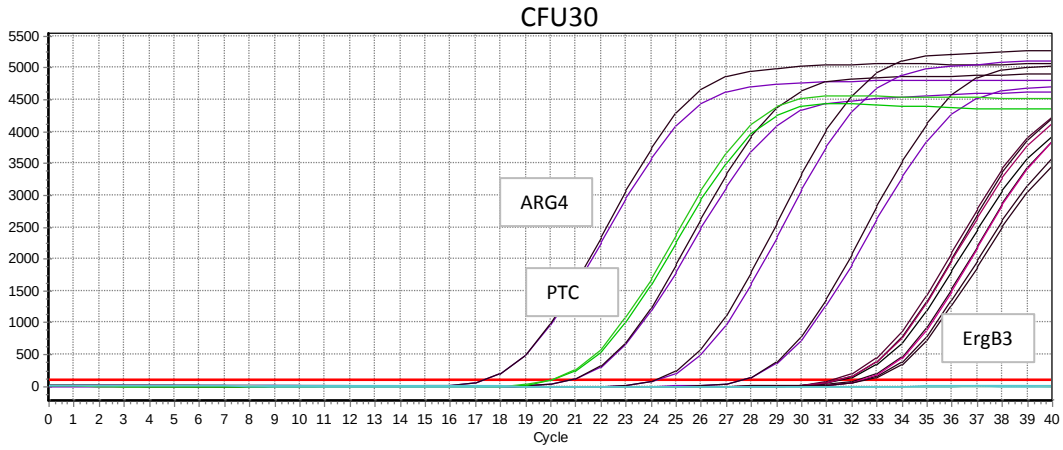


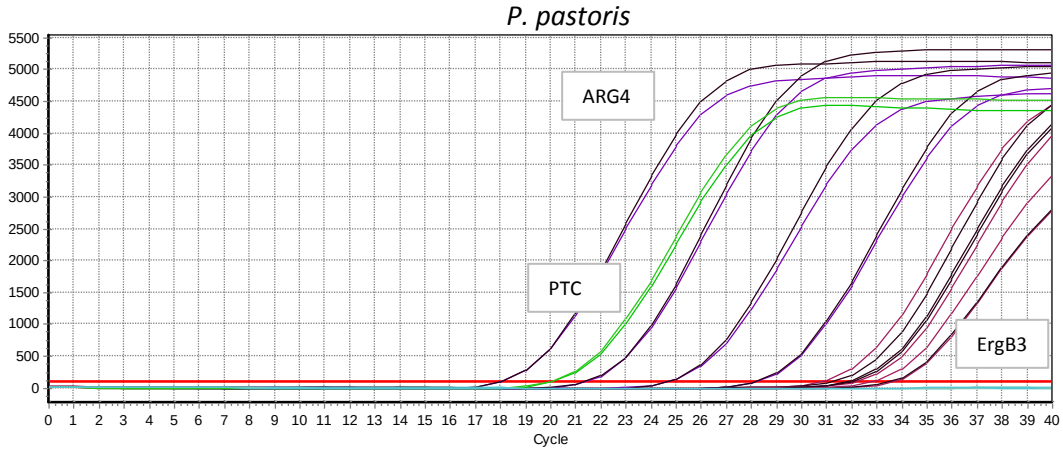
Figure 42 Colony 29 - amplified products of ARG4 and ErgB3 give a signal at a Ct of approximately 18.



Threshold: 92 (Noiseband)
Baseline settings: automatic, Drift correction OFF

Figure 43 Colony 36 - signals for the amplified products of ErgB3 appear at Ct-values higher than 30.

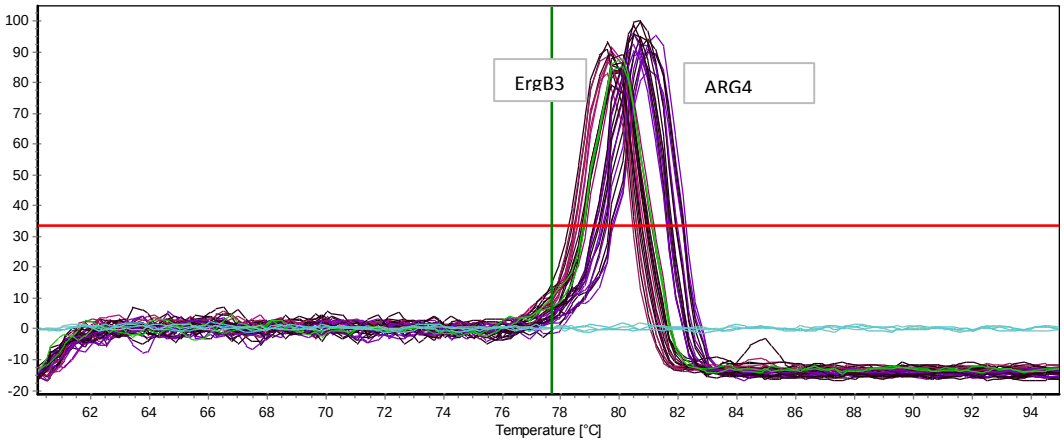
The other colonies at quantification gave signals after a Ct of 30, so did the negative control as can be seen in Figure 44.



Threshold: 92 (Noiseband)
Baseline settings: automatic, Drift correction OFF

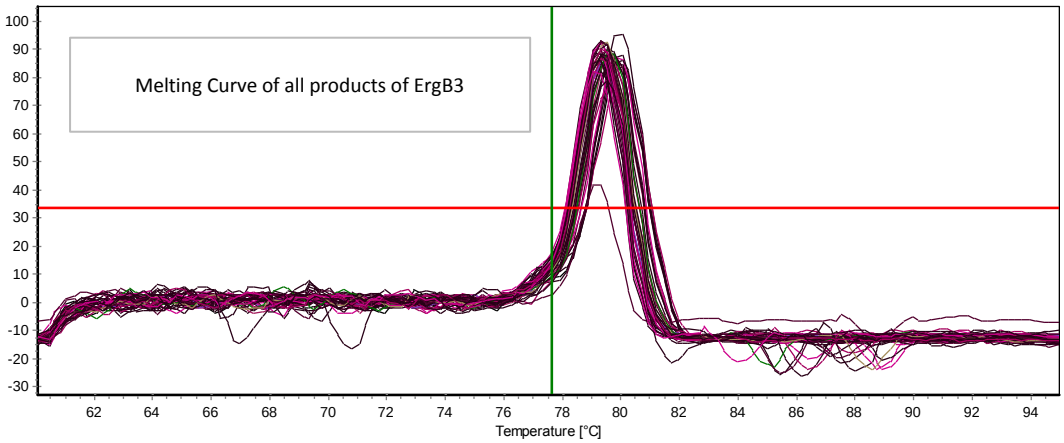
Figure 44 Negative control - signals for products of primer pair ErgB3 appearing after a Ct of 30.

To observe the amplified products of all colonies and determine if there are any differences visible the melting curves were observed. Figure 45 shows the difference between the amplified products of the two different primer pairs. In figure 46 the products of ErgB3 for all positive colonies as well as for all negative colonies are visible. The melting curves are similar to each other.



Threshold: 33%

Figure 45 Melting curves of amplified products of all colonies and both primer pairs ARG4 and ErgB3.



Threshold: 33%

Figure 46 Melting curves of products of all colonies amplified with ErgB3.

For further observation of the amplified products an agarose gel electrophoresis was carried out (Figure 47 and Figure 48). All colonies (positive and negative concerning containing the vector plus insert) showed bands all at the same height.

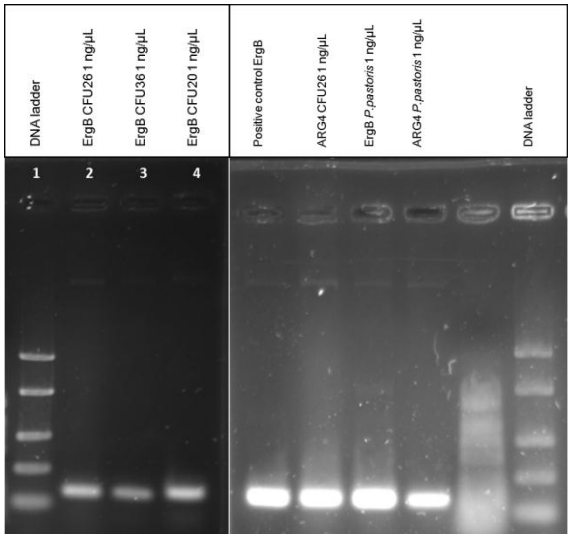


Figure 47 (on the left) Gel electrophoresis after quantification at real time PCR. Lane 1: FastRuler™ Low range DNA ladder; Lane 2: ErgB CFU 26 1 ng/µl; Lane 3: ErgB CFU 36 1 ng/µl; Lane 4: ErgB CFU 20 1 ng/µl;
Figure 48 (on the right) Gel electrophoresis after quantification at real time PCR. Lane 1: Positive control ErgB; Lane 2: ARG4 CFU 26 1 ng/µl; Lane 3: ErgB *P. pastoris* 1 ng/µl; Lane 4: ARG4 P. pastoris 1 ng/µl; Lane 5: empty; Lane 6: FastRuler™ Low range DNA ladder;

Anyhow, it can be excluded that the colonies 20, 25, 20 and 36 contain the actual vector plus insert, because the positive cells have signals at a Ct between 18 and 19. All other colonies show signals after a Ct of 30 which would mean that there is twenty times less initial DNA in the cell. So overall, it can be said, that three colonies definitely contained the insert whereas the others did not.

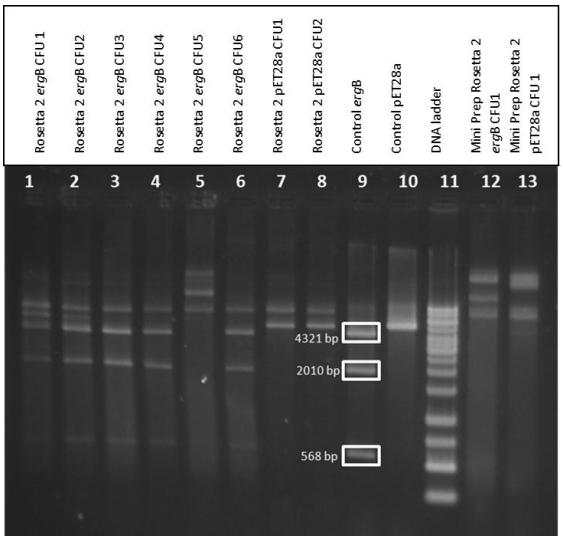
3.3. Expression of ErgB in different *Escherichia coli* expression strains

After preparation of competent cells of diverse *E. coli* expression strain the evaluated transformation efficiencies for all strains were not as high as expected (Table 24). However, after transformation a sufficient amount of transformants could be gained. Although *E. coli* Origami 2 had the highest transformation efficiency with the test plasmid pUC18 no transformants formed on the agar plates after transformation with *erg*B pET28a(+). Even changes in the experimental procedure such as lowering the antibiotic concentration and using a bigger volume for plating out the cells, did not lead to a positive result.

Table 24 Determined transformation efficiencies of all strains used for transformation.

Transformation efficiency	
Strain	**Transformation efficiency [transformants/µg DNA]**
***E. coli* Rosetta 2(DE3)**	1.32*10^4
***E. coli* Origami 2(DE3)**	1.01*10^5
***E. coli* Tuner(DE3)**	7.07*10^4
***E. coli* HMS174(DE3)**	8.56*10^4
***E. coli* BL21(DE3)pLysS**	1.88*10^7

With the help of a restriction digest of the minipreps and a following agarose gel electrophoresis it was proven that all picked transformants contained the vector plus insert (Figures 49 to 52). All colonies containing the plasmid plus insert showed three bands at the heights of 4321 bp, 2010 bp and 568 bp.



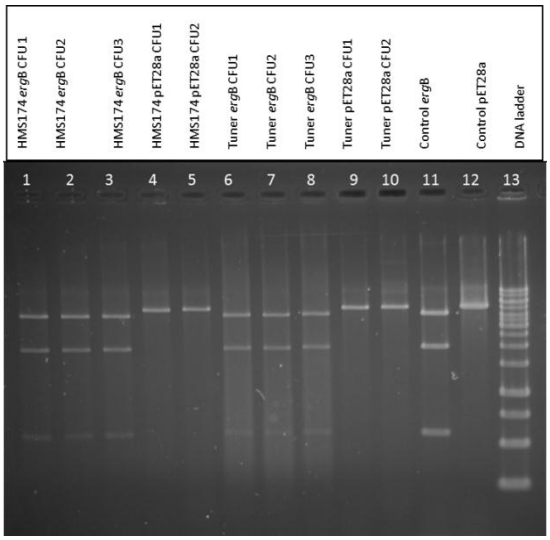


Figure 49 (on the left) Control digest of picked colonies after transformation of *erg*B pET28a(+) and pET28a(+) into *E. coli* Rosetta 2. Lane 1 to 6: CFU 1 to 6; Lane 7 and 8: CFU 1 and 2; Lane 9: Control digest of *erg*B pET28a(+); Lane 10: Control digest of pET28a(+); Lane 11: 1 kb DNA ladder; Lane 12 and 13: undigested DNA after miniprep isolation;

Figure 50 (on the right) Control digest after miniprep isolation of picked colonies after transformation of *erg*B pET28a(+) and pET28a(+) into *E. coli* HMS174(DE3) and *E. coli* Tuner(DE3). Lane 1 to 3: CFU 1 to 3; Lane 4 and 5: CFU 1 and 2; Lane 6 to 8: CFU 1 to 3; Lane 9 and 10: CFU 1 and 2; Lane 11: Control digest of *erg*B pET28a(+); Lane 12: Control digest of pET28a(+); Lane 13: 1 kb DNA ladder

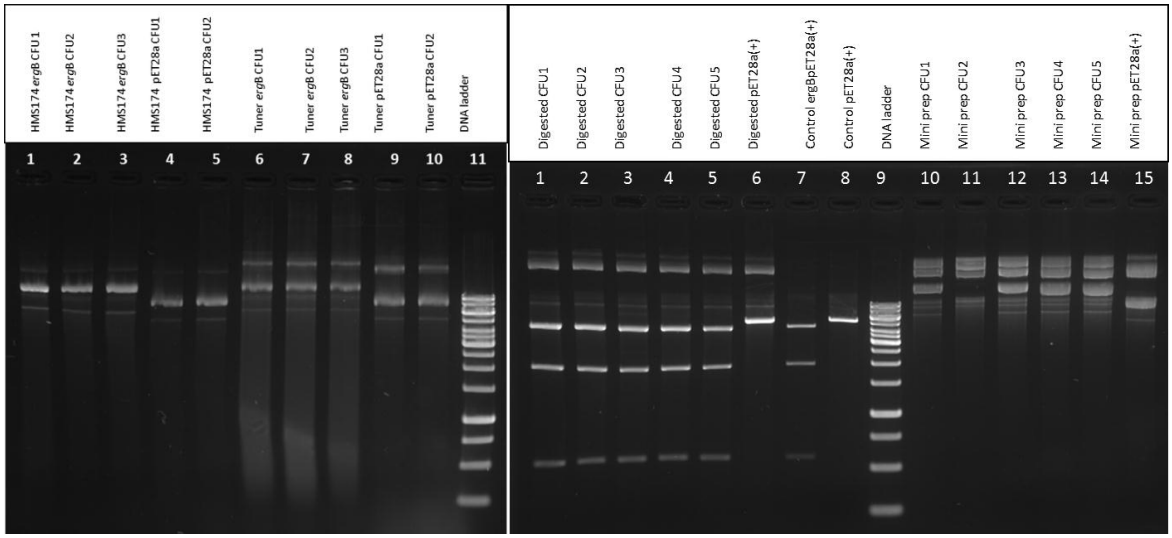


Figure 51 (on the left) Undigested minipreps. Lane 1 to 4: HMS174; Lane 6 to 10: Tuner; Lane 11: 1 kb DNA ladder;

Figure 52 (on the right) Gel electrophoresis after control digest of isolated plasmids after transformation into *E. coli* BL21(DE3)pLysS. Lane 1 to 5: *erg*B pET28a(+) CFU 1 to 5; Lane 6: pET28a(+); Lane 7: control of digested *erg*B pET28a(+); Lane 8: control of digested pET28a(+); Lane 9: DNA ladder; Lane 10 to 14: undigested miniprep *erg*B pET28a(+) CFU 1 to 5; Lane 15: undigested miniprep pET28a(+).

After expression cell lysis was carried out to get three samples per colony: the total lysate, the supernatant containing the dissolved proteins and the pellet with the unsolvable proteins. SDS-PAGE showed that in *E. coli* Rosetta 2(DE3), BL21(DE3), HMS174(DE3) and Tuner(DE3) hardly any ErgB is soluble (Figures 53 to 59). *E. coli* Tuner(DE3) expression was induced with different concentrations of IPTG and it could be seen, that the best expression of soluble ErgB was gained with the lowest amount of IPTG (Figures 57 to 59). The negative controls for these strains (colonies containing the empty vector pET28a(+)) showed that no other protein with the same size as ErgB was produced. In the negative control for *E. coli* ArcticExpress(DE3) it could be seen that another protein is produced whose band is nearly at the same height as the expected band for ErgB. However, in the supernatant of the colony that contained the vector plus insert a higher amount of protein can be seen than in the unsolvable fraction of the same colony as well as in the negative control (Figure 53). As a consequence, another experiment was started focusing on the expression in *E. coli* ArcticExpress(DE3) and the reproducibility of the results in the previous experiment.

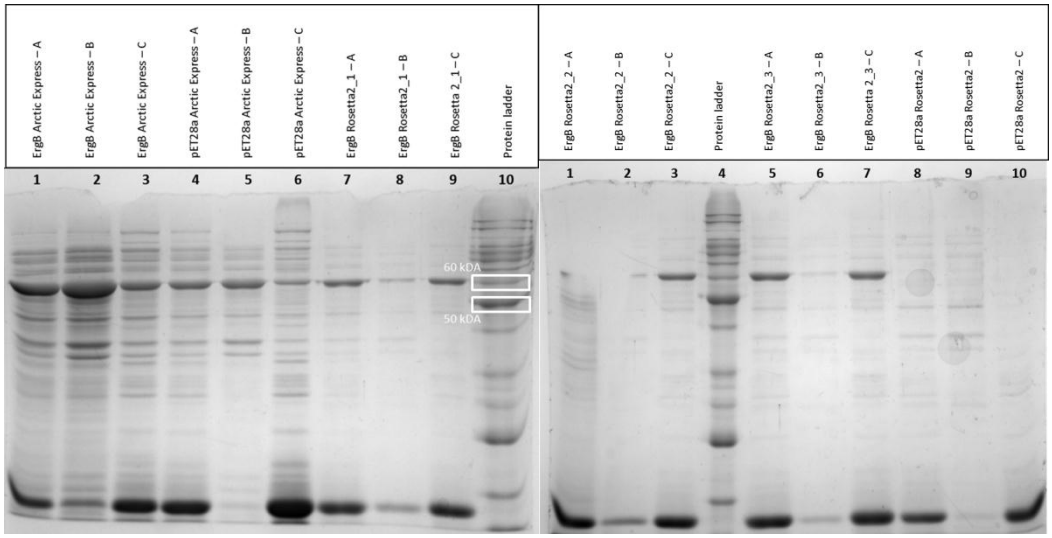


Figure 53 (on the left) SDS-PAGE of expressed ErgB (A= whole lysate; B= Supernatant with dissolved proteins; C= pellet with unsolvable proteins). Lane 1 - 3: ErgB expressed with *E. coli* ArcticExpress(DE3) A, B, C; Lane 4 - 6: pET28a(+) *E. coli* ArcticExpress(DE3) A, B, C; Lane 7 - 9: ErgB expressed in *E. coli* Rosetta 2 colony 1 A, B, C; Lane 10: Bench Mark Protein ladder

Figure 54 (on the right) SDS-PAGE of expressed ErgB (A= whole lysate; B= Supernatant with dissolved proteins; C= pellet with unsolvable proteins). Lane 1 - 3: ErgB in *E. coli* Rosetta 2 colony 2 A, B, C; Lane 4: Bench Mark Protein ladder; Lane 5 - 7: ErgB expressed in *E. coli* Rosetta 2 colony 3 A, B, C; Lane 8 - 10: pET28a(+) *E. coli* Rosetta 2 A, B, C;

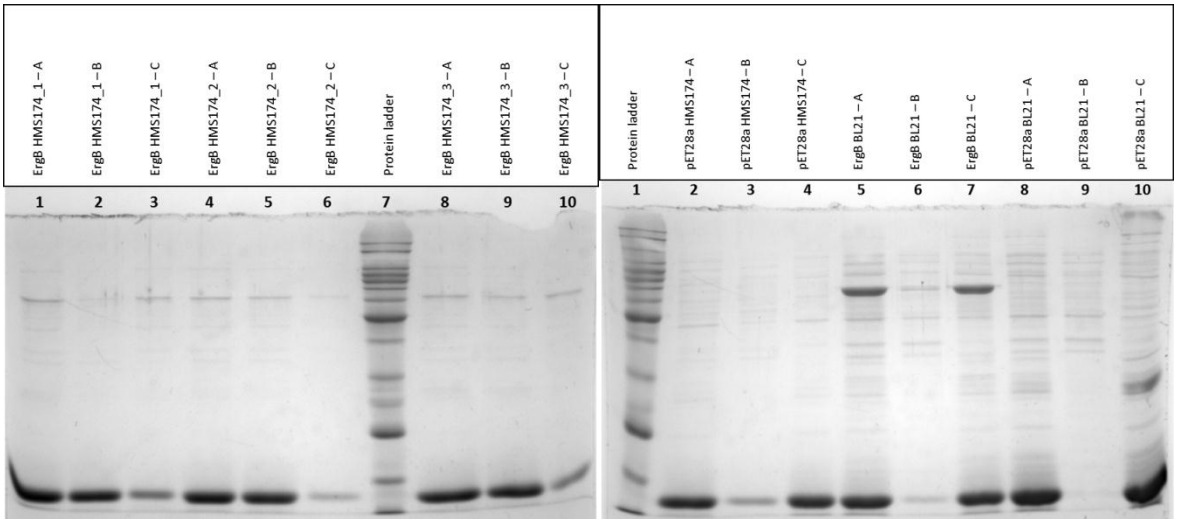
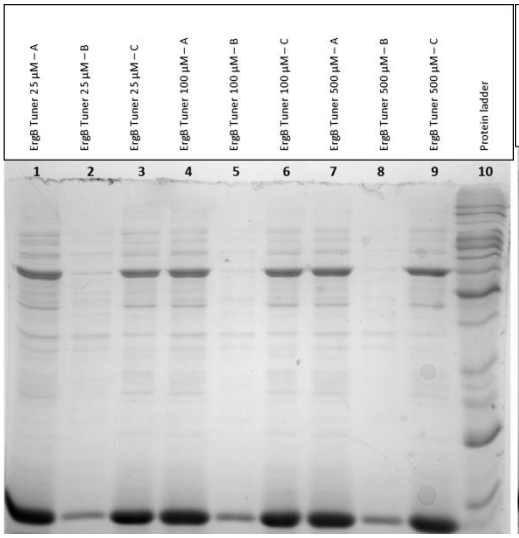


Figure 55 (on the left) SDS-PAGE of expressed ErgB (A= whole lysate; B= Supernatant with dissolved proteins; C= pellet with unsolvable proteins). Lane 1 - 3: ErgB expressed in *E. coli* HMS174 colony 1 A, B, C; Lane 4 - 6: ErgB in *E. coli* HMS174 colony 2 A, B, C; Lane 7: Bench Mark Protein ladder; Lane 8 - 10: ErgB in *E. coli* HMS174 colony 3 A, B, C

Figure 56 (on the right) SDS-PAGE of expressed ErgB (A= whole lysate; B= Supernatant with dissolved proteins; C= pellet with unsolvable proteins). Lane 1: Bench Mark Protein ladder; Lane 2 - 4: pET28a(+) E. coli HMS174 A, B, C; Lane 5 - 7: ErgB expressed in *E. coli* BL21 A, B, C; Lane 8 - 10: pET28a(+) *E. coli* BL21 A, B, C;



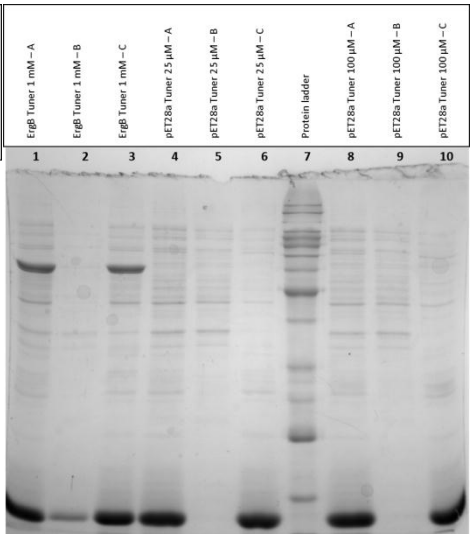


Figure 57 (on the left) SDS-PAGE of expressed ErgB (A= whole lysate; B= Supernatant with dissolved proteins; C= pellet with unsolvable proteins). Lane 1 - 3: ErgB expressed in *E. coli* Tuner, induction with 25 µM IPTG; A, B, C; Lane 4 - 6: ErgB in *E. coli* Tuner, induction with 100 µM IPTG; A, B, C; Lane 7 - 9: ErgB in *E. coli* Tuner, induction with 500 µM IPTG; A, B, C; Lane 10: Bench Mark Protein ladder

Figure 58 (on the right)SDS-PAGE of expressed ErgB (A= whole lysate; B= Supernatant with dissolved proteins; C= pellet with unsolvable proteins). Lane 1 - 3: ErgB expressed in *E. coli* Tuner, induction with 1 mM IPTG; A, B, C; Lane 4 - 6: pET28a(+) *E. coli* Tuner, induction with 25 µM IPTG; A, B, C; Lane 7: Bench Mark Protein ladder; Lane 8 - 10: pET28a(+) *E. coli* Tuner, induction with 100 µM IPTG; A, B, C

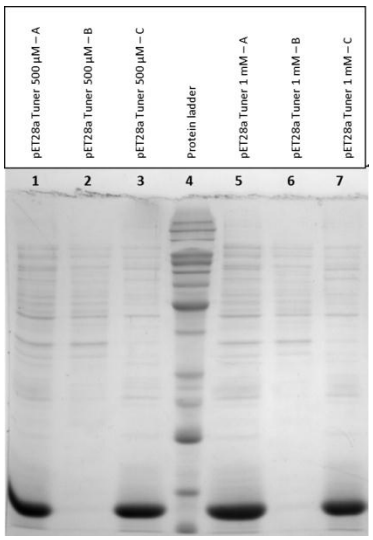


Figure 59 SDS-PAGE of expressed ErgB (A= whole lysate; B= Supernatant with dissolved proteins; C= pellet with unsolvable proteins). Lane 1 - 3: pET28a(+) *E. coli* Tuner, induction with 500 µM IPTG; A, B, C; Lane 4: Bench Mark Protein ladder; Lane 5 - 7: pET28a(+) *E. coli* Tuner, induction with 1 mM IPTG; A, B, C;

After another expression of *E. coli* ArcticExpress(DE3) the harvested cells containing the insert *erg*B pET28a(+) formed only small pellets which was obvious at measurement of OD_{600}. After comparison with the pellet size of previous expression experiments it could be

seen that the pellets were always quite small. In other words the amount of harvested cells in this experiment had nothing to do with any mistakes causing unfavourable conditions during expression of ErgB. Additionally, microscopic pictures showed that *E. coli* colonies containing the insert *erg*B pET28a(+) tend to form chains, whereas colonies with the empty vector did not. Unfortunately this fact could not be explained by any literature. Anyhow all harvested cells from all colonies were used for cell lysis and SDS-PAGE to observe the amount of expressed enzyme ErgB. SDS-PAGE analysis showed that the samples containing the vector *erg*B pET28a(+) did not express enough soluble enzyme for analysis. Considering that the low amount of harvested cells at expression lead to negative results concerning the production of ErgB, the experiment was repeated a third time.

Different optimisations concerning conditions during incubations periods were established. Although incubation periods during the whole expression experiment were prolonged and the conditions perfected, the cell number of cells containing the insert for ErgB expression sank to a half after induction and incubation for 24 h. When these cells were not induced, cell growth was normal. Normal growth was also documented for cells that contained the empty vector pET28a(+) with and without induction. These results can be seen in Table 25 and Figure 60. Regarding the gels of SDS-PAGE (Figures 62 to 65) it can be seen that there is hardly any soluble protein at a height of approximately 58 kDa after induction with IPTG. Without induction a lot of protein can be seen in the soluble fraction for all colonies containing the insert. Comparing these expression patterns with those of the negative control (empty pET28a(+)) similarities are visible. Overall, it can be said that *E. coli* ArcticExpress(DE3) expresses a native protein, which can be seen on the SDS-PAGE at the same height as the expected ErgB. After induction with IPTG neither the native protein nor ErgB are expressed in the soluble fraction which can also be the consequence of the sinking OD_{600}.

Table 25 Summary of OD-measurements during expression of *E. coli* ArcticExpress(DE3).

OD_{600} Expression in *E. coli* ArcticExpress(DE3)				
	pre culture	main culture at 0 h	main culture before induction	main culture after induction and incubation
Sample	**OD_{600}**	**OD_{600}**	**OD_{600}**	**OD_{600}**
A1-0.1 mM	1.987	0.644	1.305	0.528
A1-1 mM	1.951	0.600	1.286	0.461
A1- 0 mM	1.939	0.569	1.249	1.959
A2-0.1 mM	1.836	0.480	1.189	0.535
A2-1 mM	1.979	0.626	1.332	0.442
A2-0 mM	2.000	0.663	1.348	1.987
A3-0.1 mM	1.921	0.592	1.290	0.667
A3-1 mM	1.896	0.565	1.284	0.584
A3-0 mM	1.914	0.580	1.375	2.032
pET28-0.1 mM	2.022	0.725	1.460	1.812
pET28-1 mM	1.971	0.682	1.445	1.780
pET28-0 mM	1.921	0.654	1.423	2.081

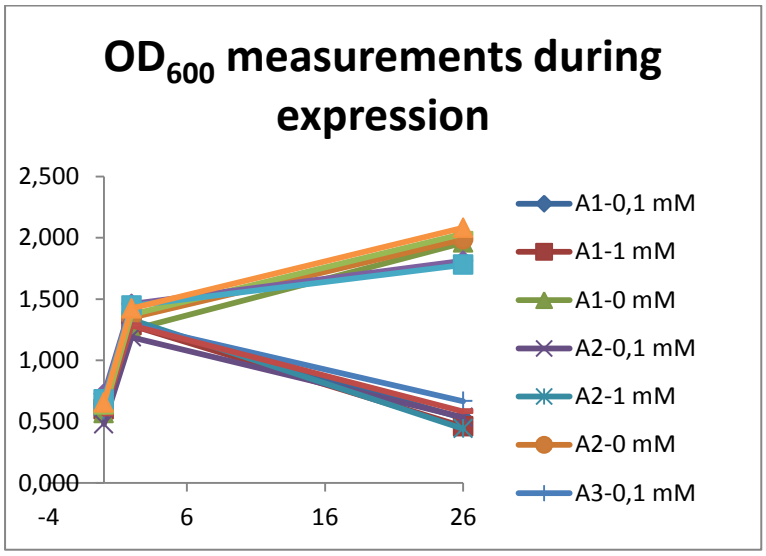


Figure 60 OD_{600} measurements of all samples during expression process of *E. coli* ArcticExpress(DE3).

Microscopic pictures taken during expression, showing the reduction of cell density during process can be seen in Figure 61.

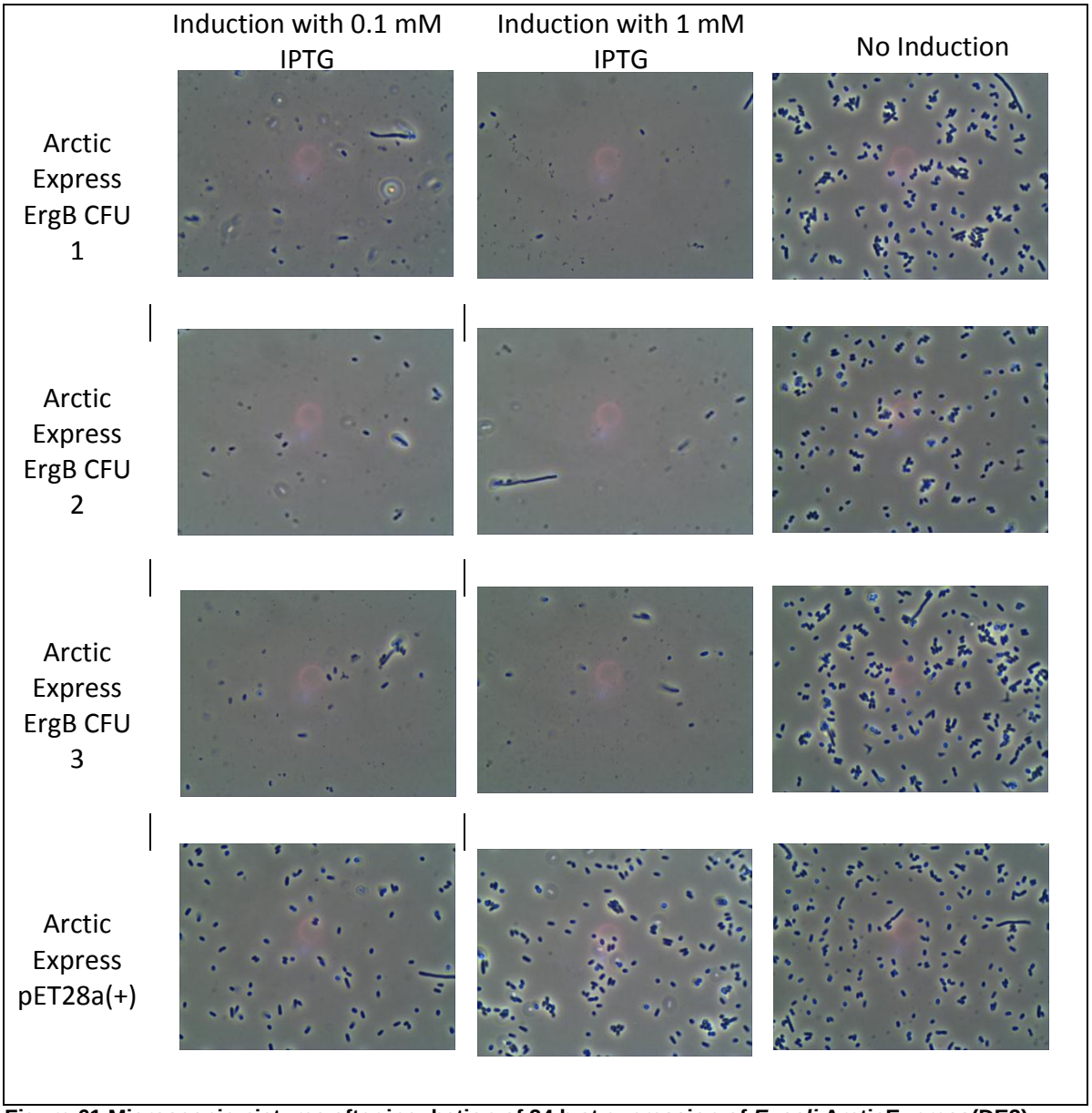
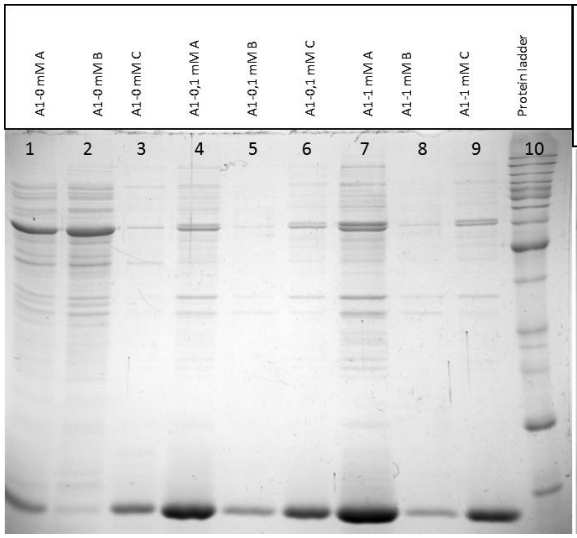


Figure 61 Microscopic pictures after incubation of 24 h at expression of *E. coli* ArcticExpress(DE3).



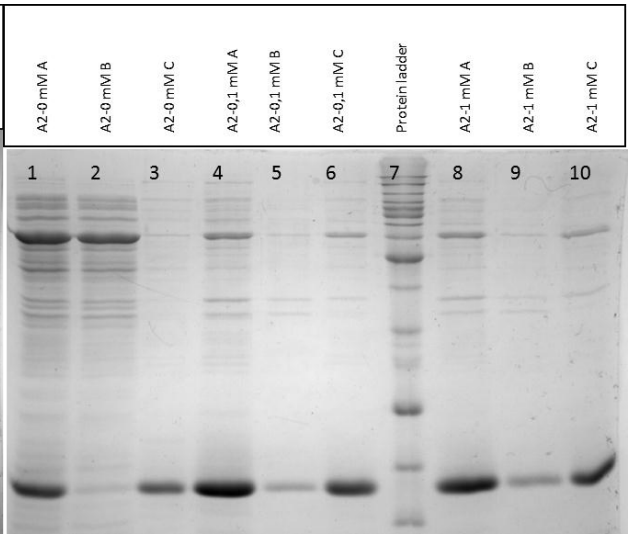
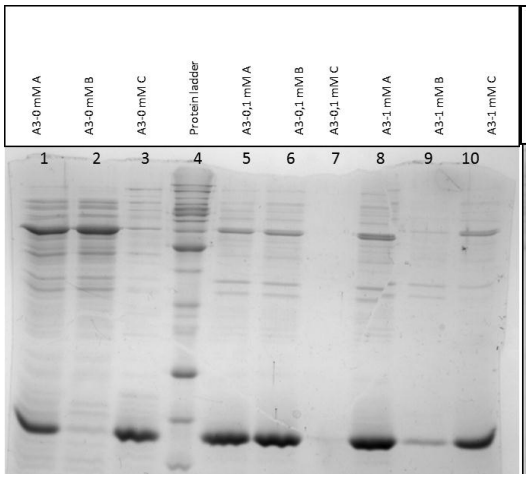


Figure 62 (on the left) SDS-PAGE of expression of ErgB in *E. coli* ArcticExpress(DE3) CFU 1. Lane 1-3: without induction; Lane 4-6: induced with 0.1 mM IPTG; Lane 7-9: induced with 1 mM IPTG; Lane 10: Bench Mark Protein Ladder;

Figure 63 (on the right) SDS-PAGE of expression of ErgB in *E. coli* ArcticExpress(DE3) CFU 2. Lane 1-3: without induction; Lane 4-6: induced with 0.1 mM IPTG; Lane 7: Bench Mark Protein Ladder; Lane 8-10: induced with 1 mM IPTG;



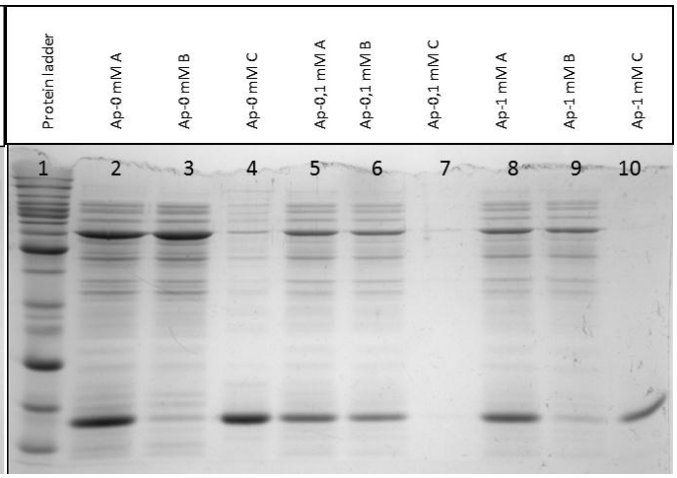


Figure 64 (on the left) SDS-PAGE of expression of ErgB in *E. coli* ArcticExpress(DE3) CFU 3. Lane 1-3: without induction; Lane 4: Bench Mark Protein Ladder; Lane 5-7: induced with 0.1 mM IPTG; Lane 8-10: induced with 1 mM IPTG

Figure 65 (on the right) SDS-PAGE of expression of ErgB in *E. coli* ArcticExpress(DE3) - negative control: empty vector pET28a(+). Lane 1: Bench Mark Protein Ladder; Lane 2-4: without induction; Lane 5-7: induced with 0.1 mM IPTG; Lane 8-10: induced with 1 mM IPTG;

Concerning expression with *E. coli* BL21(DE3)pLysS the OD_{600} of the cultures during expression were observed as can be seen in Table 26 and figure 66. The results of these measurements showed that cell growth was normal for all samples as well as for the negative control. However, SDS-PAGE analysis showed that hardly any soluble ErgB was expressed (Figure 67 and Figure 68). Summing up this means that although growth of cells was as expected still not enough enzyme was able to be expressed.

Table 26 Summary of OD-measurement during expression of ErgB in *E. coli* BL21(DE3)pLysS.

	main culture at 0 h	main culture after 2 h	main culture after 3 h before induction	main culture after induction and incubation
Sample	**OD_{600}**	**OD_{600}**	**OD_{600}**	**OD_{600}**
CFU 1	0.067	0.357	0.908	1.479
CFU 2	0.064	0.407	0.984	1.654
CFU 3	0.065	0.327	0.875	1.406
pET28a(+)	0.065	0.336	0.875	1.738

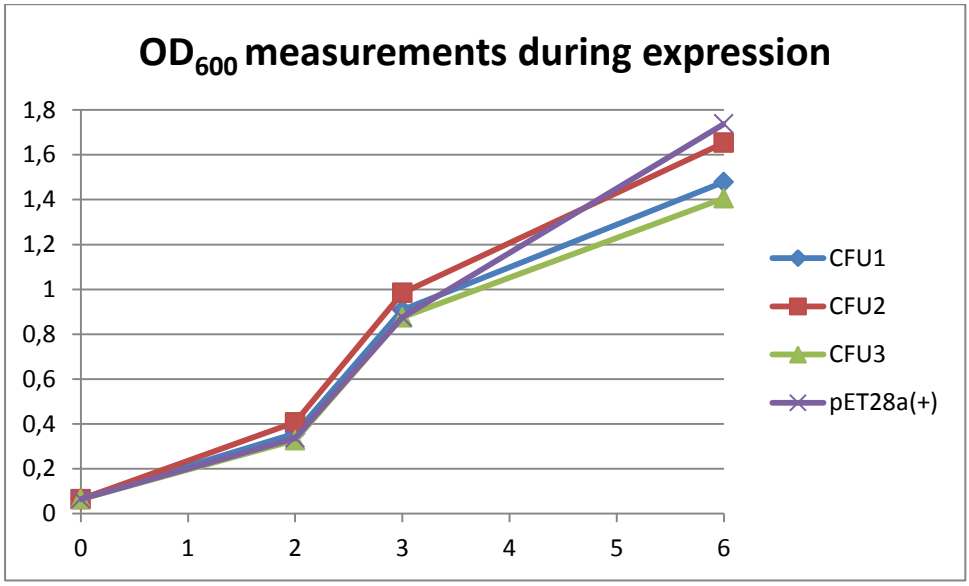


Figure 66 OD measurements of all samples during expression process of *E. coli* BL21(DE3)pLysS.

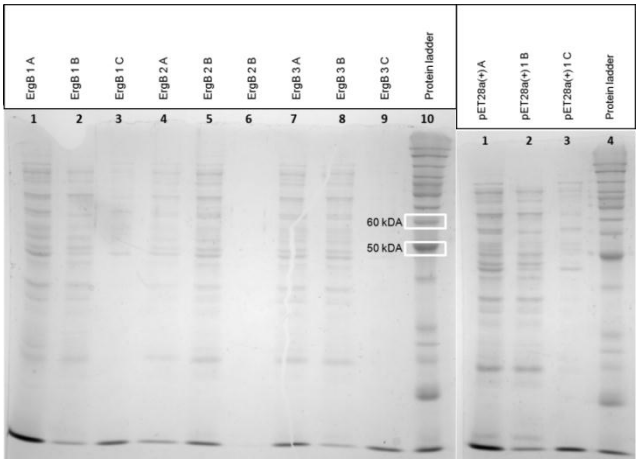


Figure 67 (on the left) Expression of ErgB in *E. coli* BL21(DE3)pLysS. Lane 1-3: Expression of Colony 1 sample A, B and C; Lane 4-6: Expression of Colony 2 sample A, B and C; Lane 7-9: Expression of Colony 3 sample A, B and C; Lane 10: Bench Mark Protein Ladder;

Figure 68 (on the right) Expression of ErgB in *E. coli* BL21(DE3)pLysS - negative control. Lane 1-3:Expression with pET28a(+) sample A, B and C; Lane 4: Bench Mark Protein Ladder;

4. Discussion

For expression of the enzymes ErgA and ErgB the expression system *Pichia pastoris* was chosen. A lot of advantages concerning the expression of foreign proteins make this strain widely used. These advantages include easy techniques for molecular and genetic manipulation as well as intracellular and extracellular secretion. Extracellular secretion allows simple techniques for assessing the produced enzymes, because additional techniques of purification, cell lysis and removal of cell debris are not necessary (Macauley-Patrick et al., 2005). Additionally, *P. pastoris* secretes only low levels of own proteins, which makes it easier to work with the foreign enzyme (Werten et al., 1999). Furthermore, this expression system is able to carry out a lot of eukaryotic posttranslational modifications. They include glycosylation, disulphide bond formation etc. These modifications can help enzymes to fold properly which as a consequence leads to an active enzyme (Cereghino and Cregg, 2000). Since structural features of both enzymes, ErgA and ErgB, are not known yet, *P. pastoris* was chosen as a possible expression system, that should help to stabilize the structure and the activity of the expressed enzymes. Transformation of *erg*A pGAPZα C into *P. pastoris* worked right away. For the transformation of ergB pGAPZα C a modified protocol was used (Wu and Letchworth, 2004, Lin-Cereghino et al., 2005, Cregg et al., 2009). The protocol was a mix of different transformation procedures for *P. pastoris* including LiCl-method and classic electroporation. However, the amount of colonies described in the literature could not be reached. As a consequence, not enough screening processes, gaining a colony with good expression results, could be carried out. Screening was carried out in the form of colony PCR. Colony PCR is a simple and quick technique to define which colonies contain the vector plus insert after transformation. Disadvantages of this PCR are that the results are not that reliable and that the method is not really sensitive (Sheu et al., 2000). Unspecific primer binding can happen and there might be false positive results although the colony does not contain the plasmid. More often it happens that primers randomly do not bind at all and there is a negative result although the colony does contain the plasmid (false negative). Additionally, unspecific primer binding can lead to more bands as expected at gel electrophoresis and leads to insecure results. Summing up it means that the results, gained with colony PCR are not reliable enough to define one colony that should be used for further expression experiments.

In the previously described expression experiments a number of colonies were chosen after transformation to express the enzymes. The used colonies were controlled with the help of colony PCR. However in other experiments usually the screening process is bigger. Hundreds

of colonies are tested for a positive expression of the foreign protein. As described above the screening process for ErgB was restricted because of the little number of colonies available after transformation.

Evaluation of expressed proteins was carried out via SDS-PAGE. After expression of ErgA expected bands were visible. ErgB was not clearly detectable. SDS-PAGE analysis is quite an easy technique to detect proteins of a known size without a lot of equipment. On the other hand the method is not very specific and as a consequence, detection of a certain protein is not always that easy (Zerbs et al., 2009).

Another possible method would be a western blot. A western blot allows specific detection of the wanted protein. To be able to get that result the sequence of the protein should be known, antibodies have to be made, special equipment and material is needed and the actual procedure is longer than for a simple SDS-PAGE. However, in the case of not being sure where the protein can be found on a SDS-PAGE gel a western blot is the method of choice.

Another way of getting an idea of the expression level is the determination of activity. When enough enzyme is expressed and it is correctly folded an activity is measurable. Concerning ErgB no activity was measured. The reason for that might have been that no enzyme was expressed or that it was not folded properly leading to inactivity. The activity of three colonies expressing ErgA was measured.

Copy number of the expression cassette, site and the mode of integration, mRNA 5'- and 3' untranslated regions, the start codon, A and T composition of cDNA, secretion signals, protease activity, host strain physiology, media, conditions during growth and parameters of fermentation, all influence protein production (Sreekrishna et al., 1997) and therefore affect the level of activity. The copy number plays an important role concerning expressions levels of foreign proteins. Depending on the protein and the expression system used the optimal copy number has to be found. Although it seems reasonable that a higher copy number leads to a higher yield of produced protein, this is not necessarily true. For some systems a copy number of one leads to best results concerning protein expression (Cregg et al., 1985). Sometimes a higher copy number even has negative effects on it (Sreekrishna et al., 1997). To be able to find this optimum a screening process with different copy numbers should be carried out. For determination of copy number in this work a rough assessment was made by plating out all used colonies on agar plates with increasing zeocin concentrations. It was thought that a higher copy number gives the cell a better resistance to a specific antibiotic and therefore, it can grow on higher concentrations (Scorer et al., 1994). With this method

differences concerning copy number could be seen. A more reliable method, namely real time PCR, proved these results to be wrong.

For real time PCR genomic DNA extraction had to be carried out. A good extraction method gaining high enough amounts of DNA and a good final purity of extracted DNA are essential for quantification with real time PCR (Cankar et al., 2006, Hoffman and Winston, 1987). In the previously described experiments there were big differences in concentration and 260/280 levels between different colonies after extraction. 260/280 values describe the ratio of absorbance at 260 nm and 280 nm. These values represent purity of DNA or RNA. For DNA a value around 1.8 is expected.

Real time PCR is considered a reliable and exact method for quantification of DNA and determining the copy number of a transformed plasmid by comparing it with a native gene, of which only one copy exists. However, in this work no clear results (no difference between a copy number of 1 or 2) could be made. As expected, the assay carried out was less sensitive as the one described by Abad et al. (2010). Reasons for this inaccuracy were that no standard for ErgA and ErgB was available. For defined results a standard with only one single copy of the transformed plasmid has to be found. For time reasons these further developments were not carried out. As a consequence it was chosen to compare the amplified products of ErgA and ErgB directly to the amplified product ARG4. Although the amplified products had similar length and GC-contents they are not exactly the same and can therefore not be compared one to one giving only a rough assessment and imprecise results. However the assessment with real time PCR was good enough to differ between colonies containing the actual insert and those without. An alternative to real time PCR is Southern Blot. This method is very reliable but more equipment, material and time for each experiment is needed (Southern, 1975).

Concerning the quantification of copy number of *erg*B pGAPZα C signals after a Ct (cycle threshold) of 30 appeared for all negative colonies and also for native *P. pastoris*. In general, it is considered that after a Ct of 30 only by-products e.g. from unspecific primer binding or spreading of the DNA that should be amplified, can appear. It can be seen that no spreading of the DNA happened because the negative controls (water instead of DNA) were negative as expected (no fluorescence signals). Additionally, all of these colonies had nearly the same pattern of curves. Then this led to the consideration that the primers must bind somewhere else in the *Pichia* genome without ErgB being integrated. This unspecific primer binding would have led to the signals at real time PCR. Another fact was that the melting curves of the products of these colonies were similar to those of the products of ErgB3 in the other

colonies. Furthermore, gel electrophoresis of all colonies (positive and negative concerning containing the vector plus insert) showed that all samples had the same mobility. This could have happened if the unspecific product had similar length and similar GC-content as the actual product. However, BLAST-search (NCBI) has not given any results that the primers specifically bind somewhere else in the 4 chromosomes of the *Pichia* genome. Additionally, the whole sequence of the amplified product of ErgB3 also could not be found. Having said this unspecific primer binding can be excluded. The fact that signals only appeared after a Ct (cycle threshold) of 30 confirmed that the vector plus the insert are actually not in the cell of the negative control.

Apart from the just previously described problems the assay was a good tool to proof that the insert has at least been integrated once into *P. pastoris*, since colony PCR is not a reliable method. This fact was even proven. At colony PCR for ErgA all colonies being proven to be positive concerning containing the vector actually contained the plasmid. For determination of copy number all samples from the previously done colony PCR were used. Although colony PCR results showed that a few colonies were negative, i.e. they did not contain the vector and insert, real time PCR proved these results to be wrong. Additionally, colony PCR showed that one colony for ErgB contained the insert. However a negative result for this colony was gained at real time PCR. These results proof that colony PCR might be a quick method getting a rough idea of transformation results but should definitely not be the method of choice for a concrete and secure result.

Overall, it can be concluded that despite all advantages of *P. pastoris* concerning expression of foreign proteins it was not the proper expression system in this experiment. Although production of ErgA worked and its activity was proven, a higher amount of the protein was able to be expressed with an *E. coli* expression system. Regarding the production of ErgB in *P. pastoris* it can be stated that expression did not work at all. Reasons for these final results could be certain features and disadvantages of the expression system *P. pastoris* that have counter-productive effects on expression of foreign proteins such as proteolytic activities (Joshi and Sahni, 2010). Other reasons for the non-sufficient and failed production of ErgA and ErgB might lie in codon optimization. The codon optimized genes were directly used for transformation processes. Usually different codon optimized sequences are used to get a higher possibility for a positive expression of the foreign protein (Huang et al., 2012). Positive results concerning expression might also be reached with different promoters (Cereghino and Cregg, 2000, Macauley-Patrick et al., 2005), copy number variation (Cregg et al., 1985, Sreekrishna et al., 1997) and bigger screening processes (Wang et al., 2012).

Expression strains *E. coli* HMS174(DE3), BL21(DE3), BL21(DE3)pLysS, ArcticExpress(DE3), Tuner(DE3) and Rosetta 2(DE3) were not the right systems for expression of ErgB. After expression it was seen that the produced enzyme was not soluble and also contained insoluble inclusion bodies. Lowering of the expression level (Weickert et al., 1996) and fusion with other proteins (Waugh, 2005) might help making an expressed protein soluble. Reduction of expression temperature is also considered to have a positive effect (Schein and Noteborn, 1988), although in this experiment it did not work as can be seen for the expression of ErgB in *E. coli* ArcticExpress(DE3). A reason for this might be an improper folding. Although *E. coli* is frequently used because of its low costs, easy techniques and availability of multiple vectors and hosts with different features there are also a few disadvantages concerning expression of eukaryotic proteins. *E. coli* lacks accessory proteins like chaperones, posttranslational modification proteins and maturation proteins (Yin et al., 2007, Daly and Hearn, 2005, Jana and Deb, 2005). As a consequence, eukaryotic proteins might not fold properly after expression with *E. coli*.

Furthermore, in this work there was a bigger focus on the expression of ErgB in *E. coli* ArcticExpress(DE3). Based on the results described in chapter 3.3, it was thought that a soluble amount of ErgB was expressed after induction but that the enzyme had toxic effects on the metabolism of the cells leading to poor cell growth and even cell death. As a consequence the expression strain *E. coli* BL21(DE3)pLysS was used. However, another researcher at the company Biomin gained the same results with *E. coli* ArcticExpress(DE3) cells containing a plasmid with another gene. After induction the cell density also decreased extensively. This suggests that the amidase ErgB must not necessarily have a toxic effect on the metabolism but that the expression strain *E. coli* ArcticExpress(DE3) is very sensitive to any kind of foreign insert. Additionally, it can be considered that IPTG might be toxic to the cells (Zerbs et al., 2009). Expression in *E. coli* ArcticExpress(DE3) also showed a native protein band on the SDS-gels making an exact identification of ErgB impossible.

5. Conclusion

P. pastoris was chosen as a competent expression system for the production of ErgA and ErgB because of its several advantages including easy molecular and genetic manipulation, production of high levels of foreign proteins, the possibility of eukaryotic posttranslational modifications and the choice between intracellular and extracellular secretion. After transformation of the vectors with the specific inserts low amounts of ErgA and no ErgB

could be expressed. As a control for transformation colony PCR was carried out. Since false negative and false positive results are quite common for this analytical method real time PCR was carried out to determine the copy number. Although the assay was not developed to find out exact copy numbers, it still showed that a few colonies contained at least one copy of the specific plasmids. As a consequence *P. pastoris* was defined as not being appropriate for the expression of sufficient amounts of ErgA and any expression at all of ErgB.

Based on the previous results different expression strains of *E. coli* with different advantages and properties were used for the production of ErgB. Although transformation worked no soluble protein was expressed.

6. Futur aspects

Problems during identification of the specific protein on an SDS-Gel after expression posed a few problems in this work. Therefore, western blots should be considered for future experiments. They enable an easier and more specific detection of expression levels of ErgA and ErgB. For this method antibodies are needed as described above. Without the production and purification of the enzyme no specific antibodies can be produced. Alternatively antibodies against the His-tag, which is fused to each protein for the opportunity of affinity purification, can be used.

Since not enough ErgA was produced with the expression system *P. pastoris* and no positive results could be gained concerning the production of ErgB with *P. pastoris* and different strains of *E. coli*, another expression system needs to be used for the production of both enzymes leading to higher expression levels, which allow further purification and characterization. A possible system might be *Bacillus subtilis*. This organisms enables large scale productions, high cell densities, easy and inexpensive cultivation as well as high secretory capacity leading to a direct export of the expressed protein into the extracellular medium, better folding conditions and prevention of the formation of inclusion bodies (Westers et al., 2004, Vavrova et al., 2010).

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Appendix

Table 27 Sequences of ErgA and ErgB

Nucleotide Sequence	
ErgA	5'- AATGCCATTGGTTGTTTTGTCCGACGGTACTAGAATCCACGTTGAGACTTCCGGTAACGGTGTTCCAGCTTTG GTTCCTTGTGTTGGTTCTTCCGTTCCATTCGAGAGAACTTTCGGTGAAGAGTTGAAAACTGACATCCAGTACAA CTTCGTTGAGGTTAGAGGTACTTCCAGATCTGACGGTGAACCTTCTGAAGTTGCTTCCTTGGACAGAATCTCTG ACGACTTGGAAGAGGTTAGACAGTTGTTGGGTTTGGACAAGGTTATCGCTTTGGGTCAGTCCAGAAACGGT ATGATGGCTGCTCATTACGCTCAGAAGTACCCAAACTCCGTTTTGCACTTGGTTACTATCGGTACTCCAGCTTCC TTGTCCATGATCAAGAACGAAGAGTACTGGAACGCTTTCGCTGACGACGAGAGAAAGAGATTGAGAGCTGA AAACGACGCTGCTATGGAAAGAGAGGGTTTGTTGGACTTGGACAACTTGAACACTGCTGAGAAGATCGTTA GATTGTTCGATTTGGAGGGTGCTGTTTACTTCTACGACCCAACTACTTTGATGAACGACTGGTGGGACGCTTC TTTGTTGTCCAGAACTTTTGAGGTTGTTATGGCTTCCAACATGGGTTGGGCTGACTTTGATTTGGTTCAGACAT TGCAGAACTCCGACGTTCCTGCTTTCGTTACTTTCGGTAAGTACGACTTCATGGTTTCCCCATTGCCAAAGCCAG GTAACCCAGTTGATGGTAAGGCTGGTTTGTTCGAGGACATCCCAGGTGTTAGAGTTGAGGTTTTCGAGAAGT CTGGTCACTTCCCATACTGGGAACAAGAGCAAGAGTTCGCTAGAAGATACAGAGACTGGGTTGCTACTTTGCC AGAGTCTGCTGTTAGAGCTGCTGAAGCTATGACTCCAAACGGTATTAGACAACATCACCATCACCACCA-3'
ErgB	5'- CTCGAGAAGAGAGTTAGATACAAGAGAGCTTTCGCTGTTGCTATCACTGCTGGTGTTGCTTTGGCTTTGTCTG GTTGTTCTGACGAAACTTCCGCTCCACAGACTACTCCAATGACTGCTCTTCAACCAGCTTCCGCTATGCAAGGTG ATGTTGCTAATGGTGAAGCTCCATTGGAGTTGACTATCAAGCAGGTTAAGGACGCTTTTGCTCACGGTGCTTA CACTTCCGAGCAATTGACTAAGGCTTACTTGAACAGAATCGACAGATACGAGCCAGCTTACAACGCTTTGACTT TCATGAACGAGCACGCTTTGGCTGACGCTAGAGCTTCTGATGAAGCTAGAGCTGCTGGTAAGCCACAAGGTG CTTTGGCTGGAGTTCCAGTTTTGATCAAAGAAGCTATGAACACTAAGGGTTTCCCTATGACTGCTGGTTGGGC TCCATTGTCTCCAGAATTGGGTGGTTTGTCTTTGATGCCAGATAAGGACGCTCCAATGGTTAGAAGAATCAGA GATGCTGGTGCTATCATCTTGGGTAAGACTAACATCCCAGTTTTCTGTGTTAGAGGTGACGCTAACGATTCTT GGGCTGGTCCAACTTACAATGCTGTTGACAGATCCTTGATCCCAGGTGGTTCTTCTGCTGGTTCTGCTACTGCT GTTGCTGCTAACTTCGGTGTTATTGCTTTGGGTGCTGAGACTATGGGTTCCTTGACTTTGCCAGGTTCTGCTCA GTCCATCGTTGTTATGAAGCCAACTTTCGGTATGGTTGCTAACTTGGGTGACAACCCAATGGCTGTTGACACTA CTGATACTGTTGGTCCAATGACTAGATCCGTTGAGGACGCTGCTACTGTTTTGGATGTTTTGGCTGGTGTTGA TCCAGAGGACCCAAAGACTAAGGCTTCTGAAGGTCATATTCCTCCAGGTGGTTTCACTTCTTCCTTGAACAACG AATCCTTGAACGGTGCTAGAATCGGTATCTTGTCCCCAGGTTACAGAAAGGACTTGGTTAGATCCCAAGAAGA GACTGCTCTTTACGACAACGCTGTTAACGAGTTGAGAACTAGAGGTGCTACTTTGGTTGACAACGCTTTCGTT GGTTCTGGTTTGGACGGTTTGCCAAGAAACATGTTGCCAACTTCCATGACTCACGACTGGAACGAGTTCTTGA AGGACTTCTCTGGTTCCGGTATTTCCTCCACTGCTGATTTGGCTGCTAAGTTGAACCCAGAGGACTTGAAAGAA GGTTCCCCTGGAGCTTTCTTGGCTGATGATCCAGCTAAGCCAGATATGTCCGAGTACAACAACTTGGCTGACCA GTACAGAGCTGTTTTCGCTTCCATCGTTAAGCAGTACGACTTGGACGGTTTCGTTTTGCCAACTATGGTTGGTG AGATCCCTAGATTGCACCCAGATTGGGAAAAGTGGGACGACATGTTGACTAACCCAGAGGTTAACTACACTG GTATGCCATCCGTTGTTGTTCCAGCTGGTAGATACGAAAACGGTTCCCCATTTGCTGTTGCTTTCGTTGGACCA GATTGGTCCGATTCCAGAATGTTGTCTTTCGCTTACGACTACGAGCAGGCTACTCACCATAGAATCGTTCCAAC TTTGACTACTGACTTGCCATCTTCTCATCACCATCACCACCACTAATCTAGA-3'