Expression of Secreted Branched Chain Aminotransferase by *Pichia pastoris*

by

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A Thesis Submitted
in Fulfilment of the
Requirements for the Degree of

Master of Science
in Molecular Microbiology

at

Graz University of Technology

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2014

STATUTORY DECLARATION

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Acknowledgement

Hereby, I would like to thank everyone who supported and accompanied me during this thesis.

First of all I want to say thank you to Ms. Dr. Margit Winkler and Mr. Dr. Anton Glieder who made this thesis possible for me at ACIB.
Thank you to Mr. Dr. Andrea Camattari for his commitment, support and advice and that he always took time for discussions.
A big thank you to Ms. Mag. Katrin Weinhandl for the good supervision, support and the great constant help whether in the laboratory or in the analysis of data, whose interpretation and the nice acceptance in the work group.
Furthermore, I would like to thank Ms. BSc Gerlinde Offenmüller for her help in finding chemicals, instructions in diverse application systems and the great talks; simply for the fun time we had in the office.
Thank you to Mr. Dr. Helmut Schwab and all participants at the Schwab seminar as well as the people at the Tuesday breakfast for the great and productive discussions and suggestions.
I also want to say thank you to Ms. Svitlana Münzer, who autoclaved the flasks and bottles, which I have needed. Thank you for the nice talks and for the help in finding laboratory materials.

Moreover, I would like to thank my cubicle neighbours and colleagues from the 3rd floor for the pleasant time, their expertise, support and talks/discussions.

Finally, my greatest thanks is especially for my family for their support at all levels, without which I would not have been in a position to carry out the study in that way.

Abstract

The production of recombinant proteins in *Pichia pastoris* is beneficial for industrial applications. On the one hand, *Pichia pastoris* offers a good secretion efficiency and on the other hand, a high yield of biomass and thereby a high yield of protein per litre in the medium is achievable. It is desirable to secrete proteins into the medium, since *Pichia pastoris* hardly secretes endogenous proteins and therefore, it is easier and cheaper to isolate the protein of interest in high purity.

The transaminase BCAT (branched chain aminotransferase - BCAT) from *Escherichia coli* is producible as secreted protein by *Pichia pastoris* CBS7435 Mut^S. Deletion variants in respect to cell wall associated proteins were evaluated in terms of potential increase of secretion efficiency. Additionally, Och1, representing a protein involved in N-glycosylation, which affects the secretion efficiency as well, was inactivated. The aim was to make the cell wall leaky by these deletions and thereby to improve secretion.

In addition to the branched chain aminotransferase BCAT, the lipase B from *Candida antarctica* (*Ca*lB) was used as model protein for this research.

In order to determine how these gene deletions of cell wall proteins affect the enzymatic activity and the secretion of the model proteins, single knockout strains as well as double knockout strains were tested.

Additionally, a new selection marker was tested for *Pichia pastoris*. For the generation of this selection marker, the acetamidase (*amd*S) derived from *Aspergillus nidulans* was chosen as a target, since it was already known to work well for *Saccharomyces cerevisiae*. It was determined that the strong and constitutive *GAP* promoter is important for a successful selection on acetamide. Expression of acetamidase under the control of the weaker *ILV5* promoter was not sufficient for the selection of transformants.

Contents

List of Tables

List of Figures

1 Aim

The aim of this master's thesis was to optimise the secretion of IlvE, the branched chain aminotransferase (BCAT) from *Escherichia coli* by engineering the methylotrophic strain *Pichia pastoris* in terms of cell wall plasticity; in addition, in order to extend the toolbox available for *P. pastoris* modifications, a newly discovered selection marker was applied and tested and respective deletion strains were also evaluated for *Candida antarctica Ca*lB lipase secretion.

IlvE has been expressed as an intracellular recombinant protein in *P. pastoris*, but it is necessary for the industrial applications to produce BCAT in high amounts. Therefore, in order to avoid expensive and time-consuming procedures to extract IlvE, strains were engineered to express IlvE as a secreted protein. In addition, secreted enzyme has direct access to all substrates even if whole cell cultures are applied for biotransformations.

The enzyme *Ca*lB is also a protein of interest. It is known that it is well secreted and therefore, this enzyme was also chosen to be tested and to explore engineered strains of *P. pastoris* CBS7435 Mut^S.

In order to improve the expression level of BCAT, cell wall associated proteins Cwp1 (cell wall protein 1) and Scw10 (soluble cell wall protein 10), as well as the protein Och1, which is involved in glycosylation, were knocked out: the resulting strains were tested for secretion of the desired products.

An additional selection marker was also tested, since each modification requires a selection marker. In total, three selection markers were needed to pursue the goal of this thesis: strains carrying the expression cassette for *ilv*E or *ca*lB could be selected via zeocin. Selection of the knockout strains occured by geneticin or without a selection marker. Therefore, one goal was to test an alternative selection marker (*amdSYM*), which was already known in *Saccharomyces cerevisiae* to work fine allowing selection with minimal medium containing acetamide as sole nitrogen source or counter-selection (negative selection) with medium containing fluoroacetamide.

2 Introduction

2.1 *Pichia pastoris*

Pichia pastoris is a methylotrophic yeast. This organism belongs to eukaryotes and therefore, it is able to perform eukaryotic posttranslational modifications like protein glycosylations, proteolytic processing etc. One of the advantages of *P. pastoris* compared to other organisms is that a high yield of biomass can be obtained during cultivation. Manipulation of *P. pastoris* can also be easily performed and cultivation is simple and inexpensive, which is advantageous (Cregg et al., 2000).

The methylotrophic yeast is able to use methanol as carbon and energy source. An advantage is that methanol is used for a strong induction of expression, whereas for example in large-scale procedures the disadvantages are high heat production and substantial need of oxygen (Curvers et al., 2001). In order to metabolise methanol and gain energy, the enzymes alcohol oxidase 1 and 2 are needed. These enzymes are encoded by the genes *AOX1* and *AOX2*. The *AOX1* promoter (P_{AOX1}) is a strong promoter, whereas the *AOX2* promoter (P_{AOX2}) is a weaker one. Both can be induced by methanol. It is known that these promoters and thus, also in further succession gene expression is tightly regulated (Cregg et al., 1989; Cregg et al., 1987).

An alternative option to the P_{AOX1} in order to express genes is the constitutive *GAP* promoter (glyceraldehyde-3-phosphate dehydrogenase promoter - P_{GAP}), which normally initiates the expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Waterham et al., 1997).

P_{AOX1} is tightly regulated, as already mentioned. In the presence of glucose or glycerol the promoter is repressed, in methanol it is induced. This is a big advantage because the expression of heterologous genes, which are toxic to the cell, can be regulated. Therefore, for the expression of toxines, the cells can be cultivated under repressed conditions to a certain level of biomass and for example at the stationary phase, expression can be initiated by induction. However, in spite of high yields of recombinant proteins, there are some disadvantages when using P_{AOX1}. Since methanol is flammable and high heat production can occur in large-scale use, P_{GAP} is an advisable alternative (Curvers et al., 2001; Tschopp et al., 1987; Waterham et al., 1997).

In order to secrete heterologous proteins, a secretion signal is needed. The most frequently used secretion signal is the prepro signal sequence of the α-mating factor from *Saccharomyces cerevisiae*. It is much easier and cheaper to isolate and purify the proteins of interest compared to secreted proteins in *S. cerevisiae*, because there is hardly any protein background in *P. pastoris*. Therefore, *P. pastoris* is preferably used for recombinant protein production. Additionally, secretion of heterologous proteins is the preferred option for production. An alternative would be to accumulate the proteins in the cytoplasm, but then

elaborate and probably expensive procedures are necessary in order to receive as pure protein as possible (Cregg et al., 2000; De Schutter et al., 2009; Kueberl et al., 2011).
Secretion might be also of interest for whole cell conversion systems in order to have a continuous ongoing reaction process for the extracellular production of a protein of interest, where it can be easily isolated.
Nowadays, this organism is one of the standard tools for generating recombinant proteins, also in large-scale. The use of the organism *P. pastoris* is increasing in the pharmaceutical industry, for example to produce drugs, biopharmaceutical proteins and a majority of chemicals. This yeast is widely used for recombinant protein production in order to do basic research and to investigate medical applications (Cregg et al., 2000).

2.2 Recombinant protein expression

In this thesis, the strain *P. pastoris* CBS7435 Mut^S was used as a platform for all studies (Naeaetsaari et al., 2012). As in the previous chapter already mentioned, *P. pastoris* belongs to the methylotrophic yeasts and therefore, it is able to grow on methanol as sole carbon source. Mut^S stands for ``methanol utilisation slow´´, because in this strain the *AOX1* gene is deleted. Compared to *P. pastoris* CBS7435, which contains a functional *AOX1* gene, *P. pastoris* CBS7435 Mut^S is reduced in growth and consumes less methanol during induction. However, it was also reported that *P. pastoris* CBS7435 Mut^S is beneficial in producing heterologous proteins compared to the wild type strain (Krainer et al., 2012).
The alcohol oxidase 1 (Aox1), encoded by the gene *AOX1*, performs the initial step of the methanol utilisation (MUT) pathway together with Aox2. These enzymes oxidise methanol to formaldehyde and reduce oxygen to hydrogen peroxide. Subsequently, formaldehyde can be either oxidised to carbon dioxide (methanol dissimilation pathway), generating NADH molecules or metabolised to dihydroxyacetone and glyceraldehyde-3-phosphate (GAP) (methanol assimilation pathway). The produced NADH is used in order to produce energy, which the yeast further needs for growth (Hartner and Glieder, 2006; Schroer et al., 2010). In order to produce high levels of the desired recombinant proteins, genes encoding for the particular model proteins were cloned into the shuttle vector pPpT4 α S downstream the *AOX1* promoter and the α factor secretion signal from *S. cerevisiae*. The produced constructs were linearised and transformed into *P. pastoris* CBS7435 Mut^S. The linear fragment integrated into the genome by homologous or random recombination. In the presence of glucose or glycerol the expression of enzymes which are involved in the MUT pathway are repressed. Whereas, expression of those enzymes are induced when only methanol is present or by addition of methanol when glucose/gycerol is already depleted (Hartner and Glieder, 2006). The Mut^S was used because it allows a controlled expression of

the desired model proteins by methanol induction even in deep well plates where no continuous and controlled methanol feed is feasible.

2.3 Model proteins

This project was focused on engineering *P. pastoris* strains in order to obtain high titres of extracellular BCAT and *Ca*lB as the respective model proteins. It was considered to use *P. pastoris* knockout strains, where the corresponding proteins are involved in cell wall assembly or morphogenesis, in order to evaluate if this supports high titres of the model proteins in the supernatant.

2.3.1 Branched chain aminotransferase (BCAT)

Transaminases are well characterised and very important enzymes for prokaryotic and eukaryotic metabolism. In general, transaminases with a broad substrate specificity are becoming increasingly interesting, due to the synthesis of natural as well as unnatural or nonproteinogenic amino acids (Taylor et al., 1998). These unnatural amino acids, for example TBG (L-tert-butylglycine), are important building blocks for the synthesis of pharmaceuticals (Hong et al., 2010). The branched chain aminotransferase belongs to the L-α-amino acid transaminases and is a type IV pyridoxal-5-phosphate dependent enzyme. BCAT is able to use many substrates for the transaminase reaction, for example L-glutamate, L-leucine, L-valine and L-isoleucine (Inoue et al., 1988; Lee-Peng et al., 1979; Taylor et al., 1998) - basically all branched-chain amino acids. The branched chain aminotransferase is encoded in *E. coli* by the gene *ilv*E. The enzyme performs the co-substrates‘ transamination as a multimer (hexamer) of identical subunits. The molecular weight of the monomer is around 34 kDa (Inoue et al., 1988; Taylor et al., 1998). In this thesis a codon-optimised *ilv*E gene was used. The corresponding protein has a calculated mass (http://molbiol.ru/ger/scripts/01_06.html) of 34.4 kDa per subunit.

This codon-optimised gene was cloned into the vector pPpT4 α S, which was expressed under the control of the *AOX1* promoter. The sequence of the optimised *ilv*E gene as well as the vector map (pPpT4 α S containing *ilv*E) are shown in S 1 and S 3 in ``Supplementary Materials´´.

In previous in-house studies, we found out that the TBG production in a whole cell conversion system did not work as well as expected. For the pharmaceutical industry it is important to produce high amounts of the protein branched chain aminotransferase BCAT in order to produce like already mentioned TBG or other non-natural L-amino acids. Therefore, *ilv*E was expressed as a secreted protein. The activity of this enzyme was measured by a colorimetric assay procedure (Weinhandl et al., 2012). In figure 1, there is depicted the screening method for BCAT with all involved enzymatic reactions. This assay procedure was

investigated by Weinhandl et al. (2012). L-Leucine was used as substrate and α-ketoglutarate as co-substrate in order to produce L-glutamate and 4-Me-2-oxopentanoic acid by BCAT. After producing L-glutamate, this co-product was oxidised by L-glutamate oxidase. In this reaction hydrogen peroxide and ammonia were released. Subsequently, ABTS was oxidised by HRP, whereby hydrogen peroxide served as electron donor. BCAT activity could be determined due to the resulting colour change (oxidised ABTS).

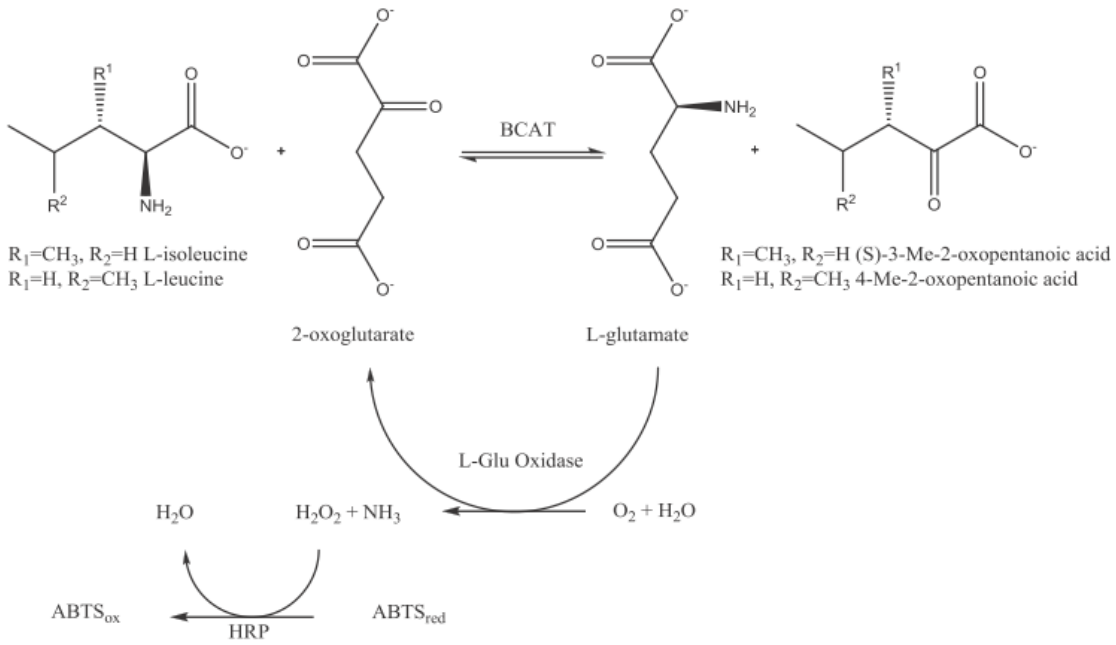

Figure 1: Colorimetric assay procedure for BCAT. In general, BCAT can syntesise L-glutamate by using e.g. L-leucine or L-isoleucine and α-ketoglurate as substrate and co-substrate. L-glutamate oxidase oxidises the co-product L-glutamate and releases H_2O_2 and NH_3. Subsequently, HRP oxidises ABTS and H_2O is released. Source: Weinhandl et al., 2012

2.3.2 *Candida antarctica* lipase B (*Ca*lB)

Candida antarctica lipase B (*Ca*lB) belongs to triacylglycerol lipases and has a α/β-hydrolase fold. It does not display interfacial activation or offers a lid domain in order to cover the active site (Martinelle et al., 1995; Overbeeke et al., 2000; Rotticci et al., 2000). The protein *Ca*lB is an important biocatalyst for many applications in industry (Blank et al., 2006) and, it is used for the production of building blocks in pharmaceuticals (Sharma et al., 2001). According to Rotticci-Mulder et al. (2001) *Ca*lB has a calculated molecular weight of 33 kDa and when expressed in *P. pastoris*, the heterologous protein was determined as a 36 kDa protein. With the help of experimental procedures involving endoglycosidase H (Endo H) and endoglycosidase F, it was detected that *Ca*lB is N-glycosylated and, thus, was leading to the higher molecular weight (Rotticci-Mulder et al., 2001).

C. antarctica expresses another lipase as well, lipase A (CalA). Although those two enzymes originate from the same organism, they are different relating to thermostability and substrate

specificity, for example *Ca*lB is calcium independent and less thermostable, whereas CalA is calcium dependent and highly thermostable (Anderson et al., 1998).

A codon-optimised *ca*lB gene (S 2) was used in this thesis. The corresponding protein has a calculated mass (http://molbiol.ru/ger/scripts/01_06.html) of 35.3 kDa instead of 33 kDa as reported in the literature.

As for BCAT, the codon-optimised gene was cloned into the vector pPpT4 α S, which was placed under the control of the *AOX1* promoter (S 4).

This enzyme was used as a model protein, because it is known to be well secreted, but known to be problematic in respect to folding (Abad et al., 2010).

A simple colorimetric assay can be used for screening. The reaction and the involved components are shown in figure 2. para-Nitrophenyl butyrate (pNPB) was used as substrate, which was converted to para-nitrophenol and butyric acid by *Ca*lB (Sigma-Aldrich, 2013).

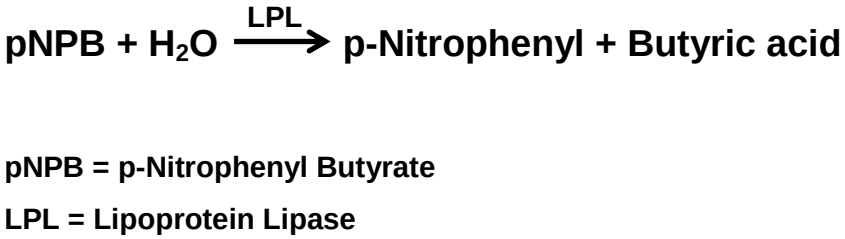

Figure 2: Colorimetric assay for *Ca*lB. *Ca*lB converts pNPB to para-nitrophenol and butyric acid. This can be measured colorimetrically. Source: Sigma-Aldrich, 2013

2.4 Cell wall composition in *Saccharomyces cerevisiae*

In this thesis the main focus was the exploration of strains with deleted cell wall protein genes. The basic idea behind this concept was to make the cell wall leaky in order to secrete more protein. Therefore, it was necessary to acquire knowledge about the cell wall assembly and morphogenesis. However, in comparison with *S. cerevisiae*, little is known about the cell wall composition and dynamics in the methylotrophic yeast, *P. pastoris*. Thus, literature was searched for information about the cell wall of *S. cerevisiae*, because this organism is well characterised and well understood. The literature research should serve as a general overview in order to get a better understanding for cell wall composition in yeast. As already mentioned, there is hardly any literature available about the *P. pastoris* cell wall and therefore, in this chapter the cell wall of *S. cerevisiae* is explained.

S. cerevisiae has a bilayered cell wall, which consists of three major components. These are mannoproteins, β-glucans and chitin. The inner layer of the cell wall contains glucans and chitin and is responsible for the mechanical strength. In contrast, the outer layer of the cell wall exhibits mannoproteins and this external layer provides limited access for example to foreign enzymes.

β-glucans consist of β-1,3-glucan chains, which are moderately branched. These branches consist of covalently linked β-1,6-glucan residues. Chitin is build up of β-1,4-linked N-acetylglucosamine (GlcNAc) residues and it is a linear polymer. It mainly appears at the bud neck and at the septum and to a lesser degree in the lateral walls. The chitin chains, with their reducing ends, are linked to the non-reducing ends of β-1,3-glucan. These linkages are present in the bud neck, whereas in the lateral walls, the reducing ends of the chitin chains are linked to the non-reducing ends of β-1,6-glucan. Furthermore, there are mannoproteins, which form the outer layer of the cell wall. These mannoproteins can either be N-glycosylated or O-mannosylated. Moreover, these cell wall mannoproteins can possess a glycosylphosphatidylinostol (GPI) molecule. Proteins containing a GPI anchor are called GPI-dependent cell wall proteins (GPI-CWPs). These GPI-CWPs are anchored to the outer cell wall layer. The linkage occurs indirectly to β-1,3-glucan, because they are attached to β-1,6-glucan, which on the other hand is linked to β-1,3-glucan and chitin. However, there are also proteins present, which are not carrying a GPI anchor. These proteins are called Pir proteins (Pir-CWPs). In contrast to the GPI-CWPs, the Pir-CWPs have a direct linkage to β-1,3-glucan (Klis et al., 2002; Lesage and Bussey, 2006; Orlean, 2012).

The cell wall acts as a barrier. On the one hand, the cell wall protects the cell against for example environmental influences and on the other hand, it is responsible for limiting the export of proteins into the medium.

2.5 *Pichia pastoris* knockout strains

In order to determine if the secretion of either BCAT or *Ca*lB can be optimised, three different knockout strains were tested in this thesis. Since little is known about the cell wall of *P. pastoris,* literature research was done regarding cell wall composition in *S. cerevisiae*. *S. cervisiae* is already well studied and therefore, in order to get an overview of the diverse components of the cell wall, which may have an effect in secretion, literature from *S. cerevisiae* was chosen. There are many suitable targets for gene deletions, which are involved in cell wall assembly or morphogenesis and further can lead to an improved secretion like for example the chitin synthase 3 (Chs3), which plays a major role in the chitin synthase pathway (Lesage et al., 2005; Merzendorfer, 2011).

Nevertheless, we decided to concentrate on the knockout strains, which were already available in-house.

The methanol-induced *P. pastoris* CBS7435 Mut^S was treated with tunicamycin (glycosylation inhibitor). The supernatant was loaded onto a gel, where two bands were detected. These two proteins were identified as Cwp1 and Scw10 by MS/MS (tandem mass spectrometry). Sequence comparisons have shown that these two proteins were potential cell wall proteins. Δ*cwp1* and Δ*scw10* strains were produced and it was investigated that

these two gene deletions effect cell wall behaviour. Furthermore, these two gene deletions may influence the secretion of proteins (A. Camattari, unpublished personal communication).

2.5.1 *Pichia pastoris* CBS7435 Mut^S Δ*cwp1*

The cell wall protein 1 (Cwp1) was first identified in *S. cerevisiae* by Shimoi et al. (1995). The gene *CWP1* encodes for a cell wall mannoprotein (Zhang et al., 2008) containing a hydrophobic sequence in the N- as well as C-terminus. In the N-terminus the hydrophobic sequence was detected as a signal sequence for secretion (Shimoi et al., 1995). The hydrophobic sequence in the C-terminus was identified as a signal for glycosylphosphatidylinostol (GPI)-anchorage. In previous experiments it was established that a deletion in this hydrophobic sequence resulted in secretion of this protein. This revealed that this C-terminal hydrophobic sequence is important for linkage to the cell wall (Ram et al., 1998; Shimoi et al., 1995). Additionally, proteins, which are linked to the cell wall by a GPI-anchor, are usually rich in serine and threonine residues. These residues are an indication for O-glycosylation (Zhang et al., 2013). Moreover, it was investigated that the deletion of *CWP1* is not lethal, as there are other homologous genes present, which can complement the function of Cwp1 (Shimoi et al., 1995). In *P. pastoris* the protein Cwp1 was determined as well, however, it has just a low similarity to *S. cerevisiae* (27.4 % similarity and 16.8 % identity). It was detected that the Δ*cwp1* strain was more resistant to zymolyase and osmotic stress compared to *P. pastoris* CBS7435 Mut^S (A. Camattari, unpublished personal communication).

So far, no enzymatic function is known for Cwp1 in several organisms. However, it is assumed to be a potential β-glucanase (A. Camattari, personal communication).

According to these data, it was of great interest to determine how far this gene deletion influences secretion of heterologous proteins.

2.5.2 *Pichia pastoris* CBS7435 Mut^S Δ*scw10*

In 1998, the soluble cell wall proteins 4 and 10 (Scw4 and Scw10) were identified in *S. cerevisiae.* These two proteins are homologue to each other and share a 63 % identity. Furthermore, both proteins have a signal sequence for ER-translocation, a Kex2 processing site, but both do not exhibit a GPI anchoring sequence. Moreover, it could be verified that these two proteins act in a synergistic manner. In the case of a double deletion in *S. cerevisiae* (Δ*scw10*/Δ*scw4*), cells showed differences in cell morphology, for example abnormal cell shape including increased size or a decrease in growth rate compared to wild type (Cappellaro et al., 1998). According to Sestak et al. the genes *SCW10* and *SCW4* encode for cell-wall glucanases/transglucosidases, which play an important role in cell wall assembly and further cell wall integrity in order to keep the cell wall on the one hand rigid and

on the other hand flexible. An increased chitin level as well as an alkali-soluble glucan level could be measured in a Δ*scw10*/Δ*scw4* double mutant. In addition, these mutants were more resistant to zymolyase as the wild type (Sestak et al., 2004). A putative exo-β-1,3-glucanase was identified in *P. pastoris*, which was homologous to Scw10 in *S. cerevisiae* (65.6 % similarity and 52.6 % identity). However, no homologue of Scw4 was detectable in *P. pastoris* via BLASTP. Nevertheless, *P. pastoris* with a deletion of *SCW10* showed also a higher resistance to zymolyase (A. Camattari, unpublished personal communication) like it could be detected in the Δ*scw*10/Δ*scw*4 double mutant of *S. cerevisiae* (Sestak et al., 2004). Therefore, this was also a knockout strain of interest because a deletion of *SCW10* resulted in a change of cell wall behaviour.

2.5.3 *Pichia pastoris* CBS7435 Mut^S Δ*och1*

The gene ``outer chain elongation 1 (*OCH1*)´´ encodes for an α-1,6-mannosyltransferase. The first discovery and investigation of this gene took place in *S. cerevisiae* (Nagasu et al., 1992; Nakayama et al., 1992). In the Golgi apparatus this enzyme triggers the hypermannosylation reaction of secreted proteins with the result of hyperglycosylated protein species at high heterogeneity level (Krainer et al., 2013). As it is already known in yeasts as well as fungi, they have the ability to alter glycoproteins with heterogeneous high-mannose glycan structures. This is disadvantageous for example for therapeutic products, since these heterogeneous proteins can trigger immune responses. Therefore, it is desirable to produce a strain which is able to secrete homogeneously glycosylated proteins (De Pourcq et al., 2010; Vervecken et al., 2004). According to Krainer et al. this purpose could be achieved by deleting the gene *OCH1.* Changes occuring by this deletion are an impaired growth compared to the *P. pastoris* wild type strain. Moreover, it could be observed that this knockout strain shows an abnormal cell morphology when grown on YPD agar plates. The chitin level in this knockout strain was investigated as well. It was determined that *P. pastoris* CBS7435 Mut^S Δ*och1* and *P. pastoris* CBS7435 Mut^S have approximately the same level of chitin, whereby the localisation of chitin differs: in the wild type strain chitin is localised mainly at the bud scar, whereas in the knockout strain it could be detected in the overall lateral cell wall (Krainer et al., 2013). Therefore, this strain was chosen as well in order to determine which effects this deletion has relating to the activity of BCAT and *Ca*lB.

2.6 *amdSYM* selection marker

There are many selection markers available in order to select genetic manipulations in yeasts. Selection markers can be categorised into auxotrophic and dominant markers. Nowadays, the use of recyclable selection markers is common. This can notably be explained by the fact that for every introduced gene modification a selection marker is essential, in order to determine if a manipulation occured or not. Therefore, recyclable selection markers are advantageous for multiple manipulations, because it can be used many times (Solis-Escalante et al., 2013).

There are several methods to remove a selection marker. A quite common method is the α recombination system derived from the bacteriophage P1. In order to use this system for removal, the marker cassette needs to be flanked by *lox*P sites. Cre-recombinase recognises those sites and catalyses the excision of the marker gene. However, Cre-*lox*P recombination system also has drawbacks. One of these limitations for example is that another transformation step is required to introduce a Cre-recombinase containing plasmid, and for that purpose a selection marker is needed as well. Moreover, Cre-*lox*P-based methods are time-consuming: on the one hand, it takes a while to select the plasmids containing the gene encoding for the Cre-recombinase and the deletion cassette. On the other hand, it is necessary for the strains to lose the plasmid containing the *cre* gene after marker removal. This kind of recyclable markers can just be used a limited time for a strain. With every removal of the marker, a single repeat (*lox*P site) will be left at the integration site, thus causing serious rearrangements in the chromosome (Delneri et al., 2000; Gueldener et al., 1996; Gueldener et al., 2002; Sauer, 1987).

Since in this thesis not only single but also double knockouts were created, more than one selection marker was necessary. In order to select for example double knockout strains containing the gene of one of the used model proteins, we thought to test another selection marker than those commonly used at ACIB with the advantage of counter-selection. This was the *amdSYM* marker, which was already known to work fine in *S. cerevisiae* (Solis-Escalante et al., 2013). The plasmid pUG-amdSYM is available at EUROSCARF (European *Saccharomyces Cerevisiae* Archive for Functional Analysis), managed by the Institute of Microbiology in Frankfurt, Germany (Euroscarf, 2009).

The plasmid contains the *TEF2* promoter and terminator from *Ashbya gossypii*. *S. cerevisiae* and *A. gossypii* have a surprising number of properties in common and are similar in several aspects. Moreover, those two organisms are related, which can be indicated in the similar susceptibility of the cell wall to glucanases (Steiner and Philippsen, 1994).

pUG-amdSYM offers a codon-optimised version of the acetamidase (*amd*S) gene from *Aspergillus nidulans* and it also exhibits *lox*P recombination sites. Cells, which have integrated this marker into the genome, express acetamidase and thus are able to use

acetamide as a source of nitrogen or carbon (Hynes, 1978; Read et al., 2007), as acetamidase converts acetamide to acetate and ammonia (Hynes and Pateman, 1970). When the marker is removed, counter-selection can be easily performed. Cells are able to grow on medium containing fluoroacetamide, a homologue of acetamide. Cells, which contain this marker, cannot grow on medium with fluoroacetamide, because acetamidase will convert it to the toxic compound fluoroacetate, and the cells would die (Van Ooyen et al., 2006). This extraordinary tool was analysed in wild, industrial and laboratory *Saccharomyces* strains and it could be proven that it works in all tested strains. As a result, it allows access to manipulate *Saccharomyces* strains genetically in a fast and simple way (Solis-Escalante et al., 2013).

3 Materials and Methods

3.1 Materials

3.1.1 Strains

Table 1: Strains used

Abbreviation	Strain name	Genotype	Source
EC Top 10	*Escherichia coli* Top10F'	F'{*lac*I^{q}Tn10(Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen™, Lofer, Austria
MutS	*Pichia pastoris* CBS7435 Mut^S	Δ*aox1*	3445*
MutS F10	*Pichia pastoris* CBS7435 Mut^S AOX α *ilv*E F10 (2 copies of *ilv*E)	pPpT4 α S, *ilv*E, Zeo^R	6436*
MutS H3	*Pichia pastoris* CBS7435 Mut^S AOX α *ilvE* F10 (1 copy of *ilv*E)	pPpT4 α S, *ilv*E, Zeo^R	produced during thesis***
MutS E8	*Pichia pastoris* CBS7435 Mut^S AOX α *ca*lB E8 (1 copy of *ca*lB)	pPpT4 α S, *ca*lB, Zeo^R	produced during thesis***
MutS Δ*scw10*	*Pichia pastoris* CBS7435 Mut^S Δ*scw10*	Δ*scw10*, Gen^R protein ID: XP_002489571.1	7143*
MutS Δ*cwp1*	*Pichia pastoris* CBS7435 Mut^S Δ*cwp1*	Δ*cwp1*, Gen^R protein ID: XP_002492554.1	7142*
MutS Δ*och1*	*Pichia pastoris* CBS7435 Mut^S Δ*och1*	Δ*och1*	6815*
MutS Δ*och1*/Δ*scw10*	*Pichia pastoris* CBS7435 Mut^S Δ*och1*/Δ*scw10*	Δ*och1*, Δ*scw10*, Gen^R	ST815**
MutS Δ*och1*/Δ*cwp1*	*Pichia pastoris* CBS7435 Mut^S Δ*och1*/Δ*cwp1*	Δ*och1*, Δ*cwp1*, Gen^R	ST793**
MutS Δ*cwp1* C11	*Pichia pastoris* CBS7435 Mut^S Δ*cwp1* AOX α *ilv*E C11	Δ*cwp1*, pPpT4 α S, *ilv*E, Zeo^R, Gen^R	ST796**
MutS Δ*cwp1* F11	*Pichia pastoris* CBS7435 Mut^S Δ*cwp1* AOX α *ilv*E F11	Δ*cwp1*, pPpT4 α S, *ilv*E, Zeo^R, Gen^R	ST797**
MutS Δ*cwp1* E7	*Pichia pastoris* CBS7435 Mut^S Δ*cwp1* AOX α *ca*lB E7	Δ*cwp1*, pPpT4 α S, *ca*lB, Zeo^R, Gen^R	ST798**
MutS Δ*cwp1* H6	*Pichia pastoris* CBS7435 Mut^S Δ*cwp1* AOX α *ca*lB H6	Δ*cwp1*, pPpT4 α S, *ca*lB, Zeo^R, Gen^R	ST799**
MutS Δ*och1* B12	*Pichia pastoris* CBS7435 Mut^S Δ*och1* AOX α *ilv*E B12	Δ*och1*, pPpT4 α S, *ilv*E, Zeo^R	ST805**
MutS Δ*och1* A4	*Pichia pastoris* CBS7435 Mut^S Δ*och1* AOX α *ilv*E A4	Δ*och1*, pPpT4 α S, *ilv*E, Zeo^R	ST804**
MutS Δ*och1* A3	*Pichia pastoris* CBS7435 Mut^S Δ*och1* AOX α *ca*lB A3	Δ*och1*, pPpT4 α S, *ca*lB, Zeo^R	ST806**
MutS Δ*och1* B7	*Pichia pastoris* CBS7435 Mut^S Δ*och*1 AOX α *ca*lB B7	Δ*och*1, pPpT4 α S, *ca*lB, Zeo^R	ST807**
MutS Δ*scw10* B7	*Pichia pastoris* CBS7435 Mut^S Δ*scw10* AOX α *ilv*E B7	Δ*scw10*, pPpT4 α S, *ilv*E, Zeo^R, Gen^R	ST800**
MutS Δ*scw10* H3	*Pichia pastoris* CBS7435 Mut^S Δ*scw10* AOX α *ilv*E H3	Δ*scw10*, pPpT4 α S, *ilv*E, Zeo^R, Gen^R	ST801**
MutS Δ*scw10* B2	*Pichia pastoris* CBS7435 Mut^S Δ*scw*10 AOX α *calB* B2	Δ*scw10*, pPpT4 α S, *calB*, Zeo^R, Gen^R	ST802**

MutS Δ*scw10* A4	*Pichia pastoris* CBS7435 Mut^S Δ*scw10* AOX α *cal*B A4	Δ*scw10*, pPpT4 α S, *cal*B, Zeo^R, Gen^R	ST803**
MutS Δ*och1*/Δ*scw10* F9	*Pichia pastoris* CBS7435 Mut^S Δ*och1* Δ*scw10* AOX α *calB* F9	Δ*och1*, Δ*scw10*, pPpT4 α S, *calB*, Zeo^R, Gen^R	ST813**
MutS Δ*och1*/Δ*scw10* G8	*Pichia pastoris* CBS7435 Mut^S Δ*och1* Δ*scw10* AOX α *cal*B G8	Δ*och1*, Δ*scw10*, pPpT4 α S, *cal*B, Zeo^R, Gen^R	ST814**
MutS Δ*och1*/Δ*cwp1* B11	*Pichia pastoris* CBS7435 Mut^S Δ*och1* Δ*cwp1* AOX α *cal*B B11	Δ*och1*, Δ*cwp1*, pPpT4 α S, *cal*B, Zeo^R, Gen^R	ST794**
MutS Δ*och1*/Δ*cwp1* A8	*Pichia pastoris* CBS7435 Mut^S Δ*och1* Δ*cwp1* AOX α *cal*B B11	Δ*och1*, Δ*cwp1*, pPpT4 α S, *cal*B, Zeo^R, Gen^R	ST795**

* strain collection data base from the institute of molecular biotechnology
** strain collection data base from ACIB
*** created during thesis, but not recorded in the strain collection data base

3.1.2 Vectors

Table 2: Vectors used

Vector name	Characteristics	Source
pJET1.2/blunt ligated with 1,500 bp fragments covering the 5‘ and 3‘ region of arms of Δ*scw10* and Δ*cwp1*, respectively	origin of replication, P_{lacUV5}, T7 promoter, Amp^R	produced by Dr. Camattari**
pPpT4 α S	F- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK-, mK+) *pho*A *sup*E44 λ- thi-1 *gyr*A96 *rel*A1 Zeo^R, shuttle vector for either *E. coli* (pUC origin) or *Pichia* expression (AOX promoter), α factor secretion signal of *S. cerevisiae*	6071*
pPpT4 GAP S	F- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK-, mK+) *pho*A *sup*E44 λ- thi-1 *gyr*A96 *rel*A1 Zeo^R, shuttle vector for either *E. coli* (pUC origin) or *Pichia* expression (*GAP* promoter)	6072*
pUC19	F´ *tra*D36 *pro*AB+ *lac*Iq *lac*ZΔM15 Δ(*pro-lac*AB) *sup*E *hsd*R17 *rec*A1 *gyr*A96 thi *end*A1 *rel*A1 λ-	730*
pUG-amdSYM	bacteriophage P1 cre-*loxP*, P_{TEF}, Amp^R (*E. coli*)	EUROSCARF, Frankfurt, Germany

* strain collection data base from the institute of molecular biotechnology
** produced by Dr. Camattari, but not recorded in the strain collection database

3.1.3 Restriction enzymes

Table 3: Restriction enzymes used

Restriction enzyme	Characteristics	Cutting region	Source
*Bgl*II	10 U/µL	A^GATCT	Thermo Scientific – Austria GmbH, Vienna, Austria
*Not*I	10 U/µL	GC^GGCCGC	Thermo Scientific – Austria GmbH, Vienna, Austria
*Sfi*I	10 U/µL	GGCCNNNN^NGGCC	Thermo Scientific – Austria GmbH, Vienna, Austria
*Sma*I	10 U/µL	CCC^GGG	Thermo Scientific – Austria GmbH, Vienna, Austria
*Smi*I (*Swa*I)	10 U/µL	ATTT^AAAT	Thermo Scientific – Austria GmbH, Vienna, Austria
*Spe*I (*Bcu*I)	10 U/µL	A^CTAGT	Thermo Scientific – Austria GmbH, Vienna, Austria

3.1.4 Other enzymes

Table 4: Other enzymes used

Enzyme	Characteristics	Source
L-Glutamate oxidase	5 U/mg solid	Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany
Dream Taq polymerase	5 U/µL	Thermo Scientific – Austria GmbH, Vienna, Austria
Peroxidase VI-A from horseradish (HRP)	1,000 U/mg solid	Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany
Phusion® high-fidelity DNA polymerase	2 U/µL	Thermo Scientific – Austria GmbH, Vienna, Austria or New England Biolabs, Ipswich, USA
RNase A	10 mg/mL	Qiagen GmbH, Hilden, Germany
T4 DNA ligase	5 U/µL	Thermo Scientific – Austria GmbH, Vienna, Austria
T5 exonuclease	10 U/µL	Biozym BioTech Trading GmbH, Vienna, Austria
Taq DNA ligase	40 U/µL	New England Biolabs, Ipswich, USA

3.1.5 Markers

Table 5: Markers used

MassRuler™ DNA Ladder, Mix ready-to-use	Thermo Scientific – Austria GmbH, Vienna, Austria
PageRuler™ Prestained Protein Ladder	Thermo Scientific – Austria GmbH, Vienna, Austria

3.1.6 Selection markers

All substances were filter sterilised.

Table 6: Selection markers used

Name	Stock solution	Organism	Source
Acetamide	6 g/L	*P. pastoris*	Sigma-Aldrich Corp., St. Louis, USA
Ampicillin sodium salt	100 mg/mL	*E. coli*	Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany
G-418 disulphate (geneticin)	100 mg/mL	*P. pastoris*	ForMedium™, Norfolk, UK
Kanamycin sulphate	100 mg/mL	*E. coli*	Carl Roth GmbH, Karlsruhe, Germany
Zeocin™ powder	100 mg/mL	*E. coli, P. pastoris*	InvivoGen, San Diego, USA

3.1.7 Primers used for amplification and colony PCR

Table 7: Primers used for amplification and colony PCR

Primer n°	Primer name	Use	Sequence
P13047	KanMx6 cassette_F	Colony PCR of MutS Δ*och1*/Δ*cwp1*	GATCCTTCAGTAATGTCTTGTTTC
P13048	KanMx6 cassette_R	Colony PCR of MutS Δ*och1*/Δ*cwp*1 and MutS Δ*och*1/Δ*scw10*	CTAAGGTAATCAGATCCAAGTTTC
P13194	0337_chr_F	Colony PCR of MutS Δ*och1*/Δ*cwp1*	GGAACTGTAACGGCAAGA
P13197	0229_chr_R	Colony PCR of MutS Δ*och1*/Δ*scw10*	CAGAAAGAGATTGACGAAG
P13413	0337out_Arm1_F	Colony PCR of MutS Δ*och1*/Δ*cwp1*	TGTTGGTATTGCCTTGGAA
I10005	GAPf	Colony PCR of *E. coli* pPpT4 GAP S/*amd*S	AAAGGCGAACACCTTTCC
I10006	AOX1TTr	Colony PCR of *E. coli* pPpT4 GAP S/*amd*S	ATAGGCTGATCAGGAGCAAG
I13030	amdS (T4)_fw	Amplification of *amd*S gene	GACTAAACCGATCCATGCCACAATCTTGGGAAG
I13031	amdS (T4)_rv	Amplification of *amd*S gene	GTAAGGTGTCAATTGCTCGAGTTATGGAGTAACAACGTTACC
I13032	resT4_fw	Amplification of pPpT4 α S	AGCAATTGACACCTTACGATTATTTAGAGAG
I13033	resT4_rv	Amplification of pPpT4 α S	GGATCGGTTTAGTCCTCCTTACACCTTGTCG
I13065	amdS_Spe1_fwd	Amplification of *amd*S gene	AAAACTAGTAGATCCATGCCACAATCTTGGGAAG
I13066	amdS_Not1_rev	Amplification of *amd*S gene	AAAGCGGCCGCCTCGAGTTATGGAGTAACAACGTTACC

3.1.8 Primers used for real-time PCR

Table 8: Primers used for real-time PCR

Primer n°	Primer name	Sequence
P09121	aox1_fw_RT	GAAGCTGCCCTGTCTTAAACCTT
P09122	aox1_rv_RT	CAAAAGCTTGTCAATTGGAACCA
P09123	ARG4-RTfw	TCCTCCGGTGGCAGTTCTT
P09124	ARG4-RTrv	TCCATTGACTCCCGTTTTGAG

3.1.9 Primers used for sequencing

Table 9: Primers used for sequencing

Primer n°	Primer name	Sequence
P13048	KanMx6 cassette_R	CTAAGGTAATCAGATCCAAGTTTC
P13195	0337_ARM2_R	ATGTTAGGCTTGAGAAGGA
P13196	0229_ARM1_F	GAAAAAATTGAACTCGGGG
P13330	POXpILV5_R	ACTACGGGTGGAATGTTTGG
P13414	Seq0337_Arm1_R	TAAGATTGTGCTGTTCAAGG

3.1.10 Instruments and devices

Table 10: Instruments and devices used

Instrument type	Instrument name	Source
Analytical balance	Electronic Balance ABS 220-4	Kern & Sohn GmbH, Balingen, Germany
Autoclave	V150	Systec GmbH, Wettenberg, Germany
Bench scale	FCB 3K0.1	Kern & Sohn GmbH, Balingen, Germany
Centrifuge (Rotor JA 10)	Avanti J-20XP Centrifuge	Beckman Coulter GmbH; Vienna, Austria
Centrifuge	Centrifuge 5415 R	Eppendorf AG, Hamburg, Germany
Centrifuge	Centrifuge 5810 R	Eppendorf AG, Hamburg, Germany
Certoclave	Certoclave	Certoclave Steriliser GmbH, Traun, Austria
Electrophoresis cell	Sub-Cell® GT	Bio-Rad Laboratories GmbH, Vienna, Austria
Electrophoresis instruments	PowerPac™ Basic Power Supply	Bio-Rad Laboratories GmbH, Vienna, Austria
Electroporator	MicroPulser™	Bio-Rad Laboratories GmbH, Vienna, Austria
UV-Transilluminator – Fisherbrand FT-28/312	Fisherbrand® UV-Transilluminator FT28/312	Fisher Scientific, GmbH, Schwerte, Germany
Incubator (28 °C)	Incubator with mechanical control	Binder GmbH, Tuttlingen, Germany
Incubator (37 °C)	Type BVW 50	Memmert, Schwabach, Germany
Laminar flow	Biological safety cabinet	Clean Air Products, Minneapolis, USA

Magnetic stirrer	IKA® RCT basic safety control	IKA®-Werke GmbH & Co. KG, Staufen, Germany
Micro centrifuge	Rotilabo®-mini-centrifuge	Carl Roth GmbH, Karlsruhe, Germany
Mini centrifuge	Mini Centrifuge MCF-2360	LMS Co., Tokyo, Japan
Microplate reader	Synergy Mx monochromator-based multi-mode microplate reader	BioTek Instruments, Winooski, USA
Nanodrop	NanoDrop 2000c spectrophotometer	Peqlab Biotechnologie GmbH, Polling, Austria
NuPAGE® cell	Xcell SureLock™ Mini-Cell	Novex®, Carlsbad, USA
NuPAGE® device	Power Ease 500	Invitrogen™, Lofer, Austria
Real time PCR machine	7300 Real Time PCR System	Applied Biosystems, Foster City, USA
PCR machine	Gene Amp® PCR Systems 2700	Applied Biosystems, Foster City, USA
PCR machine	2720 Thermal Cycler	Applied Biosystems, Foster City, USA
pH-electrode	Polyplast Temp Din	Hamilton Messtechnik GmbH, Höchst, Germany
pH-meter	inoLab pH 720	WTW GmbH, Weilheim, Germany
Photometer	Eppendorf Biophotometer	Eppendorf AG, Hamburg, Germany
Platform rocker	Grant-Bio PMR-30 platform rocker	Grant Instruments, Cambridgeshire, UK
Precision balance	GP3202	Sartorius Austria GmbH, Vienna, Austria
Scanner	HP Scanjet 4370 photo scanner	Hewlett-Packard GmbH, Graz, Austria
Shaker	Certomat® BS-1	Sartorius BBI Systems GmbH, Melsungen, Germany
Shaker for DWPs (28 °C)	Multitron	Infors AG, Bottmingen, Switzerland
Shaker (28 °C and 37 °C)	Orbitron	Infors AG, Bottmingen, Switzerland
Titramax	Titramax 1000	Heidolph Instruments GmbH, Schwabach, Germany
Thermomixer	Thermomixer comfort	Eppendorf AG, Hamburg, Germany
UV lamp	Vilber Lourmat electronic ballast transilluminators ECX-F20.M	Sigma-Aldrich Handels GmbH, Vienna, Austria
Vortex	Vortex-Genie2	Scientific Industries, New York, USA
Vortex (12 wells)	Vortex-Genie2	Scientific Industries, New York, USA

3.1.11 Other materials

Table 11: Other materials used

Name	Source
Bacterial cell spreader	Carl Roth GmbH, Karlsruhe, Germany
Biohit optifit tip 1,200 µL	Sartorius Biohit Liquid Handling OY, Helsinki, Finland
Baffled flask (250 mL and 2,000 mL)	Kavalierglass, Co.Ltd, Sázava, Czech Republic
Cryo.S, 2 mL, PP, round bottom, external thread natural screw cap, sterile	Greiner Bio-One GmbH, Frickenhausen, Germany
DURAN® baffled flask GL 45 (250 mL and 2,000 mL)	Duran Group GmbH, Wertheim/Main, Germany
DURAN® laboratory bottle GL 45 (100 ml, 500m, 1,000 mL and 2,000 mL)	Duran Group GmbH, Wertheim/Main, Germany
Electroporation cuvettes	Hanke Laboratory Products, Vienna, Austria
Eppendorf Research® pipette (adjustable volume), 0.1 – 2.5 µL	Eppendorf AG, Hamburg, Germany
Filter pipette tips (20 µL, 100 µL, 200 µL and 1,000 µL)	Greiner Bio-One GmbH, Frickenhausen, Germany
GL 45 screw caps	Duran Group GmbH, Wertheim/Main, Germany
Glass beads	Retsch, Haan, Germany
Half skirt 96 well PCR plate	VWR International GmbH, West Chester, USA
Injekt® Solo 5 mL	B. Braun Austria GmbH, Maria Enzersdorf, Austria
Injekt® Solo 20 mL	B. Braun Austria GmbH, Maria Enzersdorf, Austria
Ino-Loop	Simport, Beloeil, Canada
Labnet hand held pipette (20 µL, 200 µL and 1,000 µl)	Labnet International Inc, Edison, USA
MF^{TM}-membrane filter	Merck Millipore, Darmstadt, Germany
Micro tube 1.5 mL	Sarstedt AG & Co., Nümbrecht, Germany
mLINE mechanical pipette (5 µL – 100 µL)	Sartorius Biohit Liquid Handling OY, Helsinki, Finland
MµltiFlex round tips	Sorenson Bioscience Inc., Salt Lake City, USA
Nalgene® labware 500 mL PPCO centrifuge bottle	Thermo Scientific, Rochester, USA
NuPAGE® 4-12 % Bis-Tris Gel	Novex®, Carlsbad, USA
OmniTray w/lid, sterile, PS	Thermo Fisher Scientific, New York, USA
PCR – stip, 8 well, 0.2 mL	Greiner Bio-One GmbH, Kremsmünster, Austria
Petri dish	Greiner Bio-One GmbH, Kremsmünster, Austria
Pipetboy	Integra Biosciences GmbH, Fernwald, Germany
Pipette Tip Gilson® - style micro P10	Greiner Bio-One GmbH, Kremsmünster, Austria
Pipette Tip Gilson® - style 1000 µL	Greiner Bio-One GmbH, Kremsmünster, Austria
Pipette tip universal 200µL	Greiner Bio-One GmbH, Kremsmünster, Austria

Pipette with tip, sterile, 5 mL	Greiner Bio-One GmbH, St. Gallen, Switzerland
Pipette with tip, sterile, 10 mL	Greiner Bio-One GmbH, St. Gallen, Switzerland
Pipette with tip, sterile, 25 mL	Greiner Bio-One GmbH, St. Gallen, Switzerland
PP-microplate, 96 well, V-shape	Greiner Bio-One GmbH, Frickenhausen, Germany
PP-tube, sterile, cap, 12 mL	Greiner Bio-One GmbH, Frickenhausen, Germany
PP-tube,sterile, skirt, 50 mL	Greiner Bio-One GmbH, Frickenhausen, Germany
PP-tube, sterile, 50 mL	Greiner Bio-One GmbH, Frickenhausen, Germany
Proline electronic pipette (50 µL – 1,200 µL, multichannel)	Sartorius Biohit Liquid Handling OY, Helsinki, Finland
Proline plus mechanical pipette (30 µL – 300µL, multichannel)	Sartorius Biohit Liquid Handling OY, Helsinki, Finland
PS-microplate, sterile, 96 well, flat bottom	Greiner Bio-One GmbH, Frickenhausen, Germany
PS-semi-micro-cuvette, 1.6 mL	Greiner Bio-One GmbH, Kremsmünster, Austria
Replicator	Enzyscreen, Haarlem, the Netherlands
Rotilabo®-syringe filters, CME, sterile	Carl Roth GmbH, Karlsruhe, Germany
Scienceware® 96 deep-well plate	Best Lab Deals Inc, Garner, USA
Toothpick flat and round	Decor Service GmbH, Bad Radkersburg, Austria
Trifill multi channel pipettor reservoir	Camlab, Cambridge, UK
Tube strips 0.2 mL with domed cap strips	Peqlab, Erlangen, Germany
Vivaspin 500 VS0102	Sartorius Stedim Biotech GmbH, Göttingen, Germany

3.1.12 Kits

Table 12: Kits used

Kit	Source
GeneJET™ Plamid Miniprep Kit	Thermo Scientific, Vienna, Austria
QIAquick® Gel Extraction Kit	Qiagen GmbH, Hilden, Germany
QIAquick® PCR Purification Kit	Qiagen GmbH, Hilden, Germany

3.1.13 Software

Table 13: Software used

Software	Source
7300 System SDS Software	Applied Biosystems
ApE – A Plasmid Editor	Biologylabs
Gen5 1.11 – Data Analysis Software	BioTek
HP Image Zone	Hewlett-Packard GmbH
HP Solution Center	Hewlett-Packard GmbH
Launch Doc-ItLS – Image Analysis Software	UVP
Nanodrop 2000	Thermo Scientific
SeqBuilder	Lasergene (test version)

3.1.14 Reagents

Table 14: Reagents used

Name	Source
1,4-Dithiothreitol (DTT)	Carl Roth GmbH, Karlsruhe, Germany
2,2´-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS)	Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany
6x Mass Ruler DNA Loading Dye	Thermo Scientific, Vienna, Austria
α-Ketoglutarate	Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany
D-Glucose-monohydrate (D)	Carl Roth GmbH, Karlsruhe, Germany
D-Sorbitol	Carl Roth GmbH, Karlsruhe, Germany
L-Glutamate	Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany
L-Leucine	Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany
p-Nitrophenyl butyrate (pNPB)	Sigma-Aldrich, Chemie GmbH, Schnelldorf, Germany
Acetic acid	Carl Roth GmbH, Karlsruhe, Germany
Ammonium acetate	Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany
Aqua bidest. ``Fresenius´´	Fresenius Kabi Austria GmbH, Graz, Austria
Bacto™ agar	Becton, Dickinson and Company, Sparks, USA
Bacto™ peptone	Becton, Dickinson and Company, Sparks, USA
Bacto™ yeast extract	Becton, Dickinson and Company, Sparks, USA
Bicine	Carl Roth GmbH, Karlsruhe, Germany
Biotin (B)	Carl Roth GmbH, Karlsruhe, Germany
Biozym LE agarose	Biozym BioTech Trading GmbH, Vienna, Austria
Bovine serum albumin (BSA) [2 mg/ml]	Thermo Scientific Pierce, Rockford, USA
Chloroform	Carl Roth GmbH, Karlsruhe, Germany
di-Potassium hydrogen phosphate (K_2HPO_4)	Carl Roth GmbH, Karlsruhe, Germany
di-Sodium hydrogen phosphate (Na_2HPO_4)	Carl Roth GmbH, Karlsruhe, Germany
Difco™ yeast nitrogen base w/o amino acids	Becton, Dickinson and Company, Sparks, USA
Dimethyl sulphoxide (DMSO)	Carl Roth GmbH, Karlsruhe, Germany
Deoxyadenosine triphosphate (dATP)	Carl Roth GmbH, Karlsruhe, Germany
Deoxycytidine triphosphate (dCTP)	Carl Roth GmbH, Karlsruhe, Germany
Deoxyguanosine triphosphate (dGTP)	Carl Roth GmbH, Karlsruhe, Germany
Deoxynucleotide triphosphate (dNTP)	Thermo Scientific, Vienna, Austria
Deoxythymidine triphosphate (dTTP)	Carl Roth GmbH, Karlsruhe, Germany
Ethidium bromide	Carl Roth GmbH, Karlsruhe, Germany
Ethylene glycol	Carl Roth GmbH, Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth GmbH, Karlsruhe, Germany
Formaldehyde	Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany
Glycerol	Carl Roth GmbH, Karlsruhe, Germany
HEPES	Carl Roth GmbH, Karlsruhe, Germany
Hydrochloric acid (HCl)	Carl Roth GmbH, Karlsruhe, Germany
Isopropanol	Carl Roth GmbH, Karlsruhe, Germany

J. T. Baker ® Ethanol absolute	VWR International GmbH, Vienna, Austria
Liquid nitrogen	Air Liquide Austria GmbH, Graz, Austria
Magnesium chloride ($MgCl_2$)	Carl Roth GmbH, Karlsruhe, Germany
Magnesium sulphate (Mg_2SO_4)	Carl Roth GmbH, Karlsruhe, Germany
Methanol	Carl Roth GmbH, Karlsruhe, Germany
Nicotinamide adenine dinucleotide (NAD)	Carl Roth GmbH, Karlsruhe, Germany
NuPAGE® LDS sampling buffer (4x)	Invitrogen™, Lofer, Austria
NuPAGE® MOPS SDS running buffer (20x)	Novex® Lofer, Austria
NuPAGE® sample reducing agent	Invitrogen™, Lofer, Austria
PEG-8000	Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany
Platesealer, silverseal, aluminium	Greiner Bio-One GmbH, Kremsmünster, Austria
Potassium chloride (KCl)	Carl Roth GmbH, Karlsruhe, Germany
Potassium dihydrogen phosphate	Carl Roth GmbH, Karlsruhe, Germany
Potassium hydroxide (KOH)	Carl Roth GmbH, Karlsruhe, Germany
Power SYBR® Green Master Mix (2x)	Applied Biosystems, Warrington, UK
Pyridoxal-5-phosphate (PLP)	Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany
Roti®-Phenol/C/I	Carl Roth GmbH, Karlsruhe, Germany
Roti®-Quant	Carl Roth GmbH, Karlsruhe, Germany
Rotilabo®-sealing film, microtest plates	Carl Roth GmbH, Karlsruhe, Germany
Silver nitrate (Ag_2NO_3)	Merck Millipore, Darmstadt, Germany
SimplyBlue™ SafeStain	Invitrogen™, Lofer, Austria
Sodium carbonate (Na_2CO_3)	Carl Roth GmbH, Karlsruhe, Germany
Sodium chloride (NaCl)	Carl Roth GmbH, Karlsruhe, Germany
Sodium hydroxide (NaOH)	Carl Roth GmbH, Karlsruhe, Germany
Sodium dihydrogen phosphate (NaH_2PO_4)	Carl Roth GmbH, Karlsruhe, Germany
Sodium dodecyl sulphate (SDS)	Carl Roth GmbH, Karlsruhe, Germany
Sodium thiosulphate ($Na_2S_2O_3$)	Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany
Triton X-100	Carl Roth GmbH, Karlsruhe, Germany
Tryptone/peptone	Carl Roth GmbH, Karlsruhe, Germany
TRIS	Carl Roth GmbH, Karlsruhe, Germany
Y-PER® plus, dialyzable yeast protein extraction reagent	Thermo Scientific, Rockford, USA

3.1.15 Solutions and buffers prepared

Table 15: Solutions and buffers prepared

Name	Components
70 % ethanol	70 % (v/v) ethanol
10 % glycerol*	100 g/L glycerol
60 % glycerol*	600 g/L glycerol
10x acetamide**	6 g/L acetamide
500x B**	200 mg/L biotin
10x D*	220 g/L D-glucose-monohydrate
1x MOPS buffer	50 mL/L 20x MOPS buffer
1x PBS buffer*	8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4, pH 7.4
1x TAE buffer	20 mL/L 50x TAE buffer
50x TAE buffer	242 g/L TRIS, 5.71 % (v/v) acetic acid, 18.6 g/L EDTA
10x YNB*	134 g/L Difco™ yeast nitrogen base with ammoniumsulphate w/o amino acids
4 M ammonium acetate*	308.32 g/L ammonium acetate
1 M DTT**	152.2 g/L DTT
1 M potassium phophate buffer (PPB)*	30 g/L K_2HPO_4, 118 g/L KH_2PO_4, pH 6
100 mM sodium phosphate buffer pH 7.5 (SPB)*	21.76 g/L Na_2HPO_4, 2.6 g/L NaH_2PO_4, pH 7.5
1 M sorbitol*	182.18 g/L D-sorbitol
1 M TRIS*	121,14 g/L TRIS
300 mM TRIS/HCl pH 7*	36.34 g/L TRIS, pH 7 adjusted with HCl
BCAT assay solution**	for one microtiter plate: 1 mL 100 mM L-leucine, 1 mL 100 mM α-ketoglutarate, 1.6 mL 100 mM SPB pH 7.5, 100 µL 2.5 mM PLP, 100 µL HRP [1mg/mL], 100 µL L-glutamate-oxidase [1 U/mL], 5 mL 5 mM ABTS ➔ all chemicals are solved in SPB
BEDS solution**	1.632 g/L bicine-NaOH, pH 8.3, 3 % (v/v) ethylene glycol, 5 % (v/v) DMSO, 182 g/L D-sorbitol
*Cal*B assay solution	for one microtiter plate: 200 µL substrate solution (16.8 µL pNPB 98 % + 183.2 µL DMSO) 20 mL buffer (300 mM TRIS/HCl pH 7)
Developing solution	6 % (w/v) Na_2CO_3, 0.0004 %(w/v) $Na_2S_2O_3$, 0.05 % formaldehyde
Fixation solution	50 % (v/v) ethanol, 12 % (v/v) acetic acid, 0.05 % (v/v) formaldehyde
Gibson assembly master mix	320 µL 5x ISO reaction buffer, 0.64 µL T5 exonuclease, 20 µL Phusion® high-fidelity DNA polymerase, 160µL Taq DNA ligase, 699.36 µL ddH_2O ➔ aliquot 15 µL each into PCR tubes ➔ store at –20 °C
Hepes buffer**	11.92 g/L HEPES, 9 g/L NaCl, pH 7

Isothermal reaction buffer (5x)	1.5 g PEG-8000, 3,000 µL 1 M Tris/HCl pH 7.5, 150 µL 2 M $MgCl_2$, 300 µL 1 M DTT, 60 µL 100 mM dATP, 60 µL 100 mM dCTP, 60 µL 100 mM dGTP, 60 µL 100 mM dTTP, 300 µL 100 mM NAD, up to 6 mL with ddH_2O ➔ aliquot 100 µL each into sterile tubes ➔ store at –20 °C
Sensitising solution	0.02 % (w/v) $Na_2S_2O_3$
Staining solution	0.2 % (w/v) $AgNO_3$, 0.076 % (v/v) formaldehyde
TE buffer**	1.21 g/L TRIS-HCl, pH 7.5, 0.29 g/L EDTA, pH 8.0
Terminating solution	12 % (v/v) acetic acid
Yeast lysis buffer**	20 mL Triton X-100, 100 mL 10 % SDS, 20 mL 5 M NaCl, 2 mL 0.5 M EDTA, 10 mL 1 M TRIS-HCl pH 8, distilled water to 1 L
Washing solution	50 % (v/v) ethanol

* sterilisation at 121 °C

** filter sterilised

3.1.16 Media

All media were sterilised at 121 °C. Antibiotics were added after cooling the autoclaved media to ~60 °C. The components biotin, acetamide, $MgCl_2$ and $MgSO_4$ were filter sterilised.

Table 16: Media used

Medium name	**Components**
2xTY liquid medium	16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl
BMD-1 liquid medium	200 mL/L PPB, 100 mL/L 10x YNB, 50 mL/L 10x D, 2 mL/L 500x B
BMD-1 (-YNB) + acetamide liquid medium	200 mL/L PPB, 100 mL/L 10x acetamide, 50 mL/L 10x D, 2 mL/L 500x B
BMD-1 (-YNB) + acetamide plates	200 mL/L PPB, 100 mL/L 10x acetamide, 50 mL/L 10x D, 2 mL/L 500x B, 15 g bacto agar
BMM-2 medium	200 mL/L PPB, 100 mL/L 10x YNB, 10 mL/L MeOH, 2 mL/L 500x B
BMM-10 medium	200 mL/L PPB, 100 mL/L 10x YNB, 50 mL/L MeOH, 2 mL/L 500x B
LB liquid medium	5 g/L NaCl, 10 g/L tryptone/peptone, 5 g/L yeast extract
LB amp liquid medium	5 g/L NaCl, 10 g/L tryptone/peptone, 5 g/L yeast extract, 100 µg/mL ampicillin
LB kan liquid medium	5 g/L NaCl, 10 g/L tryptone/peptone, 5 g/L yeast extract, 50 µg/mL kanamycin
LB zeo liquid medium	5 g/L NaCl, 10 g/L tryptone/peptone, 5 g/L yeast extract, 25 µg/mL zeocin
LB plates	5 g/L NaCl, 10 g/L tryptone/peptone, 5 g/L yeast extract, 15 g/L bacto agar

LB amp plates	5 g/L NaCl, 10 g/L tryptone/peptone, 5 g/L yeast extract, 15 g/L bacto agar, 100 μg/mL ampicillin
LB kan plates	5 g/L NaCl, 10 g/L tryptone/peptone, 5 g/L yeast extract, 50 μg/mL kanamycin
LB zeo plates	5 g/L NaCl, 10 g/L tryptone/peptone, 5 g/L yeast extract, 15 g/L bacto agar, 25 μg/mL zeocin
SOC liquid medium	20 g/L tryptone, 0.58 g/L NaCl, 5 g/L yeast extract, 2 g/L $MgCl_2$, 0.18 g/L KCl, 2.46 g/L $MgSO_4$, 3.46 g/L D
YPD liquid medium	10 g/L bacto yeast extract, 20 g/L bacto peptone, 100 mL/L 10x D
YPD plates	10 g/L bacto yeast extract, 20 g/L bacto peptone, 15 g/L bacto agar, 100 mL/L 10x D
YPD gen plates	10 g/L bacto yeast extract, 20 g/L bacto peptone, 15 g/L bacto agar, 100 mL/L 10x D, 100 μg/mL geneticin
YPD gen/zeo plates	10 g/L bacto yeast extract, 20 g/L bacto peptone, 15 g/L bacto agar, 100 mL/L 10x D, 100 μg/mL geneticin, 100 μg/mL zeocin
YPD zeo plates	10 g/L bacto yeast extract, 20 g/L bacto peptone, 15 g/L bacto agar, 100 mL/L 10x D, 100 μg/mL zeocin

3.2 Methods

3.2.1 Cloning

3.2.1.1 Cloning of the gene *ilv*E or *ca*lB into pPpT4 α S

The codon-optimised gene *ilv*E or *ca*lB was cloned into the vector pPpT4 α S, which is a shuttle vector of *E. coli* and *P. pastoris*, and was expressed under the control of the *AOX1* promoter. The plasmid containing *ilv*E or *ca*lB was linearised with *Smi*I and afterwards transformed into the various *P. pastoris* strains.

For specific information see the following chapters.

3.2.1.2 Plasmid isolation and DNA purification

The kit GeneJET™ from Thermo Scientific was used for plasmid isolation. For the purification of PCR products, restriction enzyme approaches, etc., the kit QIAquick PCR Purification Kit from Qiagen was used. The kit QIAquick Gel Purification was used for purification of DNA from gel slices. All the kits are shown in table 12.

3.2.1.3 Isolation of the genomic DNA from yeast – *P. pastoris*

In order to isolate the genomic DNA from *P. pastoris* for colony PCR applications, an optimised version of the Bust n‘ Grab protocol from Harju et al. (2004) was used.

The following steps were changed:

1. All centrifugation steps were performed at 16,100 g
2. 100 µL of lysis buffer was added
3. Tubes were immersed in liquid nitrogen for 2 min and transferred to the thermomixer (95 °C) for 1 min
4. 100 µL of chloroform was added and vortexed for 2 min
5. Suspension was centrifuged for 5 min
7. Incubation was performed at room temperature for 15 min
8. Suspension was centrifuged for 7 min; supernatant was removed via automatic pipette

3.2.1.4 Polymerase chain reaction

The polymerase chain reaction (PCR) conditions depend on the used DNA polymerase. The conditions for each DNA polymerase are shown in table 17. Dream Taq polymerase was used for colony PCR to assess the correctness of transformants. DNA fragments, which were used for further experiments, were amplified by Phusion polymerase. The reason to use Phusion polymerase is the proof reading activity, which keeps the rate of mutations at a minimum level. For all PCRs a total volume of 50 µL was used.

Table 17: PCR conditions

Condition	Dream Taq	Phusion
Initial denaturation	95 °C, 2 min	98 °C, 30 sec
Denaturation	95 °C, 30 sec	98 °C, 10 sec
Annealing	55 °C – 60 °C*, 30 sec	55 °C – 60 °C*, 30 sec
Extension/elongation	72 °C, time is dependent on the length of fragment (1 min/kb)	72 °C, time is dependent on the length of fragment (30 sec/kb)
Final elongation	72 °C, 5 min	72 °C, 5 min
Final hold	4 °C, ∞	4 °C, ∞
Number of cycles	25	30

* dependent on primer melting temperature (Tm was calculated by ApE or CLC Workbench)

In this thesis, different PCR techniques were used: these are the general PCR and the colony PCR. The general PCR was used for amplification of genes mainly from plasmids and also from genomic DNA. Colony PCR was performed for example to determine if a gene deletion also occured on another position as the wild type or to prove if a gene was cloned in a vector etc. The reagents for those types of PCR are shown in table 18 and table 19.

Table 18: PCR reagents

Reagent	Concentration/Units	Final Concentration
Template	100 ng plasmid DNA 500 ng – 1 µg genomic DNA	100 ng plasmid DNA 500 ng – 1 µg genomic DNA
Polymerase	2 U/µL (Phusion)	0.02 U/50 µL (Phusion)
dNTPs	10 mM	1 mM
Primer forward	5 µM	0.2 µM
Primer reverse	5 µM	0.2 µM
Buffer	5x HF Buffer	1x HF Buffer
ddH_2O	up to 50 µL	up to 50 µL

In order to isolate DNA (template for colony PCR) from *E. coli*, one colony was picked and resuspended in 50 µL ddH_2O. The cells were broken at 95 °C for 10 min. Subsequently, the suspension was centrifuged for 10 min at 16,100 g.

Genomic DNA from *P. pastoris* was isolated like it is written in 3.2.1.3 Isolation of the genomic DNA from yeast – *P. pastoris*.

Table 19: Colony PCR reagents

Reagent	Concentration/Units	Final Concentration
Template	100 ng plasmid DNA 500 ng – 1 µg genomic DNA	100 ng plasmid DNA 500 ng – 1 µg genomic DNA
Polymerase	5 U/µL (Dream Taq)	0.05 U/50 µL (Dream Taq)
dNTPs	10 mM	1 mM
Primer forward	5 µM	0.2 µM
Primer reverse	5 µM	0.2 µM
Buffer	10x Dream Taq Buffer	1x Dream Taq Buffer
ddH_2O	up to 50 µL	up to 50 µL

3.2.1.5 Cloning with restriction enzymes

Generally, digestion of vectors or DNA fragments was performed for 2 h at the designated temperature (30 °C / 37 °C) with the chosen enzyme. *Bgl*II was available as a ``fast digest´´ enzyme and was applied for 30 min at 37 °C. The restriction sites as well as U/µL are shown in table 3. The digested samples were loaded onto an agarose gel for control.

3.2.1.6 Ligation

The digested fragments were purified via a kit from Qiagen. The ligation of vector and insert was performed at a vector/insert ratio of 1:1, 1:2 or 1:3, depending on the length of the fragments, for 2 h at room temperature without shaking.

3.2.1.7 Electrocompetent *E. coli* Top10F‘ cells

In this thesis an in-house protocol was used.

The procedure is as follows:

Day 1:

Prepare two overnight cultures (ONCs) from a single colony and incubate at 37 °C with 200 rpm. Put reaction tubes, 1 L 10 % glycerol and 2 L 1 M Hepes buffer, pH 7 in the 4 °C room.

Day 2:

- Transfer 3 mL ONC in a 2 L baffled flask containing 330 mL 2xTY medium and incubate. Cool centrifuge and centrifuge beakers. In the meantime measure OD_{600} value. The cell suspension should reach an OD_{600} 0.8 -> harvest (after 5 – 6 h):

  From here, it is important to work on ice or 4 °C.

  - Transfer cell cultures from baffled flasks to centrifuge beakers and put them on ice for 1 h (4 °C room).
  - Centrifuge for 10 min, 3,000 g and 4 °C. Remove supernatants and resuspend pellets in a little amount of buffer. Pool two centrifuge beakers and refill the one to one fourth with buffer (keep pellets on ice).
  - Centrifuge for 10 min, 4,000 g and 4 °C. Remove supernatant and resuspend pellet in a little bit of buffer. Afterwards, fill up the beaker with 10 % glycerol (do not add glycerol first, because pellet will freeze).
  - Centrifuge 20 min, 4,500 g and 4 °C. Remove supernatant and resuspend pellet in 10 % glycerol (refill whole beaker).
  - Centrifuge 15 min, 4,500 g and 4 °C. Remove supernatant. Per litre medium, 2 mL 10 % glycerol are used for resuspension of the pellet. Aliquot 100 µL of the suspension in reaction tubes and freeze them in liquid nitrogen. Then, store them at –80 °C until use.
- In order to determine the transformation rate of electrocompetent EC Top10 cells, 1 µL pUC19 [10 pg/µL] is transformed.

3.2.1.8 Transformation of *E. coli* Top10F‘

An electroporation cuvette was precooled on ice. 50 µL of EC Top10 cells were pipetted into the cuvette. Furthermore, DNA with a concentration between 200 ng – 500 ng was added and incubated for approximately 1 min on ice. Afterwards, the cuvette was dried and a pulse was performed with an electroporator. EC2 (2,000 V, 25 µF and 200 Ω) was the used program. After pulsing the cells, 1 mL SOC medium was added to the cells and incubated for 1 h at 37 °C and 600 rpm for regeneration. After the incubation, the cell suspenion was diluted and plated on selective agar plates. Then, the plates were incubated at 37 °C overnight to allow growth of colonies that harbour the selection marker. Selection was performed with either ampicillin, zeocin or kanamycin.

3.2.1.9 Linearisation

A circular plasmid needs to be linearised before transformation into *P. pastoris,* in order to allow an integration into the genome. For the diverse plasmids, different restriction enzymes with only one restriction site were used for linearisation. In general, the site of linearisation is chosen upstream of the sequence, where it is intended to integrate. The linearisation occured for 2 h or 30 min at 30 °C / 37 °C depending on the used enzyme.

3.2.1.10 Electrocompetent *P. pastoris* cells

Preparation of competent cells and transformation adapted from Lin-Cereghino et al. (2005) for the production of electrocompetent *P. pastoris* cells.

The following steps were different:

1. ONC of *P. pastoris* cells were grown as 20 mL ONCs
4. Culture was centrifuged at 3,200 g for 10 min
6. Cell suspension was incubated for 5 min by gently shaking it with the hand
7. Cells were resuspended in a certain volume of BEDS, which is adjusted to the OD_{600};
   for example: OD_{600} 0.8 → 800 µL BEDS
8. Cells were kept on ice until use or frozen in small aliquots at –20 °C

3.2.1.11 Transformation of *P. pastoris*

Electroporation cuvettes were cooled on ice. 500 ng of linearised plasmid DNA with a maximum volume of 15 µL was mixed with 90 µL of competent *P. pastoris* cells. The mixture was incubated for 15 min on ice. Afterwards, the cuvette was dried and a pulse was performed with an electroporator. PIC (1,500 V, 25 µF and 200 Ω) was the used program. After the pulse, 1 mL of regeneration medium (YPD/1 M sorbitol 1:1) was added and the suspension was transferred to a 12 mL tube. Then, the tube was incubated at 30 °C for 2 h. After the incubation, the cells were plated on selective agar plates. The plates were incubated at 30 °C for 48 h to allow growth of colonies that harbour the selection marker. Selection was performed with either zeocin or geneticin.

3.2.1.12 Agarose gel preparation and performance

In order to determine the size of DNA fragments, PCR products etc. the samples need to be visualised on an agarose gel. MassRuler^{TM} DNA ladder mix from Thermo Scientific was the used standard, shown in A 1 in the chapter ``Appendix´´. For only few samples, a small gel with 1 % of agarose was used. Consequently, 1 g agarose was weighed into a flask and 100 mL 1x TAE buffer was added. This mixture was heated up using a microwave (~4 min, highest performance). The flask with the dissolved agarose was cooled using tap water. Afterwards, one drop of ethidium bromide [1 mg/mL] was added. The liquid was mixed and then poured into a gel chamber. For a higher sample number, a big gel was used. Therefore, 2 g agarose with 200 mL 1x TAE buffer and two drops of ethidium bromide were used under otherwise identical treatment. Control gels were run at 80 volts (small gel) or 120 volts (large gel) for about 1 h. Therefore, an aliquot of the samples (2 µL), for example from the PCR, were mixed with 10 µL 1x mass ruler DNA loading dye. As soon as the gel was solid, samples could be loaded. For preparative gels, where the samples were further isolated from the gel, it was run with 80 volts for about 2 h to obtain a good separation of the DNA fragments. For the purification of DNA via gel the whole PCR mixture (50 µL) was mixed with 5 µL 6x mass ruler DNA loading dye and loaded onto. After running the gel, the gel was placed on the UV-lamp device. There, the desired bands were cut out with the help of a scalpel and tweezers under UV-light. The cut bands were purified with the kit from Qiagen. Furthermore, a picture was taken from the gel using the UV-Transilluminator with the software Launch Doc-ItLS.

3.2.1.13 Glycercol stocks *E. coli*

Glyercol stocks are an option in order to conserve strains for a longer period of time. Therefore, 5 mL ONC of the particular *E. coli* strains were prepared. 900 µL aliquots of the ONC were transferred into cryogenic tubes. Following, 300 µL of 60 % glycerol was added. The tubes, containing the cell suspension and glycerol, were frozen at –80 °C.

3.2.1.14 Glycerol stocks *P. pastoris*

Cryogenic tubes:

5 mL ONC of the particular *P. pastoris* strains were grown to high density. 900 µL aliquots of the ONC were transferred into cryogenic tubes and 300 µL of 60 % glycerol was added. Then, the glycerol stocks were frozen at –20 °C and on the next day at –80 °C, since it was observed previously, that a slower freezing process is advantageous in terms of keeping cells alive.

Microtiter plates:

Cells grown on omnitrays were transferred with the help of a replicator to deep well plates (DWPs) containing 300 µL YPD medium and were grown to high density. 150 µL of the cell suspensions were transferred to sterile microtiter plates and 50 µL 60 % glycerol were added. The glycerol stocks were frozen at –20 °C and on the next day at –80 °C.

3.2.2 Expression

3.2.2.1 Cultivation in deep well plates

Short protocol for methanol induction:

350 µL BMD-1 medium were pipetted into DWPs. Single transformants were picked from the selection plates and each clone was transferred into a well via toothpick. The first column always contained the control strains and the others the strains to be tested. Figure 3 shows the positions of the strains and controls. The DWPs were incubated at 28 °C, 80 % humidity and 320 rpm. After 48 h, the cell suspensions were stamped on YPD plates with the particular antibiotic. Subsequently, 250 µL BMM-2 medium was added. 50 µL BMM-10 medium was added 8 h after this induction. After 24 h and 32 h expression time, another 50 µL BMM-10 medium was added. 24 hours later (48 h after first induction), the cells were harvested. Therefore, the DWPs were centrifuged for 10 min, at 4 °C and 3,200 g. After the centrifugation, 200 µL of the supernatants (SNs) were transferred into a microtiter plate. The plates were covered with a sealing film and the samples were frozen at –20 °C until use.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	X	X	X	X	X	X	X	X	X	X	X
B	B	X	X	X	X	X	X	X	X	X	X	X
C	C	X	X	X	X	X	X	X	X	X	X	X
D	C	X	X	X	X	X	X	X	X	X	X	X
E	C	X	X	X	X	X	X	X	X	X	X	X
F	C	X	X	X	X	X	X	X	X	X	X	X
G		X	X	X	X	X	X	X	X	X	X	X
H		X	X	X	X	X	X	X	X	X	X	X

Figure 3: Scheme for the cultivation of *P. pastoris* clones in deep well plates. The wells labelled in grey contained just BMD-1 medium and were used for sterility check. In all the other wells were the clones obtained from the transformation. B: blank – *P. pastoris* CBS7435 Mut^S, C: positive control – *P. pastoris* CBS7435 Mut^S containing the gene of interest and X: transformants.

3.2.2.2 Rescreening in deep well plates

After performing the assays of the cultivated cells in DWPs and evaluating the data from that, certain clones were chosen for the rescreening in DWPs. In total, six clones were chosen; two clones which showed a low, two which showed an average and two which showed a high enzymatic activity in the assay. Every clone was remeasured in triplicates and the controls in duplicates. In order to avoid the loss of liquid or a crossover of the suspensions, the outer columns and lines as well as wells in between were omitted. The scheme of such a picked plate can be seen in figure 4. The whole procedure is the same as described in 3.2.2.1 Cultivation in deep well plates, except that this time no conservation of the clones was performed.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		X	X	X		X	X	X		B	B	
C												
D		X	X	X		X	X	X		C	C	
E												
F		X	X	X		X	X	X		C	C	
G												
H												

Figure 4: Scheme of a deep well plate for the rescreening. The outer frame and wells in between shown in grey contained just BMD-1 medium. Every clone was picked in triplicates and the strain controls in duplicates. X: clones, B: blank – *P. pastoris* CBS7435 Mut^S, C: positive control – *P. pastoris* CBS7435 Mut^S containing the gene of interest.

3.2.2.3 Screening in shake flasks

After the screening and rescreening in DWPs, a screening in shake flasks was the next step to verify the observed activity from several strains compared to the control strain. For that, two clones from the rescreening were chosen and cultivated in 20 mL ONCs with BMD-1 medium. A 50 mL main culture was inoculated with the ONC to an OD_{600} 0.1. For every strain, duplicates were performed, in other words, two main cultures were prepared for each strain. As a control *P. pastoris* CBS7435 Mut^S with a single copy of either the gene of *ilv*E or *ca*lB respectively, was cultivated under the same conditions as the tested strains, but only in one single approach per construct. The cultures were incubated at 28 °C and 120 rpm for approximately 72 h. After that incubation period, the glucose was presumably depleted. Subsequently, 5 mL BMM-10 medium was added for AOX promoted induction of protein expression. After 24 h and 48 h expression time, 0.5 mL of MeOH was added for further induction.

Before the first induction occured, 1 mL of the cell suspensions was taken and transferred into 1.5 mL reaction tubes. In parallel, OD_{600} values from the cultures were measured. The reaction tubes were centrifuged for 10 min and 16,100 g at room temperature. The SNs were transferred into new reaction tubes. The tubes containing either the supernatant or the cells (pellet) were frozen at –20 °C until further use. Those two steps (sampling and OD_{600} measurement) were repeated after 8 h, 24 h, 32 h, 48 h, 56 h and 72 h of expression. Subsequently, the pellets were thawed and the cells were lysed. The lysis of the cells occurred with Y-PER. Therefore, the pellets were resuspended in 200 µL Y-PER and incubated in a thermomixer at 22 °C and 1,000 rpm for 1 h. Following, the suspensions were centrifuged at 4 °C and 16,100 g for 10 min. The supernatants of the lysed cell suspensions (cell free extracts – CFEs) were transferred to new reaction tubes and frozen again until they were used for the BCAT or *Ca*lB assay.

3.2.3 Screening and strain analysis

3.2.3.1 Transaminase activity assay (BCAT assay) (Weinhandl et al., 2012)

First of all, the transaminase assay solution was prepared. The composition of this solution is shown in table 15. Afterwards, 20 µL of protein solution were provided in microtiter plates. In every well, containing 20 µL protein solution, 80 µL assay solution was added. The microtiter plate was incubated for 10 min at room temperature. Afterwards, the reaction kinetic was measured with the help of the plate reader. The used conditions were 30 °C, wavelength of 419 nm and measurement every minute for 1 h. Another option for the assay was incubation for 30 min and then the measurement occured every minute for a duration of 30 min at 30 °C and 419 nm.

The obtained data were evaluated with the programm Gen5 1.11 from BioTek (see table 13). Graphs containing the values of Vmax [mOD/min] (obtained from Gen5 1.11) on the Y-axis and on the X-axis the clone names were represented by Microsoft Excel.
In order to calculate the volumetric activity of BCAT from all knockout strains, the Vmax [mOD/min] values were normalised to an OD_{600} 1. The normalisation was performed because the knockout strains had different growth behaviour. The formula to calculate the volumetric activity [µmol/min/mL or U/mL] is shown in figure 5.

$$U/mL = \frac{\Delta mOD/min * V}{\varepsilon * v * d} * 10^3$$

U = µmol/min
V = total volume in L
v = sample volume in L
ε = 36000 L/mol * cm
d = coat thickness [0.25 cm]

Figure 5: Formula for the calculation of the enzymatic activity of BCAT.

3.2.3.2 *Ca*lB activity assay (Sigma-Aldrich, 2013)

The first step was to prepare the assay solution. The components for the solution are shown in table 15. 20 µL of protein solution was provided in microtiter plates and then, 180 µL substrate solution was added. The kinetic measurement was taken immediately and was monitored every minute for a duration of 30 min. The used conditions were 25 °C and 405 nm. The evaluation of the obtained data occured in the same way as it is already written in 3.2.3.1 Transaminase activity assay (BCAT assay).
In figure 6 the formula to calculate the volumetric activity of *Ca*lB from all knockout strains is shown. Moreover, Vmax [mOD/min] was normalised to an OD_{600} 1 in order to obtain comparable results, since the knockout strains were different in growth.

$$U/mL = \frac{\Delta mOD/min * V}{\varepsilon * v * d}$$

U = µmol/min
V = total volume in mL
v = sample volume in mL
ε = 9594 mL/µmol * cm
d = coat thickness [0.46 cm]

Figure 6: Formula for the calculation of the enzymatic activity of *Ca*lB.

3.2.3.3 Protein assay – Bradford

In order to determine the protein concentration of the respective clones, a Bradford assay was performed. First of all, the BSA (bovine serum albumin) standard was prepared with the following concentrations: 0 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, 50 µg/mL, 60 µg/mL, 80 µg/mL and 100 µg/mL.

Afterwards, the samples (CFEs, SNs) had to be diluted in an appropriate extent. All dilutions either were done with Y-PER (cell free extracts) or with BMD-1 medium (supernatants). 50 µL protein solution of the samples were provided in microtiter plates. Afterwards, 200 µL of Roti®-Quant solution was added and incubated at room temperature for 5 min. Then, the OD_{590} was measured.

3.2.3.4 Real-time PCR (Abad et al., 2010)

In order to determine the amount of copies of the genes *ilv*E and *ca*lB in the genome of MutS and all the other knockout strains, a real-time PCR (qPCR) was performed. For a qPCR a high concentration of the genomic DNA is necessary. Therefore, the protocol for the isolation of genomic DNA from Hoffman and Winston (1987) was used with a few differences.

Changes in the genomic DNA isolation protocol:

1. 20 mL ONC was prepared
2. Cells were centrifuged for 5 min and 2,200 g at room temperature. Supernatant was removed and cells were resuspended in 0.5 mL ddH_2O. Then, suspension was centrifuged for 5 sec at maximum speed (16,100 g).
4. In the following order, 200 µL yeast lysis buffer (recipe visible in table 15), 200 µL Roti®-Phenol/C/I (25:24:1) and 0.3 g of acid-washed beads were added.
6. Centrifugation was performed for 5 min and 16,100 g; 1 mL ice-cold 100 % EtOH was added.
7. Suspension was centrifuged for 1 min in a microfuge and supernatant was aspirated. Pellet was resuspended in 400 µL TE buffer and 5 µL RNase A [10 mg/mL] and incubated at 37 °C more than 2 h. 10 µL 4 M ammonium acetate and 1 mL ice-cold 100 % EtOH were added.
8. Centrifugation was performed for 1 min in a microfuge, supernatant was discarded and washed with 1 mL 70 % EtOH. Pellet was air-dried and resuspended in 50 µL ddH_2O.

After isolation of the genomic DNA the concentration was measured via Nanodrop. It is important that the level of purity (260 nm/280 nm) is between 1.8 – 2.0 for a successful qPCR. The primers ARG4 (P09123, P09124) and AOX1 (P09121, P09122) were diluted to 5 pmol/µL. The sequences of those primers are shown in table 8. MutS was used as

calibration strain. The strain was diluted to a final concentration of 33 ng/µL. From that dilution further dilutions were performed in 1:3 steps to receive the following concentrations of 11 ng/µL, 3.6 ng/µL, 1.2 ng/µL, 0.4 ng/µL and 0.1 ng/µL. Additionally, as a one-copy reference a further dilution was performed to get a concentration of 0.66 ng/µL. All strains, which should be tested, were diluted to a concentration of 0.66 ng/µL as well. Subsequently, two different master mixes containing the particular primer pairs were prepared. The composition of those master mixes is listed in table 20. The volume of one master mix is sufficient for eight unknown strains and the calibration curve.

Table 20: Composition of master mix

Component	Volume [µL]	Final concentration
Power SYBR Green Master Mix (2x)	360	1x
Forward primer [5 pmol/µL]	36	250 nM
Reverse primer [5 pmol/µL]	36	250 nM
ddH_2O	216	

36 µL of the particular master mix was provided in PCR stripes. 6 µL of DNA (calibration curve and unknown strains) as well as ddH_2O (blank) were added, then mixed by gently shaking and afterwards, the stripes were centrifuged. Subsequently, 18 µL of the samples were pipetted in a qPCR plate (half skirt 96 well PCR plate) two times in order to obtain results from a double determination. The pipetting scheme can be seen in figure 7. After pipetting all samples in a qPCR plate, a special qPCR-foil was placed on the plate to close it. Afterwards, the plate was centrifuged for 1 min at 3,000 g. Finally, the plate was placed in the real-time PCR device and the following program was used for the real-time PCR run.

Stage 1:	95 °C	10 min	(1 repetition)
Stage 2:	95 °C	15 sec	} (40 repetitions)
	60 °C	1 min	
Stage 3:	95 °C	15 sec	} (1 repetition)
	60 °C	30 sec	
	95 °C	15 sec	

The data were evaluated by 7300 System SDS software and Microsoft Excel.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Arg0	Arg0	Arg33	Arg33	Arg11	Arg11	Arg3.6	Arg3.6	Arg1.2	Arg1.2	
C		Arg0.4	Arg0.4	Arg0.1	Arg0.1	Arg0.66	Arg0.66	Arg1	Arg1	Arg2	Arg2	
D		Arg3	Arg3	Arg4	Arg4	Arg5	Arg5	Arg6	Arg6	Arg7	Arg7	
E		Arg8	Arg8	Aox0	Aox0	Aox33	Aox33	Aox11	Aox11	Aox3.6	Aox3.6	
F		Aox1.2	Aox1.2	Aox0.4	Aox0.4	Aox0.1	Aox0.1	Aox0.66	Aox0.66	Aox1	Aox1	
G		Aox2	Aox2	Aox3	Aox3	Aox4	Aox4	Aox5	Aox5	Aox6	Aox6	
H		Aox7	Aox7	Aox8	Aox8							

Figure 7: Pipetting scheme in the real-time PCR plate. The wells of the outer frame were left out to avoid evaporation of the liquid, shown in grey. Everything was performed in duplicates. Arg0 and Aox0 contained ddH_2O and were used as negative control. Arg0.66 and Aox0.66 were the references. The calibration strain with the certain concentrations were from B4 – C5 and from E4 – F7. The wells C8 – E3 contained the unknown samples Arg1 – 8 and the wells F10 – H5 contained the unknown samples Aox1 – 8.

3.2.3.5 SDS-PAGE

It is important to test if the cells are lysed or not in order to determine if the protein concentration in the supernatants is due to cell lysis or because of an improved secretion. Therefore, a SDS-PAGE was performed to verify that and exclude the possibility of lysed cells. Furthermore, it is possible to compare the expressed proteins in the supernatants with the expressed proteins in the cell free extracts. Aliquots were taken from the collected samples, which were obtained from the screenings in shake flasks, see 3.2.2.3 Screening in shake flasks. Afterwards, the SNs and CFEs were treated differently. The CFEs were diluted to a concentration of 2 µg/10 µL using Nanodrop. For a supernatant approach, 6 µL of SN was filled up with ddH_2O to 10 µL. Subsequently, 1 µL sample reducing agent and 4 µL LDS sampling buffer were added to 10 µL of SN or CFE and filled up with ddH_2O to 16 µL. In the case of a viscous supernatant approach 1 µL 1 M TRIS was added. The samples were denaturated at 72 °C for 10 min. The denaturated samples were loaded onto a NuPAGE® gel. The run conditions for a gel were 200 V, 120 mA and 25 W for 50 minutes. 1x MOPS buffer was used as running buffer. PageRuler^{TM} prestained protein ladder from Thermo Scientific was used as size standard (see appendix - A 2).

3.2.3.6 Silver staining

All the used solutions are listed in table 15. In this thesis the used silver staining method was a combination of Blum et al. (1987) and Gromova & Celis (2006).

The run gel was incubated in the fixation solution overnight. Afterwards, it was washed three times for 20 min with the washing solution and then incubated for 30 min with ddH_2O. Subsequently, the gel was incubated for 1 min in the sensitising solution and after this, it was washed with ddH_2O three times for 20 sec. After this step occured the staining for 20 min with the staining solution, followed by washing steps with ddH_2O (two times for 20 sec). The gel was developed with the developing solution until to the desired image. A further washing step was performed just for a few seconds with ddH_2O. Afterwards, the development of the gel was stopped with the stop solution. In this solution the gel could be stored for a longer period in the fridge. The stained gel was scanned with HP Scanjet 4370.

3.2.3.7 Growth curve with BMD-1 (-YNB) + acetamide

Two ONCs with different media, inoculated with MutS, were prepared. Those media were BMD-1 and BMD-1 (-YNB) + acetamide. The main cultures (50 mL) were inoculated to an OD_{600} 0.1. Samples were taken for the OD_{600} measurement four times a day (4 h span) for a duration of two days.

3.2.3.8 Growth curve with different concentrations of acetamide

BMD-1 (-YNB) media with different concentrations of acetamide were prepared for this procedure, containing either 30 mg/mL, 60 mg/mL, 150 mg/mL, 300 mg/mL or 600 mg/mL of acetamide. One colony of MutS was inoculated into the various media and incubated overnight (ONCs). From those ONCs 50 mL of the diverse media were inoculated to an OD_{600} 0.1 (main cultures). For the OD_{600} measurement samples were taken after 0 h, 4 h, 8 h, 12 h, 24 h, 28 h, 32 h and 48 h.

3.2.3.9 Assembly of *amd*S gene in pPpT4 α S vector

For the amplification of the *amd*S gene from the vector pUG-amdSYM, the primers I13030 and I13031 were used. The vector pPpT4 α S was amplified with the primers I13032 and I13033. The primers are listed in table 7. After the PCR, the fragments were loaded onto a gel to see if there are bands of the correct size visible. Afterwards, the PCR products were purified via gel or via column (Qiagen kit). After the purification the concentration of the fragments was measured by Nanodrop. In total 5 µL of vector and insert in a ratio of 1:3 were added to a 15 µL Gibson assembly master mix and incubated at 50 °C for 60 min. It is important that the vector has at least a concentration of 100 ng. In order to get a high concentration of the ligated product, several approaches were performed.

Gibson cloning is a method to combine fragments with each other via overlapping sequences, which are also amplified afterwards like in a PCR. Detailed information of how the Gibson cloning works, can be found in the paper ``Enzymatic assembly of DNA molecules up to several hundred kilobases´´ from Gibson et al. (2009).
After the incubation the construct was purified and then digested with *Smi*I at 30 °C for 2 h to obtain a linear fragment. The reaction was heat-inactivated at 65 °C for 20 min. The linearised construct (500 ng) was tranformed into MutS. The regeneration phase was performed in two different ways. One time, aliquots of the cell suspension were plated on BMD-1 (-YNB) + acetamide plates and incubated for two days at 28 °C. On the other time, the suspensions were centrifuged (5 min, room temperature, 500 g) to obtain the pellets, which were resuspended afterwards in 1 mL PBS in order to wash the pellets and get rid of any available nitrogen sources. Finally, cells were plated on three BMD-1 (-YNB) + acetamide plates.

3.2.3.10 Assembly of *amd*S gene in pPpT4 GAP S vector

This time, the primers I13065 and I13066 (table 7) were used for the amplification of the *amd*S gene from pUG-amdSYM. The primers contained *Spe*I and *Not*I restriction sites. The pPpT4 GAP S vector was stored in EC Top 10 and it was isolated by the kit GeneJet™. After amplification and isolation of the particular constructs, they were digested with *Not*I. The restriction approaches were purified via gel or via column (Qiagen kit). Afterwards, another digestion was performed. This time the digestion occured with *Spe*I and again, the approaches were purified via gel or via column. Ligation of digested vector (pPpT4 GAP S) and insert (*amd*S) in the ratio of 1:2 was the next step. T4 DNA ligase was the used enzyme. The ligated construct was desalinised with a MF™-membrane filter for 45 min at room temperature. 250 ng of the desalinised construct were transformed into EC Top 10 and after the regeneration, 100 µL of a 1:10 as well as a 1:20 dilution were plated on LB Zeo plates. Then, positive transformants were determined via colony PCR. The DNA of one positive clone was obtained with the kit from Qiagen. Subsequently, the fragment was linearised with *Smi*I and 500 ng of the linear construct was transformed into MutS (3 approaches). After the regeneration 200 µL and the rest (that means the remaining cell suspension was centrifuged, the SN was discarded and the cells were resuspended in the remaining medium) were plated on YPD zeo plates, BMD-1 (-YNB) + acetamide plates as well as on BMD-1 (-YNB) + acetamide (1.2 g/L) plates.

4 Results and Discussion

4.1 Characterisation of the protein of interest in single knockout strains

BCAT and *Ca*lB were used to evaluate possible effects of deletions of cell wall protein genes on titres of extracellular protein production. Electrocompetent MutS, MutS Δ*cwp1*, MutS Δ*scw10* and MutS Δ*och1* cells were produced as described in ``3.2.1.10 Electrocompetent *P. pastoris* cells´´. The protocol for production of electrocompetent MutS Δ*och1* cells contained slight differences. According to Krainer et al. the growth of MutS Δ*och*1 is impaired, therefore the ONC was grown for approximately 48 h instead of overnight. Furthermore, the main culture was inoculated to an OD_{600} 0.7 instead of an OD_{600} 0.2 in order to achieve the desired OD_{600} 0.8 – 1.0 after 4 or 5 h of incubation while being aware of the fact that a duplication of cells is not possible in this case.

The plasmid pPpT4 α S containing either the gene *ilv*E or *ca*lB (Zeo^R) was linearised with *Smi*I and afterwards *P. pastoris* MutS, MutS Δ*cwp1*, MutS Δ*scw10* and MutS Δ*och1* were transformed with 500 ng of DNA. Transformants were selected by zeocin or zeocin/geneticin. As previously mentioned, MutS Δ*och1* has lower growth rates compared to MutS, which was represented by significantly smaller colonies after a certain time. In the case of MutS Δ*scw10*, transformants could not be achieved by transforming 500 ng of DNA. Therefore, 1 µg of DNA was applied and furthermore, the regeneration step was extended, that means incubation occured for 2 h and 3 h (2 approaches were performed). Furthermore, it is also important to mention that the transformation efficiency in the strains MutS Δ*och1* and MutS Δ*scw10* was lower compared to the others (MutS and MutS Δ*cwp1*). After obtaining all transformants, the clones were screened in DWPs. In chapter 3.2.2.1 Cultivation in deep well plates cultivation and procedure of screening are described. After cultivation and induction of the *AOX1* promoter, the activity of BCAT and *Ca*lB was measured in the obtained SNs. For the procedure of either the BCAT assay or *Ca*lB assay, see chapters 3.2.3.1 Transaminase activity assay (BCAT assay) and 3.2.3.2 *Ca*lB activity assay. In total, six clones from the graphs were chosen to be further tested (3.2.2.2 Rescreening in deep well plates). After harvesting the SNs, again assays and data evaluation were performed. This time, only two clones were chosen to be further tested.

4.1.1 Characterisation of BCAT

A screening in shake flasks was performed with the two chosen clones from every strain. In contrast to the other screening procedures, the intracellular activity of BCAT in all strains was measured as well. This was performed in order to detect how much activity is still present intracellularly and, when compared with the extracellular activity, such information would suggest whether there was an improved secretion of BCAT by the diverse knockouts: a so-called ``secretion ratio´´ (given by the specific extracellular activity divided by the specific intracellular activity) reflects the tendency of the tested strains to secrete the product more efficiently. OD_{600} values were measured after 0 h, 8 h, 24 h, 32 h, 48 h, 56 h and 72 h of induction in order to normalise the volumetric activity to an OD_{600} 1. The intracellular as well as extracellular protein concentration was quantified by performing the Bradford assay. The protein concentration was also used to calculate the specific activity.

4.1.1.1 Copy number determination of *ilv*E

Since it is important to know how many copies each strain contains, in order to compare it with a specific control strain as well as with other knockout strains, qPCR was performed to determine the copy number of *ilv*E in different single knockout strains. The copy number may have an effect in the enzymatic activity. For example, a strain with two copies of a certain gene shows a higher activity compared to a strain with four copies or vice versa. This was also reported in Abad et al. (2010). There, a *P. pastoris* strain carried seven to eight copies of the gene *ca*lB, which showed a lower activity level in the SN compared to the control, which harboured only one copy.

qPCR was performed several times. The reason is that the standard deviation of the standard curve of one primer pair was mostly too high as well as some standard deviations from clones. In order to get an accurate copy number, a low standard deviation is essential. The reason for such high standard deviations can either be a contamination of the used primers or by the specific handling steps. In table 21 the copy numbers of each strain containing the *ilv*E gene are listed. As it is shown, there was no accurate control available for the certain knockout strains. Transformation of 500 ng DNA will usually lead to the integration of one copy of the expression cassette. However, in MutS Δ*cwp1* and MutS Δ*och1* more than one copy of the gene *ilv*E was determined. In MutS Δ*scw10* 1 µg of DNA was transformed, therefore, more than one copy was expected.

For the sake of calculation of the secretion ratio it does not matter how many copies one strain has, because it is just a ratio of two evaluated values. But for a precise comparison of the certain activity levels, the copy number of each strain is essential. It simply shows us a tendency of activity behaviour.

Table 21: Copy number determination of the *ilv*E gene. In this table the copy numbers from each strain containing the gene *ilv*E are listed.

Strain	Copy number
MutS H3	1
MutS F10	2
MutS Δ*cwp1* C11	3-4
MutS Δ*cwp1* F11	5
MutS Δ*och1* B12	2-3
MutS Δ*och1* A4	2-4
MutS Δ*scw10* B7	5
MutS Δ*scw10* H3	2

All in all, it can be concluded that the qPCR should be repeated, because sometimes results were obtained with a difference of one or more copies. An alternative option to determine the copy numbers is to perform standard southern blot analysis (Abad et al., 2010). It may take longer and may not be as simple as qPCR, however, it can be compared with qPCR in order to receive a proper copy number. Also Illumina sequencing is a possibility to determine copy numbers by coverage.

4.1.1.2 Best level of volumetric activity obtained in MutS Δ*och1*

The volumetric activity (normalised to an OD_{600} 1) is plotted against the expression time, which is demonstrated in figure 8a. The in-house available strain MutS F10 (two copies) was used as control and benchmark, because *ilv*E-expressing strains with either a MutS Δ*cwp1*, MutS Δ*och1* or MutS Δ*scw10* genetic background, contained more than one copy of the gene *ilv*E. In figure 8a, BCAT activity over time in the abovementioned strains, MutS Δ*cwp1*, MutS Δ*och1* or MutS Δ*scw10* is shown. Data in panel A show that the volumetric activity in the supernatant of the benchmark strain was higher compared to the MutS Δ*cwp1* strains (C11 SN and F11 SN). Furthermore, activity in the SNs increased until 32 h – 48 h of expression time and afterwards decreased. After 24 h of expression time, the clones, C11 and F11, showed a higher activity in the CFE compared to the control. Comparing the activities in the SNs with the CFEs, it seems that there is more protein (enzyme) per OD_{600} available in the CFEs of MutS Δ*cwp1* C11 and MutS Δ*cwp1* F11 in comparison with the control. This could lead to the conclusion that according to the OD_{600} value, in a volume of 1 mL culture, more BCAT is extracellularly available in MutS F10 and less secretion of BCAT occurs in MutS Δ*cwp1*. The volumetric activity of BCAT in the SN, as well as in the CFE, in MutS Δ*och1* was increased compared to MutS F10 (control), like it is shown in figure 8a (B). In the MutS Δ*och1* B12 strain, the extracellular activity was significantly higher (likewise, although at a lesser extent, in clone MutS Δ*och1* A4). A higher protein activity - intracellular and extracellular - for BCAT in Δ*och1* genetic backgrounds suggests an increased protein expression in this strain compared to the control strain. Figure 8a (C) represents the

extracellular and intracellular volumetric activity in MutS Δ*scw10*. It is visible that the activities are quite similar in the SNs of MutS F10 and MutS Δ*scw10* H3, whereas the volumetric activity of MutS Δ*scw10* B7 is different. At the beginning it shows a lower volumetric activity compared to the control. After approximately 48 h of induction, this clone has reached nearly the same activity level as the control. So, the deletion of the gene *SCW10* had hardly an effect to the volumetric activity in comparison to MutS F10.

According to these data, the best volumetric activity in the SN was achieved in the MutS Δ*och1* strain, whereas in MutS Δ*scw10* the level of the volumetric activity was nearly the same as the control. Moreover, the worst activity level was detected in MutS Δ*cwp1*, which seemed to block efficient secretion.

A low volumetric activity was expected in the CFEs, because the integrated *ilv*E gene carried upstream a secretion signal sequence. When expressed, it initiates the secretion of the particular recombinant protein. One conclusion for the measured volumetric activity in the CFEs can be that not all proteins were secreted (insufficient secretion) and accumulated within the cell.

Additionally, in figure 8b the volumetric activity (not normalised to an OD_{600} 1) of BCAT in the knockout strains is shown in order to get an overview how the volumetric activity differs when the different growth rates are not taken into account. It might be possible that clones with a high activity are underestimated when the biomass is not considered.

All in all, the BCAT assay still needs further optimisation in order to make the obtained results more reliable, since some results differed significantly. Alternatively, analytics by a chromatographic method can be applied to obtain more precise data for the most interesting strains. As it is explained in 2.3.1 Branched chain aminotransferase (BCAT), there are more enzymes involved in the assay except BCAT and therefore, no direct colorimetric measurement of the substrate conversion by BCAT can be done with this assay.

There are other transaminase assays, where the substrate conversion can be directly measured. For example ω-transaminase converts α-methylbenzylamine and pyruvate to acetophenone and L-alanine. Subsequently, acetophenone can be detected at a wavelength of 245 nm (Schaetzle et al., 2009).

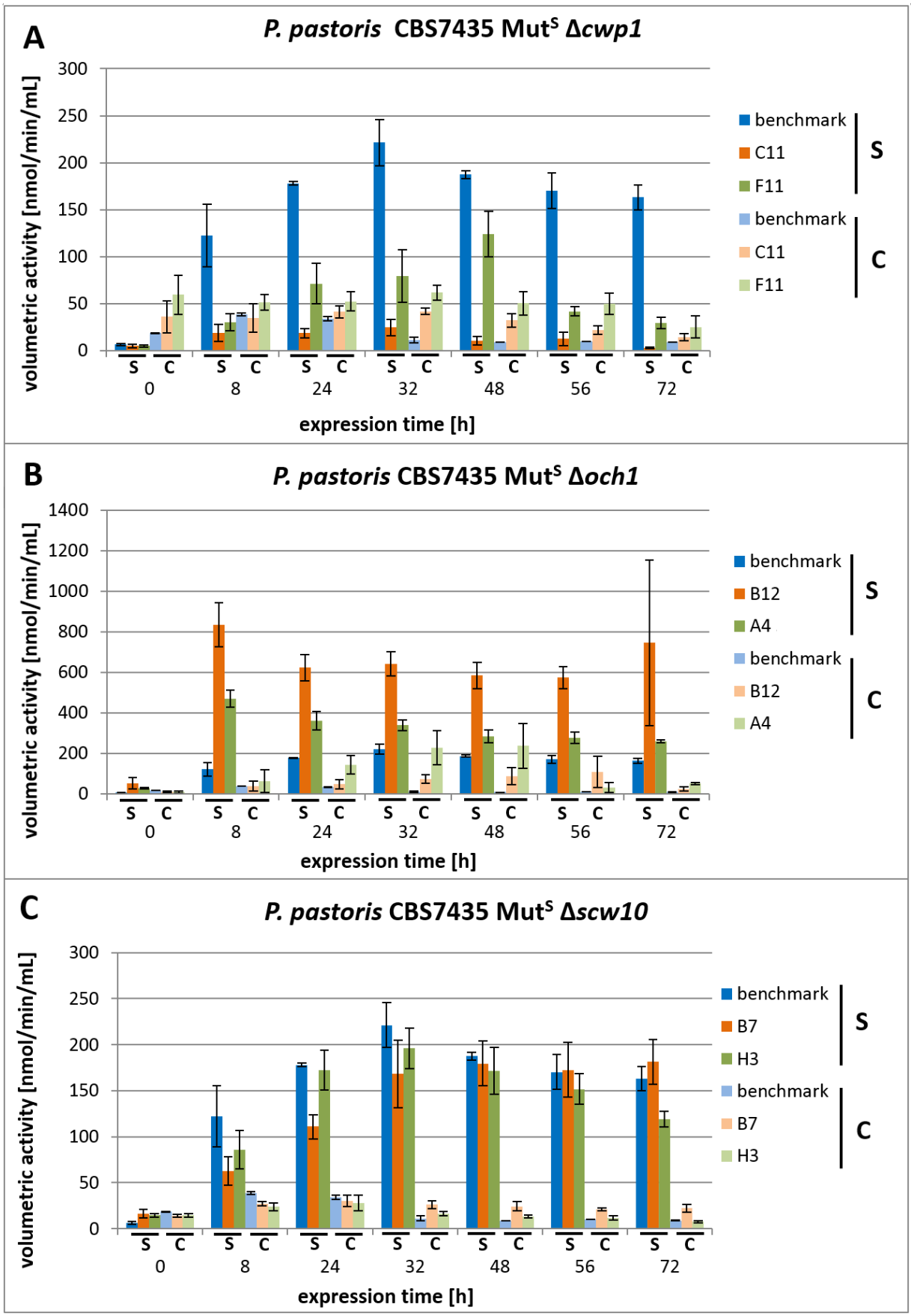

Figure 8a: Volumetric activity of BCAT in single knockout strains. The intracellular and extracellular activity of BCAT was measured for a duration of 72 h of induction. The volumetric activity was normalised to an OD_{600} 1. MutS F10 (two copies of *ilv*E gene) served as control and benchmark. The dark coloured bars indicate the activity in the supernatants (S) and the light coloured bars the activity in the cell free extracts (C). The letters with the numbers represent clones containing the *ilv*E gene in either MutS Δ*cwp1* (A), MutS Δ*och1* (B) or MutS Δ*scw10* (C).

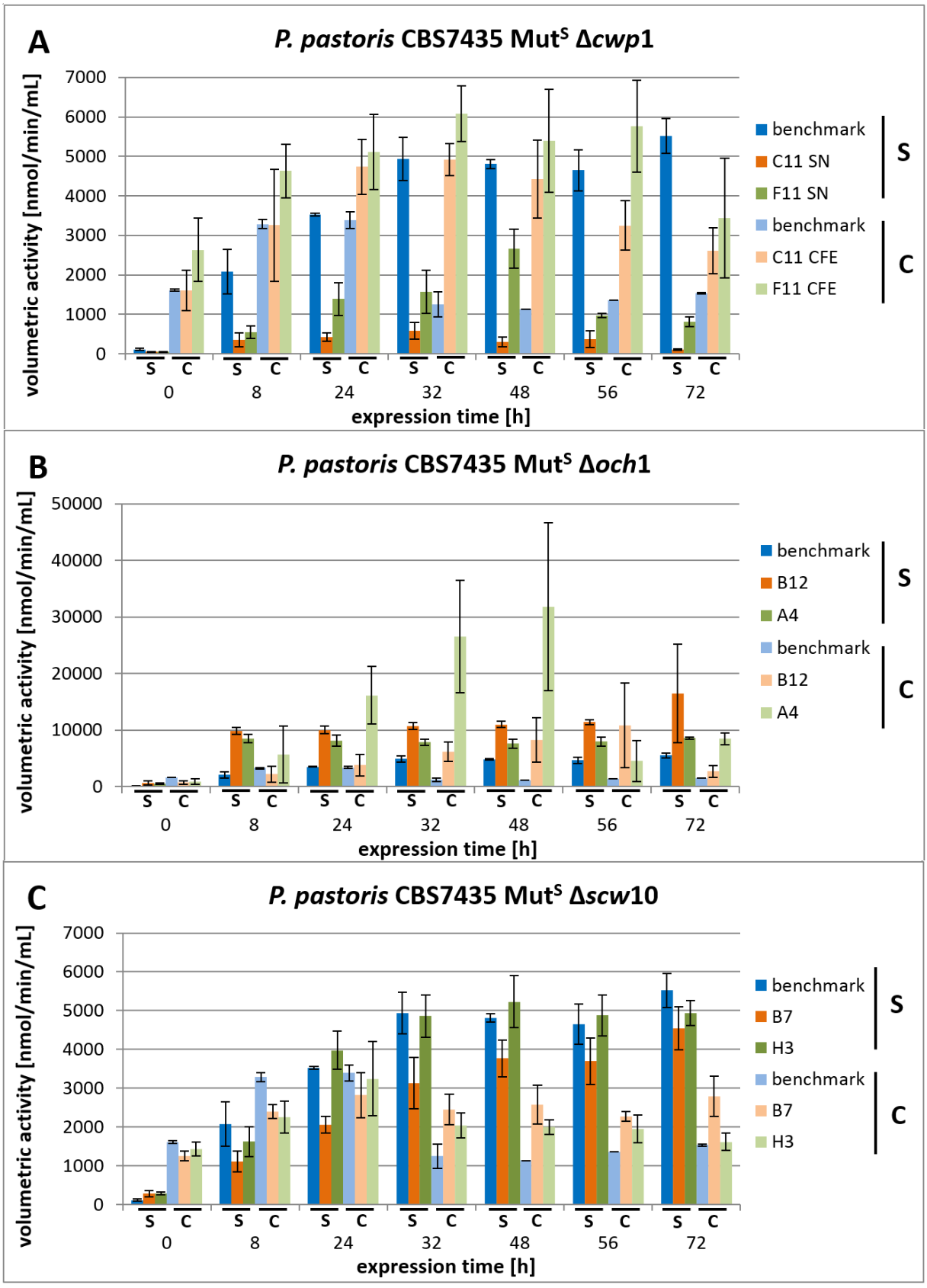

Figure 8b: Volumetric activity of BCAT in single knockout strains. The intracellular and extracellular activity of BCAT was measured for a duration of 72 h of induction. MutS F10 (two copies of *ilv*E gene) served as control and benchmark. The dark coloured bars indicate the activity in the supernatants (S) and the light coloured bars the activity in the cell free extracts (C). The letters with the numbers represent clones containing the *ilv*E gene in either MutS Δ*cwp1* (A), MutS Δ*och1* (B) or MutS Δ*scw10* (C).

4.1.1.3 Determination of the specific activity

Not only the volumetric activity of each strain was determined, also the specific activity was evaluated. It was calculated how much transaminase activity per mg protein was present in the SNs and CFEs of the particular knockout strains, to determine how the particular deletions in *P. pastoris* influence the specific activity of the produced enzyme preparations. As written previously, the protein concentration was quantified by Bradford. With this assay it is possible to determine low concentrations (20 µg/mL – 100 µg/mL) in contrast to the BCA assay, where the range for the possible measurable concentration is between 25 µg/mL - 2,000 µg/mL. Another reason for the choice of the Bradford assay was that no disturbing influence by Y-PER occured, as it was the case for BCA based measurements. The unit for the specific activity is given in µmol/min (U) per mg total protein. In figure 9 (A) the specific activity from BCAT in the Δ*cwp1* strain is shown. MutS F10 was used again as control and benchmark strain. It is visible, that the highest activity of the control in the SN took place after 32 h of induction and afterwards, the activity declined over time. The clone C11 showed a decreased specific activity in the SN compared to the control, whereas the clone F11 had nearly the same specific activity over an induction period of 48 h and diminshed thereafter. In the CFEs there was hardly any activity determined. The reason is that the amount of total protein in the CFEs is much higher compared to the total protein amount in the SNs. That means, the higher the total protein concentration the lower the specific activity. The branched chain aminotransferase was also characterised in the MutS Δ*och1* strain (figure 9 (B)). At the beginning, it seems that in the clones there was just a little amount of protein secreted, which increased over time. This can also be seen in the tendency of the specific activity. First, the specific activity was higher and then declined. Compared to the control MutS F10, MutS Δ*och1* B12 showed a lower activity per mg protein. MutS Δ*och*1 A4 had a higher activity compared to the control in the first few hours. Towards the end of the expression time, the activity in both, control and clone A4, had nearly the same level. As before, specific activity could hardly be determined in the CFEs. BCAT was tested in MutS Δ*scw10* as well. In figure 9 (C) it is shown, that the specific activity in the SNs of both clones, B7 and H3, was pretty similar to the control strain, except after 32 h. There, the control MutS F10 showed the highest activity, which could not be achieved by the tested clones. Like in the other tested strains, MutS Δ*cwp1* and MutS Δ*och1*, there was rarely specific activity detected in the CFEs.

In order to receive more accurate results for the protein concentration, an option would be to measure the protein concentration at 590 nm and 450 nm and to use the quotient for further calculations. Measurement at these two wavelengths would sensitise the Bradford-assay (Zor and Selinger, 1996). This would probably minimise the standard deviation of some results.

Comparing the intracellular with the extracellular specific activity, there can be made evidences relating to the secretion ratio for example. As mentioned before, the deletion of *SCW10* seems to have no impact in secretion in a positive or negative way, because the levels of the volumetric and specific activity were nearly the same as in MutS F10. In contrast, in the other knockout strains a similar or decreased level of specific activity could be determined.

All in all, only tendencies are shown, since the tested strains did not harbour the same copy number and no clear conclusions can be made. Therefore, the experiments should be repeated with knockout strains carrying the same amount of copies in order to allow an accurate comparison.

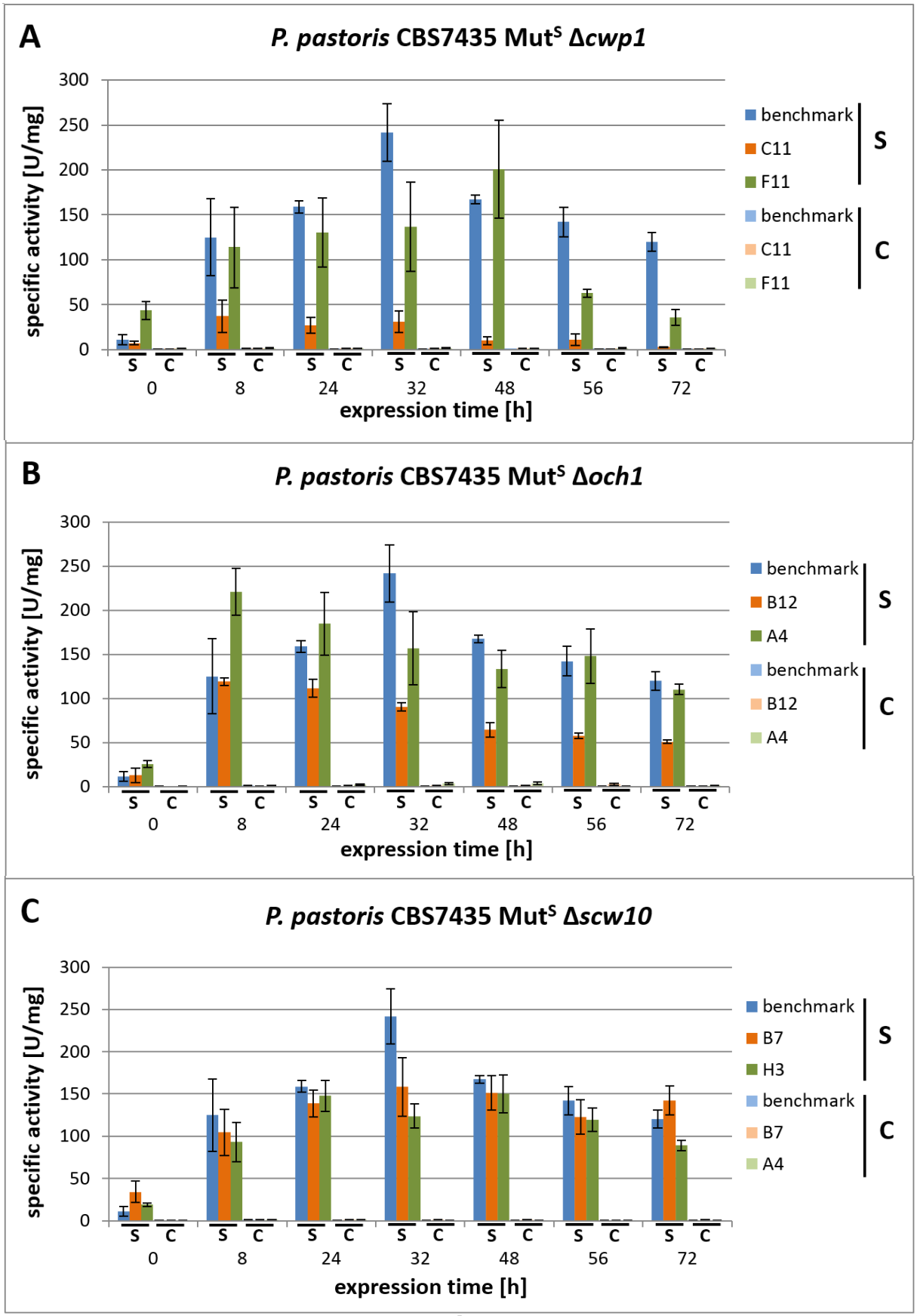

Figure 9: Specific activity of BCAT in single knockout strains. The intracellular and extracellular specific activity of BCAT was evaluated for a duration of 72 h of induction. The protein quantification was performed with the Bradford assay. MutS F10 containing two copies of the expression cassette was used as control and benchmark. The dark coloured bars indicate the activity in the supernatants (S) and the light coloured bars the activity in the cell free extracts (C). The letters with the numbers represent clones containing the *ilv*E gene in either MutS Δ*cwp1* (A), MutS Δ*och1* (B) or MutS Δ*scw10* (C).

4.1.1.4 No improved secretion in the diverse knockout strains

The secretion ratio was calculated for each clone in order to get an overview if the secretion was improved or not. The secretion ratio is the ratio between the extracellular and intracellular protein availability. It was calculated on the basis of the specific activity, see figure 10. The dark blue line indicates the control H3, which contained one single copy of the *ilv*E gene. In this case, the particular copy number does not matter, because the secretion ratio is just the ratio between BCAT activity outside and inside. If the values of the secretion ratio from the clones are higher than the control, it means that there is an increased secretion of BCAT. MutS Δ*cwp*1 C11 and F11 had a decreased secretion ratio compared to MutS H3. MutS Δ*och1* B12 and A4 showed an increased secretion ratio after 8 h of expression and afterwards decreased in contrast to the control. The secretion ratio of MutS Δ*scw10* behaved almost the same way like MutS H3. However, no conclusions can be made. The standard deviations for the calculated secretion ratio are too high for a significant statement.

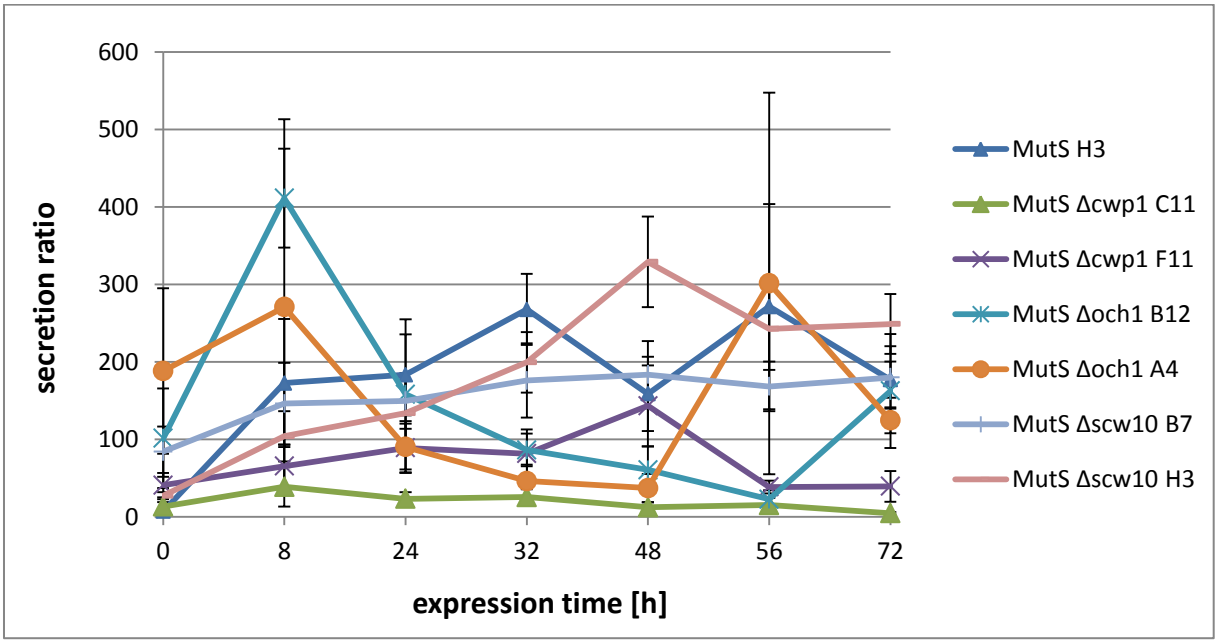

Figure 10: Secretion ratio of BCAT in single knockout strains. The secretion ratio was calculated over 72 h of expression. As control MutS H3 was used (dark blue).

4.1.1.5 Detection of BCAT by SDS-PAGE

SDS-PAGE on supernatants and cell lysates were carried out to exclude artecfacts in the observed results. The whole procedure can be read in chapter 3.2.3.5 SDS-PAGE. The gels were loaded using 2 µg of CFEs and 6 µL volume of the SNs. The gels were stained by silver staining. In order to compare the expression of the strains with each other, samples obtained after 48 h of expression were used. The period of 48 h is presumably enough to have all strains at the same growth level, because, as previously mentioned, for example, the strain Δ*och1* grows very slowly compared to the others and after 48 h they should be at the same

level. In figure 11 (A) the CFEs from the particular strains and in (B) all SNs are shown. The red arrows indicate the height of BCAT. As it is visible in the SNs, there are two bands present. It was previously determined that BCAT is partially glycosylated in *P. pastoris* and, therefore, two bands are existing. The upper band (~40 kDa) in lanes 4, 8, 9 and 10 shows the glycosylated version of BCAT (K. Weinhandl, unpublished personal communication) and the lower band (~37 kDa) displays the unglycosylated one as reported in literature (Inoue et al., 1988). It is clearly shown, that BCAT was more strongly expressed in the SN of MutS Δ*och1* (lane 9) as in the other strains. In MutS Δ*cwp1* and MutS Δ*scw10* the two bands of BCAT are hardly detectable. These results can also be confirmed by the respective enzymatic activities. As it is visible on the gel, there are more bands in lane 9 (MutS Δ*och1* B12) present, especially one very intense band (~130 kDa), compared to the other knockout strains, which means, that there was much more protein expressed. This could also be confirmed by the Bradford protein quantification and furthermore by the decreased specific activity. Additionally, it seems that this ~130 kDa band only occurs when the expression cassette is transformed into MutS Δ*och1*, as in the corresponding empty knockout strain such a band is not detectable. Also in lane 4 (MutS H3) are two intense bands between 60 kDa and 70 kDa visible and to a lesser extent in lane 5 (MutSΔ*cwp1*). It might be possible that a little bit of the sample in slot 4 ran also into slot 5, since there are the same bands with only different intensities present. Another possibility for the appearance of these bands could be culture conditions, as in shake flasks there is for example no controlled supply of oxygen or pH.

Although the codon-optimised version of BCAT has a size of 34.4 kDa, it could be detected that the protein ran higher in the SNs. This effect might come from the occured glycosylation. Another reason might be that the denaturated proteins ran slower as the standard, because the concentration in the samples was higher.

In the CFEs it could be determined that there is no clear additional band of BCAT visible. Furthermore, in the CFE BCAT can still have the α factor secretion signal, because probably there have not occured processing steps yet, so BCAT might be heavier than the determined 34.4 kDa. In order to detect BCAT in the CFE, an option would be to tag it with a His-tag for example and perform a western blot. This would be a possible and accurate method, because with a tag it is easier to detect the correct protein.

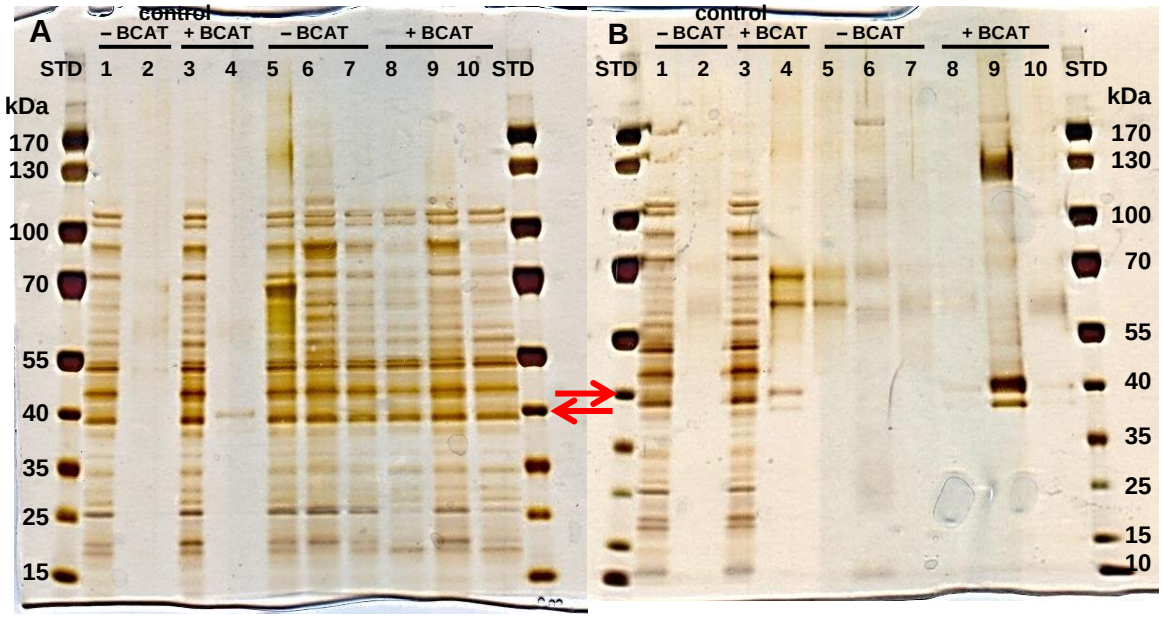

Figure 11: Silver Staining. The red arrows indicate the size of the branched chain aminotransferase (~34 kDA). All strains were based on the *P. pastoris* CBS7435 Mut^{S} (TU Graz strain collection number 3445) platform. H3 was used as control and PageRuler^{TM} prestained protein ladder as standard.
STD: standard, 1: wild type CFE, 2: wild type SN, 3: H3 CFE and 4: H3 SN - these samples are the same on both stained gels. (A) In lane 5-7 there are the samples of the CFEs from the corresponding empty knockout strains present, whereas in lane 8-10 all tested strains containing the *ilv*E gene are shown. 5: Δ*cwp1*, 6: Δ*och1*, 7: Δ*scw10*, 8: Δ*cwp1* F11, 9: Δ*och1* B12 and 10: Δ*scw10* B7. The samples from the SNs are shown in (B). Lane 5-7 represent the empty knockout strains respectively and lane 8-10 the strains expressing BCAT. 5: Δ*cwp1*, 6: Δ*och1*, 7: Δ*scw10*, 8: Δ*cwp1* F11, 9: Δ*och1* B12 and 10: Δ*scw10* B7.

Finally, MutS Δ*och1* has shown to be a very beneficial strain, because it can produce BCAT in a much higher yield as MutS Δ*cwp1* and MutS Δ*scw10*. In addition, we found out that BCAT is N-glycosylated (N91-L92-T93). Since O-glycosylation is not well characterised yet, it cannot be excluded that there are O-glycosylation sites as well (K. Weinhandl, unpublished personal communication). The localisation and the amount of glycosylation may influence the BCAT activity, because it may influence the protein folding or the active site. Since *OCH1* encodes for a α-1,6-mannosyltransferase, which is deleted in MutS Δ*och1*, the expressed BCAT might show a decreased level of glycosylation, which may explain the increased volumetric activity due to higher specific activity. However, it is impossible to do an accurate comparison of the knockout strains with each other, because as already mentioned, these strains harboured different copy numbers. And as previously stated, different copy numbers may cause different activity levels.

4.1.2 Characterisation of *Ca*lB

After performing the screening experiments in DWPs and evaluating the obtained data, two clones from each strain were chosen for further characterisation. As in 4.1.1 Characterisation of BCAT already written, the intracellular activity was measured as well, however, this time from *Ca*lB in all strains. Moreover, OD_{600} values were measured after 0 h, 8 h, 24 h, 32 h, 48 h, 56 h and 72 h of induction in order to normalise the volumetric activity to an OD_{600} 1. The intracellular as well as extracellular protein concentration was quantified by performing the Bradford assay, which was further used for the calculation of the specific activity.

4.1.2.1 Copy number determination of *ca*lB

qPCR was performed in order to determine the copy number existent in the particular knockout strains. For an accurate comparison of the diverse activities between the strains, the number of *ca*lB copies is important. This procedure was repeated many times, because the standard deviation of the standard curve of one primer pair was mostly too high as well as some standard deviations from clones. Possible causes for this inaccurate measurement can either be a contamination of the used primers, not well calibrated pipettes or by the specific handling steps. In table 22 the particular copy numbers of each strain containing the *ca*lB gene are listed. All knockout strains contained more than one copy of *ca*lB. So, in order to do a precise comparison of volumetric and specific activity, other controls have to be produced. Moreover, another experiment needs to be performed in order to compare the obtained copy numbers by qPCR. A proper method to measure the copy number is for example southern blot analysis.

Table 22: Copy number determination of the *ca*lB gene. In this table are listed the copy numbers from each strain containing the gene *ca*lB.

Strain	Copy number
MutS E8	1
MutS Δ*cwp*1 E7	1-2
MutS Δ*cwp*1 H6	2
MutS Δ*och*1 A3	1-3
MutS Δ*och*1 B7	2
MutS Δ*scw*10 B2	2
MutS Δ*scw*10 A4	2

4.1.2.2 Highest detectable volumetric activity in MutS Δ*cwp1*

In order to characterise *Ca*lB in the diverse knockout strains, the volumetric activity (normalised to an OD_{600} 1) was evaluated. In figure 12a the volumetric activity, expressed as nmol/min/mL, is plotted against the expression time. The volumetric activity is given in nmol/min/mL in order to reflect a better representation of the data. MutS E8 was used as a control, which contained one single copy of the gene *ca*lB. This control was chosen because it was supposed that the picked clones, which showed an average activity of *Ca*lB in the landscapes of the screening experiments, were single copy strains as well. In fact, the particular knockout strains had between one and three copies of *ca*lB. Detailed copy numbers are listed in table 22. In figure 12a (A), *Ca*lB was explored in MutS Δ*cwp1*. As it is displayed, the volumetric activity in the SN of the clones, E7 and H6, was higher compared to the control MutS E8. After 72 h of induction the level of the volumetric activity was similar in all three strains. It is demonstrated that the activity in the tested MutS Δ*cwp1* strains increased until 32 h – 48 h and then decreased. The volumetric activity in the CFEs was rather the same in all strains. Due to the fact that the volumetric activity is increased in the SN of the clones in contrast to the control and, additionally, the activity in the CFEs is similar, it suggests that these knockout strains express and secrete more *Ca*lB per biomass and volume. Furthermore, *Ca*lB was analysed in MutS Δ*och*1 (figure 12a (B)). The extracellular volumetric activity in all strains was increasing over time. Comparing the activity in MutS Δ*och*1 A3 and B7 with the control MutS E8, it can be seen that the activity level in the clone B7 was enhanced compared to the others. Like before, the intracellular volumetric activity was rather the same in all strains. Comparing all volumetric activities in the SNs and CFEs, it seems that MutS Δ*och1* expresses more *Ca*lB per OD_{600} and per mL than the control. In figure 12a (C) *Ca*lB is characterised in the strain MutS Δ*scw10*. In the first few hours the clones, B2 and A4, showed a decreased volumetric activity in contrast to the control, which increased over time. However, it is impossible to say that over time one of the clones had a higher volumetric activity as the control. The volumetric activity in the CFEs was pretty similar in the strains again. It seems that this deletion had no significant effect in secreting proteins. Hence, it is evident that not every knockout strain showed the desired result of an improved secretion or increased volumetric activity of *Ca*lB. Nevertheless, these are just tendencies, because the strains did not have the same number of *ca*lB copies. Therefore, an accurate comparison is impossible, because different copy numbers probably have different influences, see discussion from BCAT.

If you have to compare those obtained strains containing different copy numbers, the best strain for expressing *Ca*lB would be MutS Δ*cwp1*, because both clones showed an increased volumetric activity. In MutS Δ*och1* an increased volumetric activity could be detected as well, however to a lesser extent.

Additionally, the volumetric activity, which is not normalised to an OD_{600} 1, is shown in figure 12b. There, the different growth rates of the knockout strains are not considered in order to see how the volumetric activity differs. It might be possible that clones with a high activity are underestimated when the biomass is not considered.

All in all, the *Ca*lB assay is much easier to perform in order to receive more precise results than with the BCAT assay. The reason is because of the direct colorimetric measurement of the substrate conversion. Thus, just *Ca*lB is involved in this assay and no other enzyme like in the BCAT assay. In figure 2 it is shown how the reaction takes place.

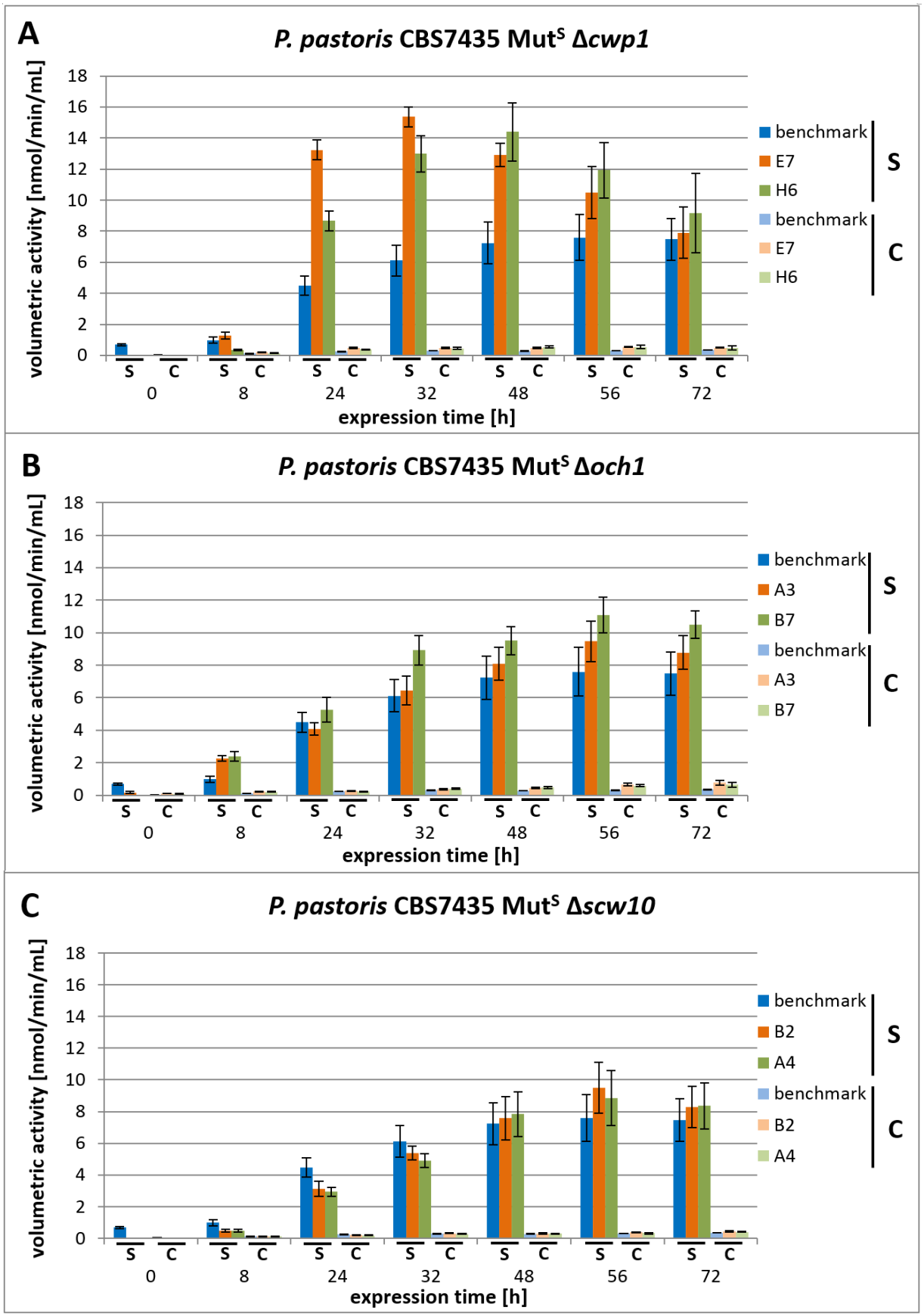

Figure 12a: Volumetric activity of *Cal*B in single knockout strains. The intracellular and extracellular activity of *Cal*B was measured for a duration of 72 h of induction. The volumetric activity was normalised to an OD_{600} 1. MutS E8 containing one copy of the gene *cal*B served as control and benchmark. The dark coloured bars indicate the activity in the supernatants (S) and the light coloured bars the activity in the cell free extracts (C). The letters with the numbers represent clones containing the *cal*B gene in either MutS Δ*cwp1* (A), MutS Δ*och1* (B) or MutS Δ*scw10* (C).

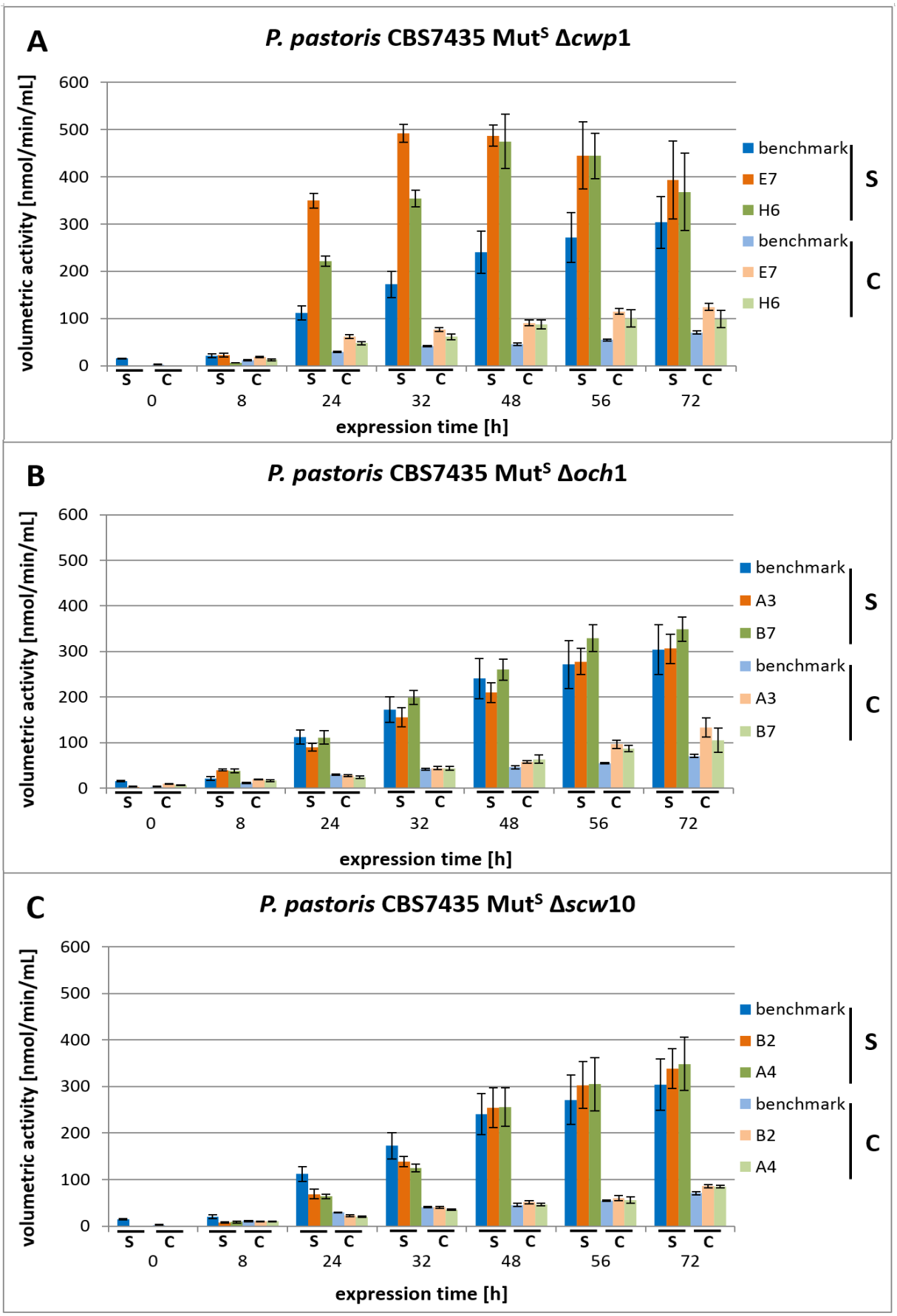

Figure 12b: Volumetric activity of *Cal*B in single knockout strains. The intracellular and extracellular activity of *Cal*B was measured for a duration of 72 h of induction. MutS E8 containing one copy of the gene *cal*B served as control and benchmark. The dark coloured bars indicate the activity in the supernatants (S) and the light coloured bars the activity in the cell free extracts (C). The letters with the numbers represent clones containing the *cal*B gene in either MutS Δ*cwp1* (A), MutS Δ*och1* (B) or MutS Δ*scw10* (C).

4.1.2.3 Specific activity was best in MutS Δ*cwp1* H6

Additionally, the activity per mg protein was determined in the SNs as well as CFEs in the respective knockout strains in order to receive an overview how the deletions affect the specific activity. The Bradford assay was used in order to determine the protein concentration in each strain. As already previously mentioned in the chapter 4.1.1 Characterisation of BCAT, this method was used because it allows the determination of low protein concentrations and it does not interfere with Y-PER.

The specific activity is represented as µmol/min/mg or U/mg and it is plotted against the expression time. In figure 13 (A) the specific activity of *Ca*lB is depicted in the strain MutS Δ*cwp1*. The specific activity in the SN of the control MutS E8 increased until 48 h of induction and slightly decreased afterwards. The clone E7 showed the same tendency with one exception, the activity decreased towards the end. MutS Δ*cwp1* H6 had the highest specific activity after 24 h of expression and then, it slightly decreased. However, it was still better as the other strains over time. The specific activity in the CFEs is not visible anymore in the graph. This is because the intracellular protein concentration is very high: the higher the protein concentration the lower the specific activity. Moreover, *Ca*lB was characterised in MutS Δ*och1* (figure 13 (B)). MutS E8 and MutS Δ*och1* B7 showed almost the same level of specific activity in the SN all the time, whereas the clone A3 showed a decreased specific activity compared to the others. In contrast, hardly any specific activity could be observed in the CFEs. Again, this is caused by a high total protein concentration within the cell. *Ca*lB was tested in MutS Δ*scw10* as well, see figure 13 (C). The specific activity in the SNs of both clones (B2 and A4) was decreased compared to MutS E8 and again, specific activity in the CFEs was hardly detectable. This leads to the conclusion that a high protein concentration is present extracellularly, which leads to the lower specific activity and not much *Ca*lB is accumulated in the cell.

As already shown in the graphs from BCAT, here again, the standard deviations are sometimes high. The causative factor is probably the protein quantification because the determination of the protein concentration occured just at a wavelength of 590 nm instead of 590 nm and 450 nm. Also the low lipase activity of *Ca*lB in the cell increases the error.

Finally, it could be determined that the best level of specific activity was achieved in MutS Δ*cwp1* H6. Moreover, it can be concluded that for *Ca*lB production MutS Δ*cwp1* would be suitable, because much more *Ca*lB was secreted, which led to the increased volumetric activity level. However, both clones of MutS Δ*cwp1* secreted different amounts of total protein, which indicates additional effects for example from different copy numbers of expression cassettes or different integration loci. Thus, no clear conclusion can be made if the observed higher *Ca*lB activity in the supernatant of the Δ*cwp1* H6 strain is an effect of the deletion of *CWP1*.

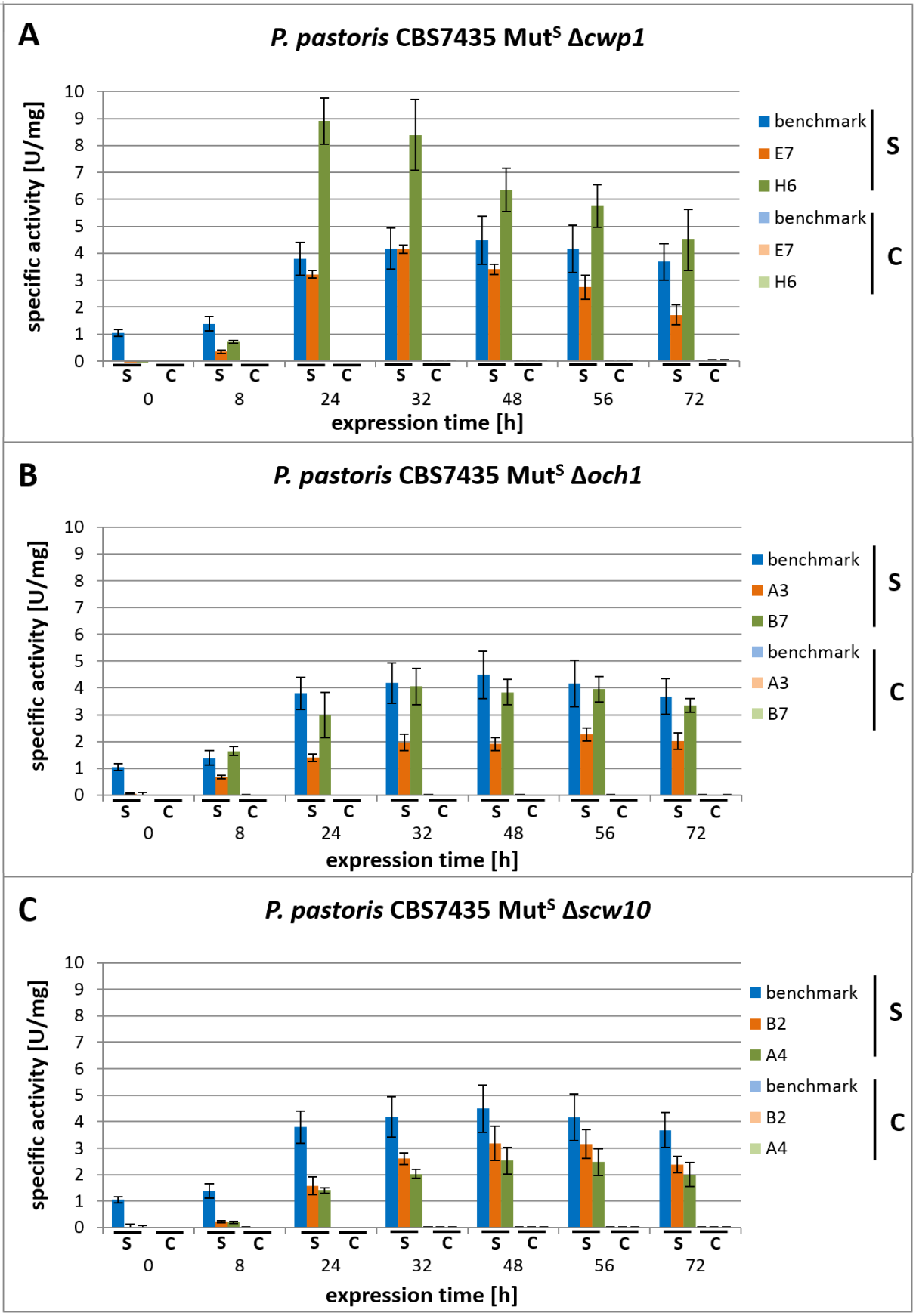

Figure 13: Specific activity of *Cal*B in single knockout strains. The intracellular and extracellular specific activity of *Cal*B was evaluated for a duration of 72 h of induction. The protein quantification was performed with the Bradford assay. As control and benchmark was used MutS E8 (one copy of *cal*B gene). The dark coloured bars indicate the activity in the supernatants (S) and the light coloured bars the activity in the cell free extracts (C). The letters with the numbers represent clones containing the *cal*B gene in either MutS Δ*cwp1* (A), MutS Δ*och1* (B) or MutS Δ*scw10* (C).

4.1.2.4 Improved secretion in MutS Δ*cwp1* H6 and MutS Δ*och1*

The secretion ratio was calculated for *Ca*lB as well in order to determine if there is an improved secretion present. The evaluation of the data is shown in figure 14. The secretion ratio is the ratio between the extracellular and intracellular protein activity. It is independent from the copy numbers, because it is just a ratio of two values. Again, it was calculated on the basis of the specific activity. MutS E8 was used as control, which is labelled in dark blue. In contrast to MutS E8, both clones of MutS Δ*och1* as well as MutS Δ*cwp1* H6 showed an increased secretion ratio, whereas all the other clones had a reduced one. According to these evaluated data, it can be concluded that MutS Δ*scw10* is impaired in secretion, whereas the secretion in MutS Δ*och1* is improved. According to MutS Δ*cwp1* there was on the one hand, one clone with an improved and on the other hand, one clone with a decreased secretion level present. Here, it might be interesting to find out in which locus the gene has integrated, because both clones harboured 1-2 copies. Therefore, the locus of integration might cause the difference in activity and secretion.

The secretion ratio of the double knockout strains are discussed in the chapter 4.2.4 Improved secretion of *Ca*lB in MutS Δ*och1*/Δ*scw10*.

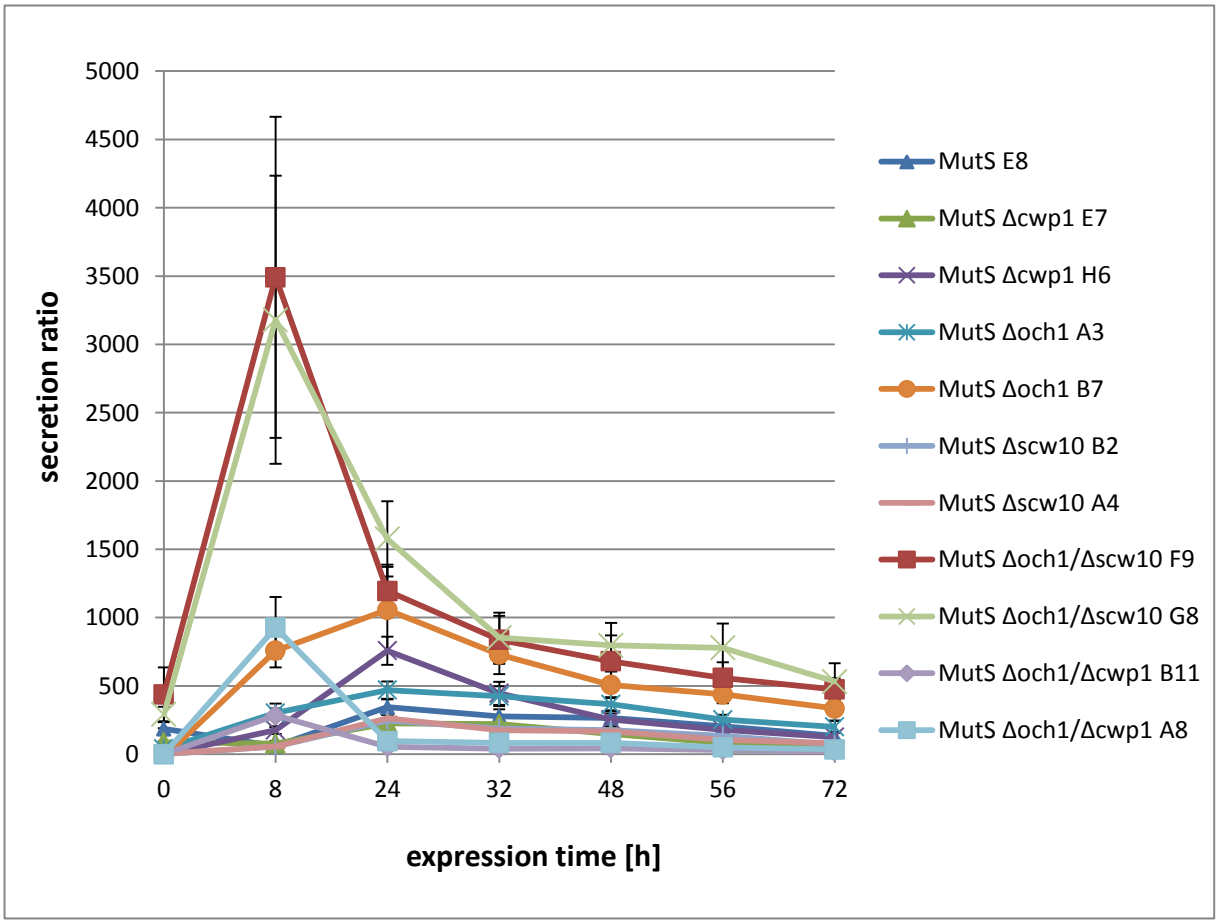

Figure 14: Secretion ratio of *Ca*lB in single and double knockout strains. The secretion ratio was calculated over 72 h of expression. As control MutS E8 was used (dark blue).

4.1.2.5 Detection of *Ca*lB by SDS-PAGE

*Ca*lB was also detected by SDS-PAGE in order to avoid the possibility of artefacts and to detect the size of the protein. Silver staining was the used method to stain the gels. The silver staining procedure is a very sensitive method in order to detect proteins in SDS-PAGE gels. Only one clone from each knockout strain was used for this experiment in order to confirm the presence of *Ca*lB. MutS E8 was used as control and in addition the knockout strains without the *ca*lB gene were applied as well. It should be noted as well that samples were used after 48 h of expression, so that all the strains were at the same growth level. In figure 15 (A) the CFEs from the particular strains are demonstrated and in (B) all SNs. The size of *Ca*lB is given by the red arrows. As it is visible on the gel, only slight differences can be observed in the intensity of the bands in the single knockout strains, which can also be reflected in the specific activity. The clones had a pretty similar level of specific activity and nearly the same protein concentration. In MutS Δ*och1* B7 (lane 11) a ~130 kDa band is visible, whereas in the corresponding empty knockout strain (lane 6) a ~130 kDa band cannot be detected. It seems that this band only occurs when an expression cassette is tranformed into MutS Δ*och1* leading to the secretion of this unknown protein. In the silver stained gel for CFEs, a correct detection of *Ca*lB could not be guaranteed. A reason might be, that many *Ca*lB proteins are not fully processed at this time, which means, that there are still many *Ca*lB proteins left containing the α factor secretion signal, which would lead to a different size as expected. In order to determine *Ca*lB in the CFEs, a near choice would be to apply a western blot. If the protein is tagged, it is quite easy to detect the tagged protein by a labelled probe (for example antibody). On the gel containing the SNs, *Ca*lB can be clearly identified. The codon-optimised *Ca*lB has an expected size of 35.3 kDa, however, it ran until ~40 kDa. The difference in size can probably be explained by glycosylation, because as already known, *P. pastoris* is able of several posttranslational processes. Additionally, it is reported that *Ca*lB is N-glycosylated, when expressed in *P. pastoris* (Rotticci-Mulder et al., 2001). It might also be that the denaturated proteins ran slower as the standard, because the concentration in the samples was higher. Moreover, the difference in size can be caused by buffer for example: standard and samples were mixed with different buffers, which may have caused differences.

Finally, the obtained results suggest that MutS Δ*cwp1* is very suitable for secreting *Ca*lB, since the volumetric activity of *Ca*lB was improved compared to the other knockout strains and, additionally, according to the evaluated secretion ratio, secretion tend to be higher. Moreover, the clone H6 achieved the best specific activity level, meaning that, the extracellular protein background was lower compared to the extracellular available *Ca*lB.

The results obtained by silver staining from the double knockout strains are discussed in the chapter 4.2.5 Detection of *CalB* by SDS-PAGE.

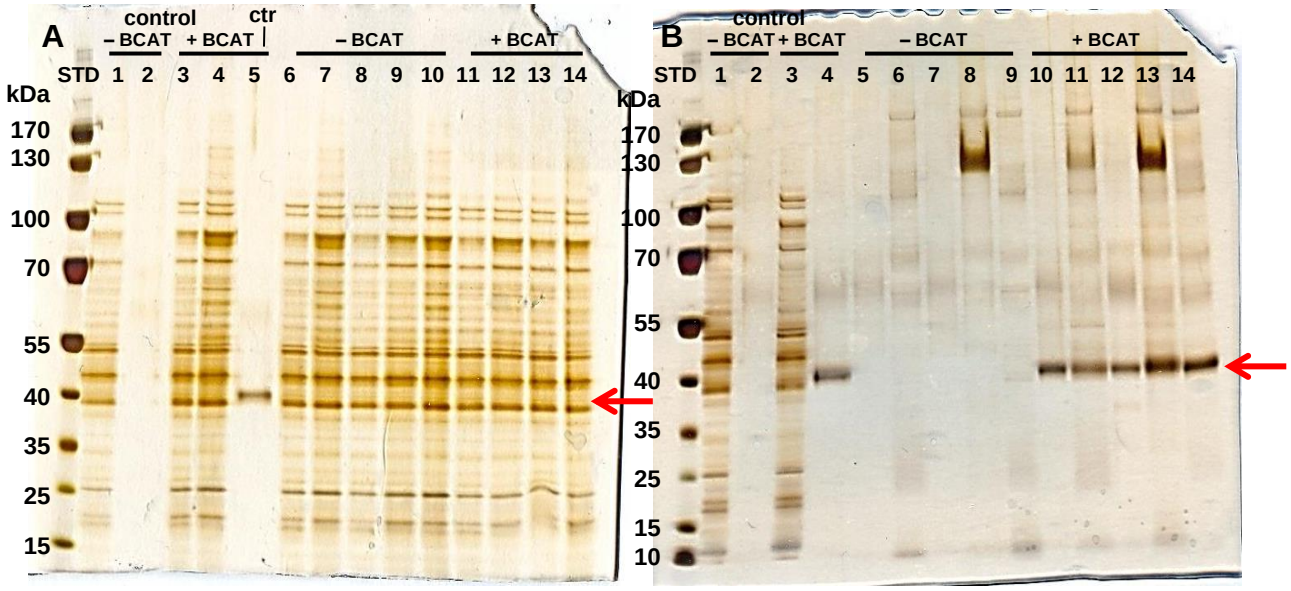

Figure 15: Silver staining. The red arrows indicate the size of *Cal*B (~35.3 kDa). All strains were based on the *P. pastoris* CBS7435 Mut^S (TU Graz strain collection number 3445) platform. E8 was used as control (ctr) containing the gene *cal*B and PageRuler^{TM} prestained protein ladder as standard. In (A) the CFEs are demonstrated. In lane 6 - 10 the corresponding empty knockout strains are demonstrated and in lane 4 and 11-14 the tested strains containing the gene *cal*B. STD: standard, 1: wild type CFE, 2: wild type SN, 3: E8 CFE, 4: Δ*och1*/Δ*cwp1* B11, 5: E8 SN, 6: Δ*cwp1*, 7: Δ*och1*, 8: Δ*scw10*, 9: Δ*och1*/Δ*scw10*, 10: Δ*och1*/Δ*cwp1*, 11: Δ*cwp1* E7, 12: Δ*och1* B7, 13: Δ*scw10* B2 and 14: Δ*och1*/Δ*scw10* G8. The SNs are represented in (B). Lane 1-3 are the same as in (A). The respective empty knockout strains are demonstrated in lane 5-9 and the strains expressing *Cal*B in lane 10-14. 4: E8 SN, 5: Δ*cwp1*, 6: Δ*och1*, 7: Δ*scw10*, 8: Δ*och1*/Δ*scw10*, 9: Δ*och*1/Δ*cwp1*, 10: Δ*cwp1* E7, 11: Δ*och1* B7, 12: Δ*scw10* B2, 13: Δ*och1*/Δ*scw10* G8 and 14: Δ*och1*/Δ*cwp1* B11.

4.2 Characterisation of *Cal*B in double knockout strains

Since BCAT and *Cal*B were already tested in single knockout strains, another consideration was to characterise the model proteins in double knockout strains. Every gene deletion leads to another effect in cell wall behaviour. So, it was also of interest to determine the outcome of two deletions of genes, which are involved in the assembly or morphogenesis of the cell wall. Therefore, such double knockout strains had to be produced. MutS Δ*och1* had no selection marker. For that reason 500 ng linearised DNA of plasmids containing a knockout expression cassette for either the gene *SCW10* or *CWP1*, which are selected by geneticin, were transformed into MutS Δ*och1*. As it was already known for the Muts Δ*och1* strain, here, colonies took longer for growth as well. Colony PCRs were performed in order to determine if the knockout cassette integrated in the correct locus. Additionally, the amplified PCR products were sent for sequencing to LGC Genomics GmbH, Berlin, Germany in order to confirm these knockout strains. The used primers are listed in table 7 and table 9. In summary, the double knockout strains MutS Δ*och1*/Δ*scw10* and MutS Δ*och1*/Δ*cwp1* were successfully produced.

Another possibility for a double knockout strain would have been MutS Δ*scw10*/Δ*cwp1*. MutS Δ*scw10* and MutS Δ*cwp1* both had a Gen^R. Therefore, the kanamycin cassette in the pJET vector containing either the disrupted gene *scw10* or *cwp1* had to be substituted by another selection marker. Since I have just worked with geneticin/kanamycin and zeocin as markers, a flipper cassette containing a zeocin marker was chosen. Therefore, this zeocin marker would be recyclable, because the marker will be removed by a recombinase. This construct was produced successfully and transformed into either MutS Δ*scw10* or MutS Δ*cwp1*. However, none of the obtained transformants had the second deletion in the correct locus. Due to the lack of time, the focus was placed on the two other already confirmed knockout strains. Since the integration of either the deletion cassettes has not occured in the correct locus, another possibility might be to transform the deletion cassettes in a Δ*ku70* strain. The protein Ku70 is involved in the non-homologous recombination pathway: a knockout strain in *KU70* locus has a reduced non-homologous recombination, which in turn leads to an accurate integration of the deletion cassette at the targeted site (Naeaetsaari et al., 2012). As already mentioned above, 500 ng of linearised pPpT4 α S *ca*lB was transformed into the respective double knockout strains. It is important to mention that the regeneration time was extended (2 h as well as 3 h incubation) in order to obtain transformants. In this case the plasmid containing the gene *ilv*E was not transformed due to time restrictions and moreover, due to the inaccurate performance of the BCAT assay.

A *Ca*lB screening in DWPs (3.2.2.1 Cultivation in deep well plates) was performed with the obtained transformants in order to determine their activity. A landscape of all clones was created with the measured data (evaluated by Gen5 1.11 and Microsoft Excel). As it was described in 3.2.2.2 Rescreening in deep well plates, six clones were chosen to be further tested. Subsequently, two of the six clones were selected to be further characterised, see 3.2.2.3 Screening in shake flasks.

4.2.1 Copy number determination of *ca*lB in double mutants

In order to get knowledge whether all the obtained results are comparable with each other and moreover, if an accurate control is available, the copy number of the certain double knockout strains was determined by qPCR. In table 23 the determined copy numbers of the gene *ca*lB are listed. All strains except MutS Δ*och1*/Δ*cwp1* A8 contained one copy.

Finally, in this case the volumetric and specific activity was comparable between those strains apart from the clone A8, which had integrated two copies of the expression cassette.

Table 23: Copy number determination of the gene *cal*B in double mutants. In this table the copy numbers from MutS E8 and the double knockout strains containing the gene *ca*lB are listed.

Strain	Copy number
MutS E8	1
MutS Δ*och*1/Δ*cwp*1 B11	1
MutS Δ*och*1/Δ*cwp*1 A8	2
MutS Δ*och*1/Δ*scw*10 F9	1
MutS Δ*och*1/Δ*scw*10 G8	1

4.2.2 Volumetric activity increased in both double knockout strains

The volumetric activity was determined in order to characterise *Ca*lB activity produced by the certain double knockout strains. The volumetric activity was calculated with the formula shown in figure 6. It was normalised to an OD_{600} 1 to allow an adequate comparison. After a period of 0 h – 72 h of expression, the volumetric activity in the SNs of both clones, MutS Δ*och1*/Δ*scw10* F9 and MutS Δ*och1*/Δ*scw10* G8, were much higher over time compared to the control and benchmark MutS E8, shown in figure 16a (A). Whereas in the CFEs it was difficult to determine the volumetric activity, because in the control as well as in the clones, there was hardly any activity detectable. In consideration of both volumetric activites (SNs and CFEs), it is possible to conclude that the clones of this double knockout strain have much more *Ca*lB in the extracellular than in the intracellular environment, which further means, that the secretion of *Ca*lB seems to be improved. The volumetric activity in MutS Δ*och1*/Δ*cwp1* was characterised as well. As shown in figure 16a (B), both clones showed an improved activity in the SNs compared to the benchmark MutS E8. In the CFEs there was hardly any activity detectable again. The volumetric activity in the SNs of MutS Δ*och1*/Δ*cwp1* B11 and A8 was not as high as in the other double knockout strain. However, it seems that one or two copies of the expression cassette had no significant effect, as the volumetric activity was almost the same in both clones.

Comparing the volumetric activity in the double knockout strains with the single knockout strains, it can be determined that the activity in MutS Δ*cwp1* is increased in comparison to MutS Δ*scw10*, whereas in the double knockout strains it is the other way around.

Additionally, the volumetric activity (not normalised to an OD_{600} 1) is shown in figure 16b in order to get an overview how the activity differs when the growth rate of the strains is not considered. It might be possible that clones with a high activity are underestimated when the biomass is not considered.

All in all, it can be concluded that the combination of the deleted genes *scw10* and *och1* had a greater impact on *Ca*lB activity than the combination of *cwp1* and *och1*.

It seems that both double gene deletions have a synergistic effect relating to *Ca*lB activity.

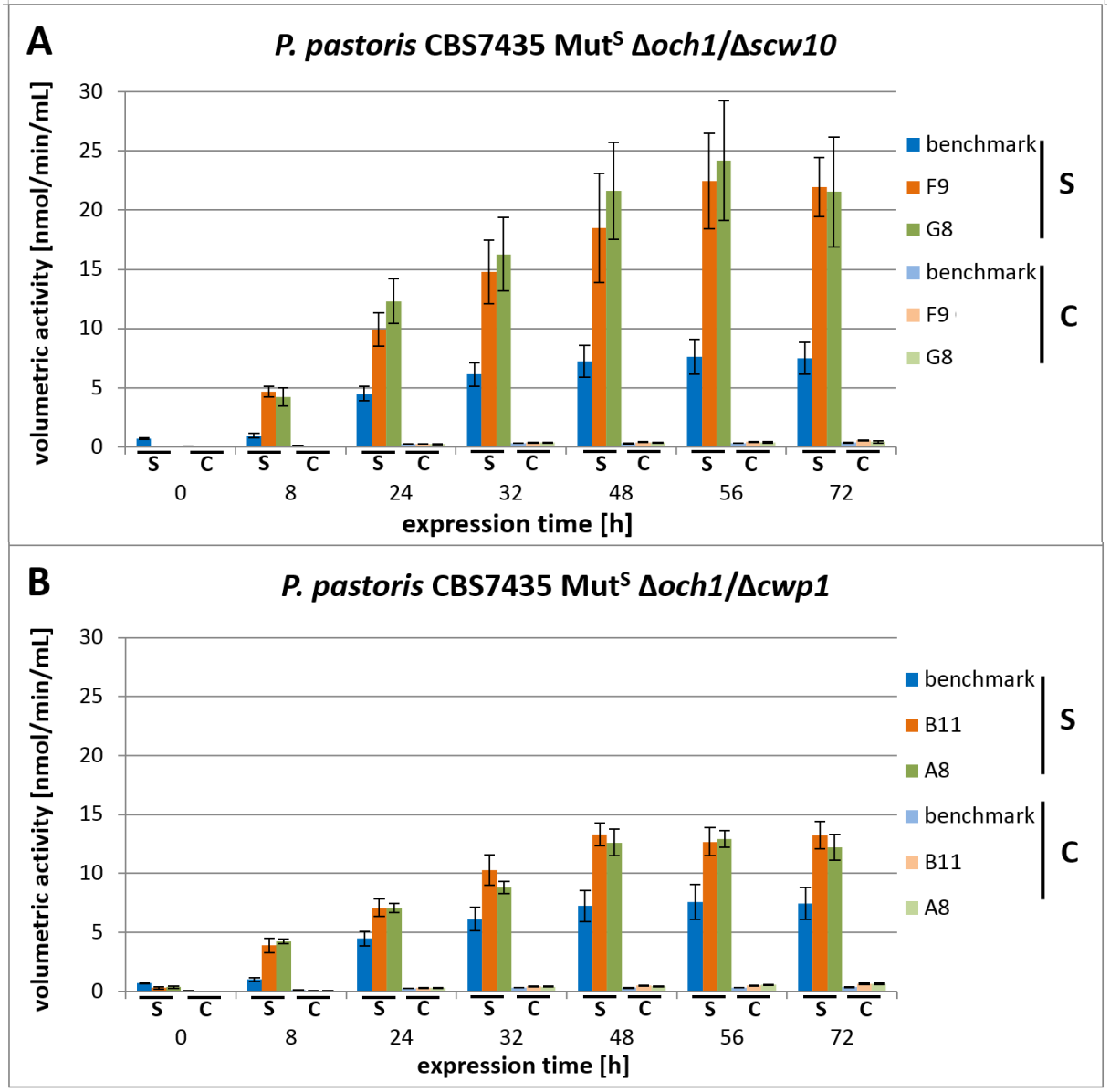

Figure 16a: Volumetric activity of *Cal*B in double knockout strains. For a period of 72 h of expression the intracellular and extracellular activity of *Cal*B was measured. The volumetric activity was normalised to an OD_{600} 1. As control and benchmark MutS E8 (one copy of the gene *cal*B) was used. The activity in the supernatants (S) is dark coloured, whereas the activity in the cell free extracts (C) is light coloured. The letters with the numbers represent clones containing the *cal*B gene in either MutS Δ*och1*/Δ*scw10* (A) or MutS Δ*och1* /Δ*cwp1* (B).

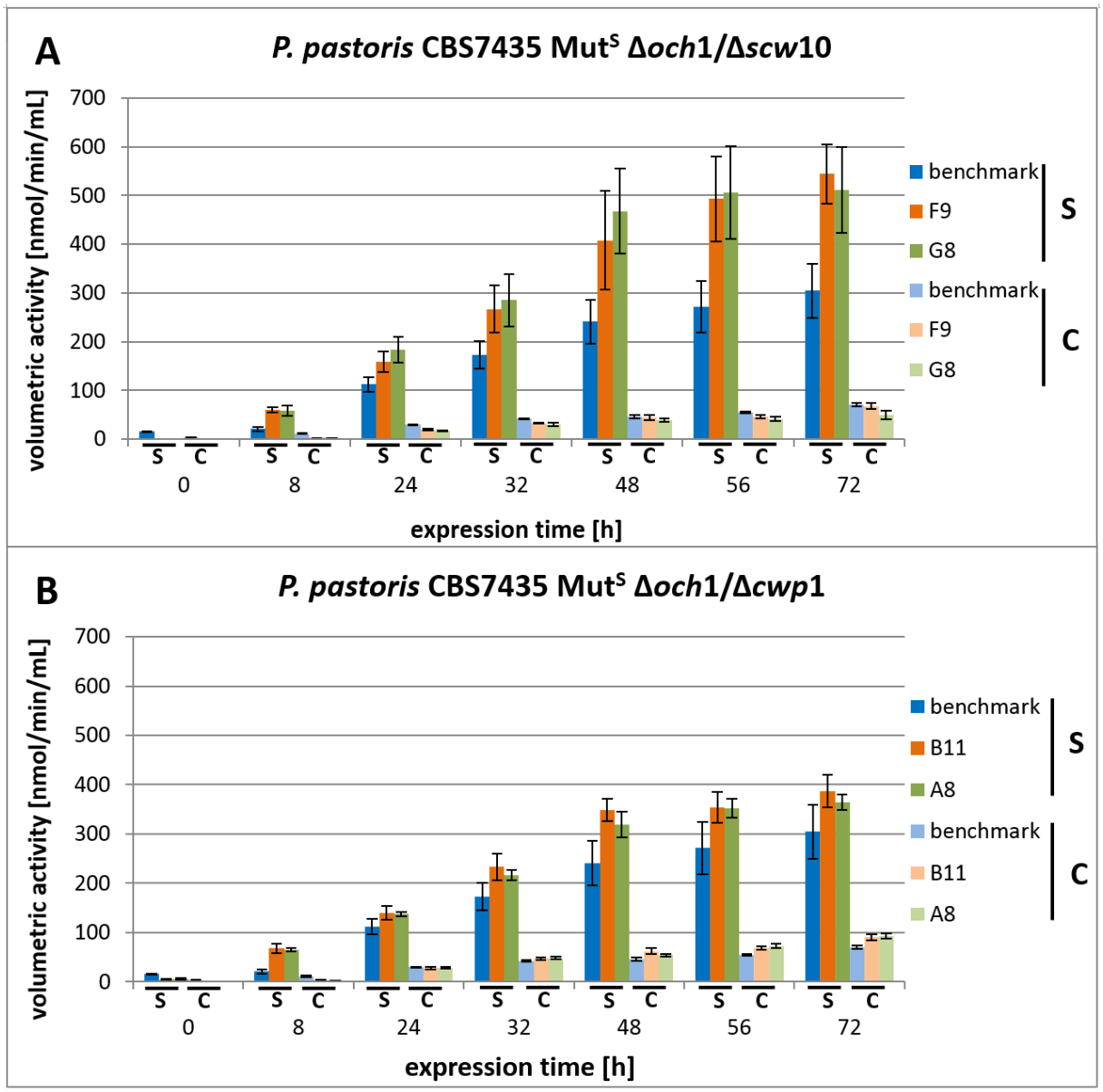

Figure 16b: Volumetric activity of *Cal*B in double knockout strains. For a period of 72 h of expression the intracellular and extracellular activity of *Ca*lB was measured. MutS E8 was used as control and benchmark. The activity in the supernatants (S) is dark coloured, whereas the activity in the cell free extracts (C) is light coloured. The letters with the numbers represent clones containing the *ca*lB gene in either MutS Δ*och1*/Δ*scw10* (A) or MutS Δ*och1* /Δ*cwp1* (B).

4.2.3 MutS Δ*och1*/Δ*scw10* showed more specific activity of secreted *Ca*lB

In addition to the volumetric activity, the specific activity of *Ca*lB was evaluated in MutS Δ*och1*/Δ*scw10* and MutS Δ*och1*/Δ*cwp1* as well. The protein concentration was quantified by the Bradford assay in order to calculate the *Ca*lB activity per mg protein. In figure 17 (A) the specific activity in MutS Δ*och1*/Δ*scw10* is shown. It can be determined that the activity in clone G8 was increased compared to the control and also the clone B11 showed a higher activity after time. Since there are a lot of other intracellular proteins present, the specific activity in CFEs is minimised. In contrast to the SNs, where much more specific activity can be determined, since *Ca*lB is present with almost no background, in the CFEs the specific activity cannot be distinguished as shown in the graph. If you compare the present activity in the double knockout strain with the appropriate single knockout strains, it can be concluded that the deletion combination affects the specific activity of *Ca*lB significantly in a positive way. The specific activity of *Ca*lB was analysed for the MutS Δ*och1*/Δ*cwp1* strain as well. The clone B11 had a reduced specific activity in the SNs over time compared to MutS E8 (figure 17 (B)). This might be due to a higher content of other proteins in the SN perhaps due to an increased leakyness of the deletion strain. The clone A8 and the control MutS E8 had a pretty similar activity level in view of the given standard deviations. As expected, in the CFEs there was hardly any activity again. Like for the volumetric activity, here again, the specific activity was improved in MutS Δ*och1*/Δ*scw10*. It seems that the deletion of *SCW10* and *OCH1* had a significant effect relating to the evaluated activities in a way which is not known yet.

It is important to mention, that all these tested strains (except clone MutS Δ*och1*/Δ*cwp1* A8) and the control had the same copy number, which means, that the obtained results are comparable with each other. As already previously mentioned, here again, the standard deviations are sometimes quite high. In order to receive more accurate results for the protein concentration and further to minimise the standard deviations, a possibility would be to measure the protein concentration at 590 nm and 450 nm.

Finally, it can be concluded that the double knockout strain MutS Δ*och1*/Δ*scw10* seems to be beneficial, because on the one hand, an increased volumetric activity and on the other hand, an increased specific activity could be determined. However, it is important to be aware that the specific activity of MutS Δ*och1*/Δ*scw10* differs in comparison with the obtained result from the silver stained gel.

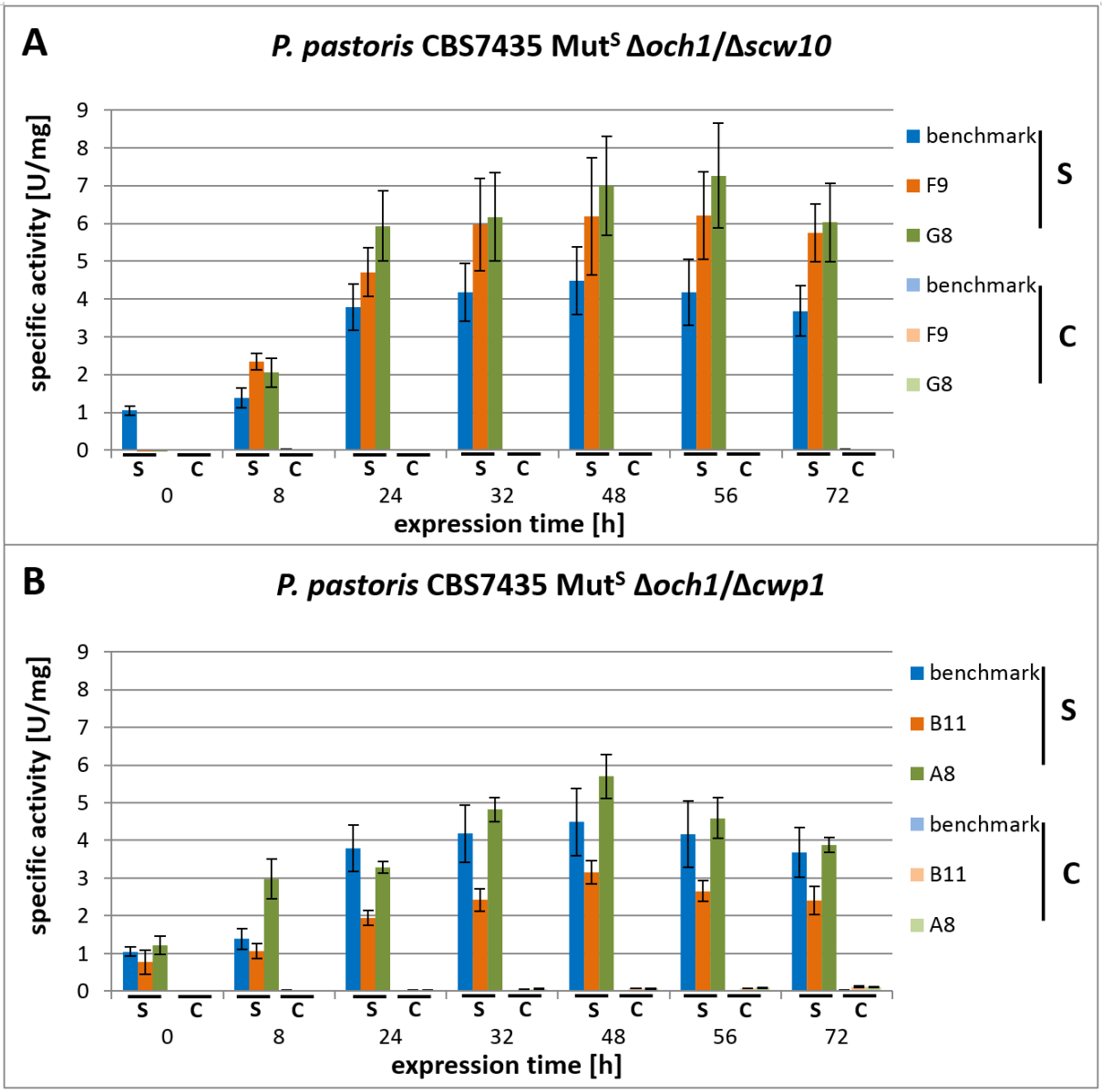

Figure 17: Specific activity of *Cal*B in double knockout strains. The specific activity of *Cal*B in the SNs as well as CFEs was evaluated for a period of 72 h of expression. MutS E8 (one copy of *cal*B gene) served as control and benchmark.The activity in the supernatants (S) is indicated as dark coloured bars, whereas the activity in the cell free extracts (C) is light coloured. The letters with the numbers represent clones containing the *cal*B gene in either MutS Δ*och1*/Δ*scw10* (A) or MutS Δ*och1*/Δ*cwp1* (B).

4.2.4 Improved secretion of *Cal*B in MutS Δ*och1*/Δ*scw10*

The secretion ratio was calculated in order to determine if the secretion is improved in the double knockout strains compared to the control MutS E8. As it is shown in figure 14, the secretion ratio of both MutS Δ*och1*/Δ*scw10* clones was greater than the control, whereas the Δ*och1*/Δ*cwp1* clones had a quite similar secretion ratio to MutS E8. These results suggest an improvement of *Cal*B secretion in MutS Δ*och1*/Δ*scw10* and moreover, that this strain is beneficial for *Cal*B expression and secretion, since the volumetric as well as the specific activity and secretion ratio of *Cal*B was increased compared to the other double knockout strain.

4.2.5 Detection of *Ca*lB by SDS-PAGE

Another experiment was performed in order to confirm the presence of *Ca*lB in the diverse knockout strains and furthermore, to exclude the presence of artefacts. Therefore, SDS-PAGE was used with subsequent silver staining. This method is very sensitive and therefore, defined bands could be detected. In figure 15 are shown all strains expressing *Ca*lB. *Ca*lB (codon-optimised) has a determined size of 35.3 kDa which is indicated by red arrows. In figure 15 (A) *Ca*lB in the diverse CFEs is shown. In each slot 2 µg of protein was loaded. In lane 5 the SN of MutS E8 as control and in lane 3 the CFE are shown. However, *Ca*lB cannot be identified in the CFEs. All bands present in the diverse knockout strains are also visible in MutS and MutS E8 with the exception of different intensities. The SNs are shown in figure 15 (B). The red arrow indicates *Ca*lB. As it is visible in lane 13 and 14, the supposed band for *Ca*lB is more intense than the others. This might be the evidence, that much more *Ca*lB was secreted compared to the protein background and, further leading to an increased specific activity in MutS Δ*och1*/Δ*scw10*. Whereas in MutS Δ*och1*/Δ*cwp1*, the intensity of the bands is rather the same or lower, expecting that, according to the specific activity more amount of total protein compared to *Ca*lB was extracellularly present, which led to the lower or same level of specific activity as the control. However, these obtained results could not be confirmed by the protein quantification via Bradford. The reason might be due to the inaccurate performance of the Bradford assay or another possibility might be that the determination of the protein concentration by Nanodrop was inaccurate. Referring to previous in-house experiences, the protein concentrations obtained by Bradford and Nanodrop can differ significantly. Therefore, there cannot be done a proper comparison between those two methods and furthermore, between the results from the specific activity and SDS-PAGE. An intense band at ~130 kDa is visible in lane 8 (MutS Δ*och1*/Δ*scw10* – empty knockout strain), in lane 13 (MutS Δ*och1*/Δ*scw10* G8) and a less intense band in lane 11 (MutS Δ*och1* B7). It seems that this band occurs when the combination of the deleted *scw10* and *och1* genes is present, because an intense band appeared in the empty knockout strain as well as in the knockout strain containing the gene *ca*lB. This apparent band (secreted protein) might be influenced by the deleted *och1* gene as well, since in MutS Δ*och1* B7 containing the gene *ca*lB, a less intense band is also visible, however not in the corresponding empty knockout strain. It seems that there are more than one possibilities for the appearance of this band.

All in all, it could be determined that no artefact led to the certain activities. There should be performed another experiment in order to detect *Ca*lB in the CFEs. As previously suggested, a western blot would be suitable. Additionally, it might be interesting to find out which function or effect has the detected protein (~130 kDa).

4.3 Exploration of the selection marker *amdSYM* in *P. pastoris*

Beside the characterisation of BCAT and *Ca*lB in the certain knockout strains, which was the main goal, another aim was to investigate a new selection marker in *P. pastoris*. This was the *amdSYM* selection marker, which was already known for *S. cerevisiae*.

4.3.1 *P. pastoris* CBS7435 Mut^S is unable to grow on acetamide

In this experiment we aimed for getting to know if the selection marker *amdSYM* is applicable to *P. pastoris*. The first step was to perform a growth curve in order to test if *P. pastoris* grows on medium containing acetamide. Like it is shown in figure 19, it was observed that MutS is unable to grow in medium containing acetamide as sole nitrogen source compared to the control MutS grown in BMD-1 (nitrogen source is YNB in this case). This result suggests that in principle cells can be selected on acetamide.

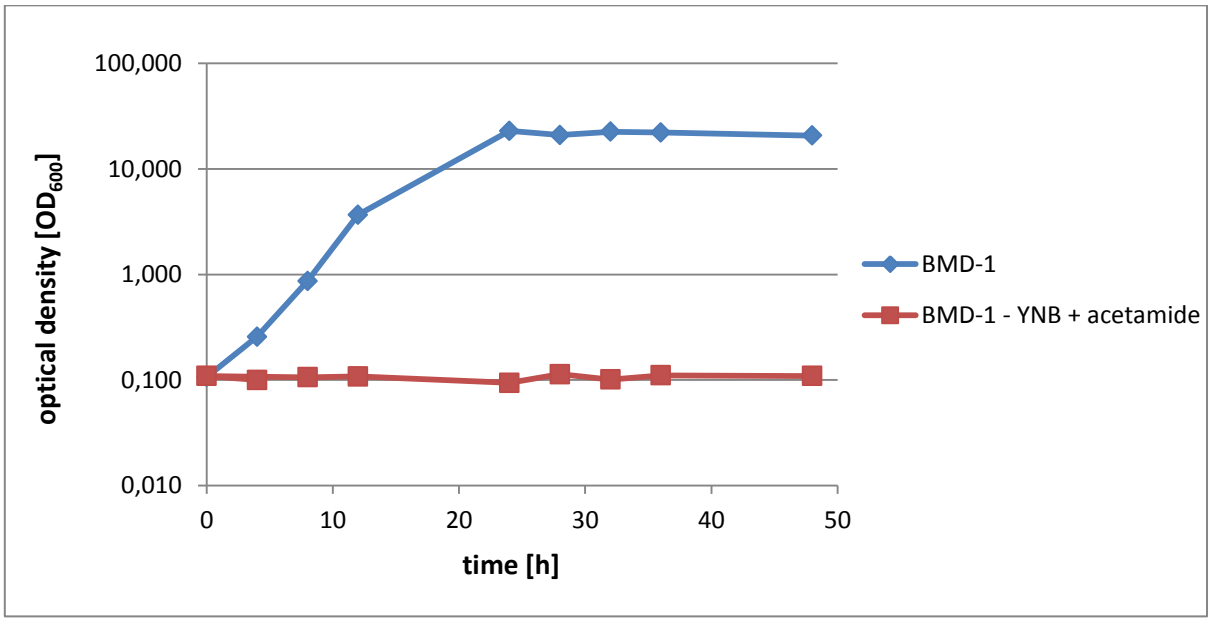

Figure 18: Growth of MutS in BMD-1 and in BMD-1 (-YNB) + acetamide. The strain MutS was grown in BMD-1 and in BMD-1 (-YNB) + acetamide. Every 4 h the OD_{600} value was measured, three times a day, for a duration of 48 h. The blue line shows the control MutS grown in BMD-1 medium. No growth was observed for MutS in BMD-1 (-YNB) + acetamide (indicated by the red line).

The next step was to produce *P. pastoris* strains expressing acetamidase (AmdS). For that, pUG-amdSYM was linearised with *Bgl*II and 500 ng of DNA was transformed into MutS. The map of pUG-amdSYM is shown in S 5 in the chapter ``Supplementary Materials´´. After the regeneration 200 µL of the cell suspension was plated on BMD-1 (-YNB) + acetamide plates. The remaining cell suspension was centrifuged for 5 min at 2,300 g and afterwards, the supernatant was discarded. The rest was plated on BMD-1 (-YNB) + acetamide plates as well. However, no transformants were obtained and just background growth was visible. So, protocol adjustments had to be made. First of all, we wanted to know at which concentration acetamide might be toxic to the cells and, furthermore, which concentration is necessary for

a successful selection. In order to test this consideration, MutS was grown in BMD-1 (-YNB) medium containing different concentrations of acetamide. It could be confirmed that MutS grows in BMD-1 medium. In BMD-1 (-YNB) medium containing different concentrations of acetamide, no significant growth of MutS was determined. The growth curves from MutS grown in diverse media are shown in figure 20.

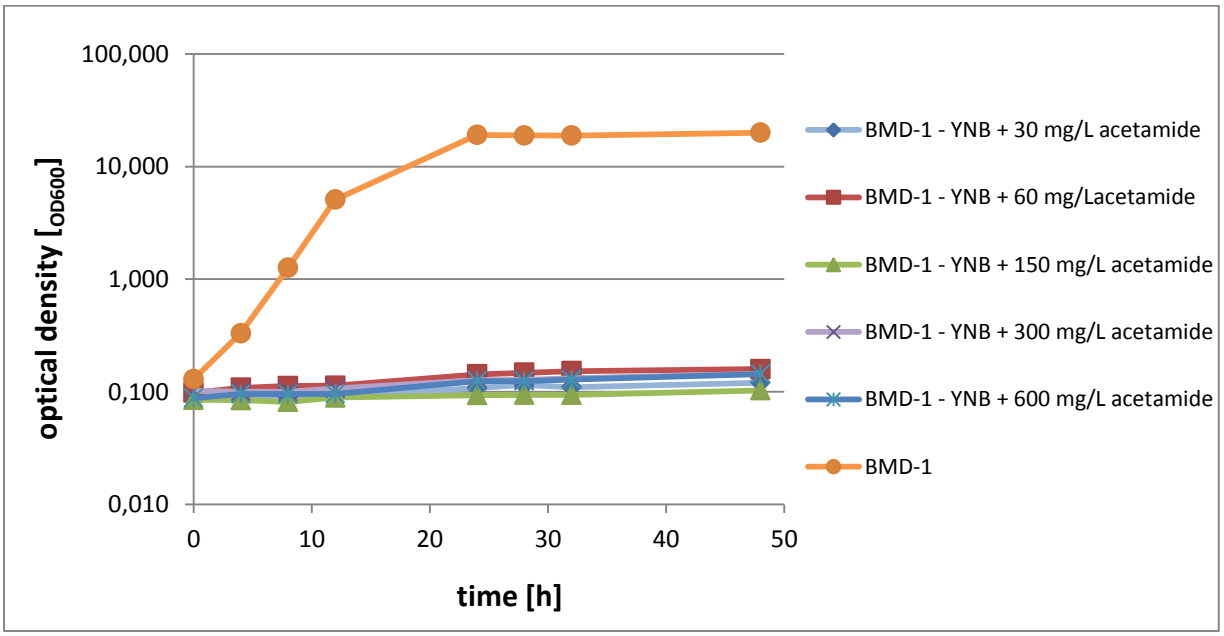

Figure 19: Growth of MutS in diverse media. In this graph the growth from MutS in BMD-1 (-YNB) + particular acetamide concentrations are shown. Every 4 h the OD_{600} value was measured, three times a day, for a duration of 48 h. The orange line is the control MutS grown in BMD-1. The other lines (light blue, red, green, purple and dark blue) correspond to the growth curves from the cells which are grown in the different acetamide concentrations.

The other consideration was to clone the *amd*S gene into the vector pPpT4 (shown in S 6) to ensure an integration into the genome. It is known that this shuttle vector works fine in *P. pastoris*. The whole procedure of producing this construct can be read in the section 3.2.3.9 Assembly of *amdS* gene in pPpT4 α S vector. The pPpT4 α S vector contained the sequence for the α factor secretion signal upstream the *ilv*E gene in order to express BCAT as a secreted protein, and acetamidase (AmdS) was tested as a marker instead of zeocin. The construct was linearised with *Smi*I, then transformed into MutS and plated on BMD-1 (-YNB) + acetamide. Like in the previous transformation, no transformants were obtained, just background growth. Therefore, MutS grown on a YPD plate was streaked out on a BMD-1 (-YNB) + acetamide plate to determine if MutS is able to grow on that plate. In contrast to the experiments with the liquid cultures, this time growth on the solid medium could be observed. Therefore, it was considered that MutS could use nitrogen sources from the however little amount of YPD (used to just regenerate cells) poured on the plates, rather than the acetamide present, which would explain the background growth visible after the transformation. In other words, the cells could regenerate efficiently and therefore, they had

enough nitrogen resources. Hence, another transformation was performed, but this time the regeneration occured with YPD/1 M sorbitol (1:1), PBS/H_2O (negative control for regeneration), PBS/1 M sorbitol (1:1) or PBS/YPD. 1 mL of cell suspension was plated on three plates containing 0.6 g/L acetamide. The obtained result was the same as before, just background growth.

A possible explanation, having explored unsuccessfully different protocols for transformation, could have been that maybe the concentration of the expressed acetamidase was too low and thus led to an inefficient conversion of acetamide. The consequence of this would be that not sufficient ammonia was produced in order to enable the cells to grow in colonies. This could be an explanation why only background growth could be detected all the time.

4.3.2 P_{GAP} allows selection by acetamide

In order to finalise this experiment, it was tested, if, in principle, an acetamidase activity could be determined in *P. pastoris*. Therefore, the *amd*S gene was cloned into pPpT4 GAP S. The map of pPpT4 GAP S is shown in S 7. For a detailed description see chapter 3.2.3.10 Assembly of *amdS* gene in pPpT4 GAP S vector. Transformants expressing AmdS under the control of the *GAP* promoter were plated on YPD zeo plates, BMD-1 (-YNB) + acetamide plates as well as on BMD-1 (-YNB) + acetamide (1.2 g/L) plates. After 48 h of incubation, colonies were already visible on YPD zeo, however, it took much longer (72 h - 96 h) to see colonies on the BMD-1 plates containing acetamide as sole nitrogen source, confirming that, the strong host promoter *GAP* is necessary in order to select cells containing the acetamide marker. In previous experiments, P_{ILV5} was used in order to express acetamidase: the *ILV5* promoter is used for the expression of e.g. zeocin resistance markers in *Pichia* and it is not as strong as P_{GAP}. A reason why transformants grew slowly on BMD-1 (-YNB) + acetamide plates might be due to toxicity. The grown colonies were smaller than normal grown MutS cells, which might be an indication that acetamide has toxic effects to the cells. Another possibility might be metabolic burden of AmdS expression driven by $P_{GAP,}$ which have led to the change in cell shape (smaller colonies).

All in all, it is stated that P_{GAP} enables the ability to select the cells by acetamide and that weaker promoters are not sufficient for selection. Moreover, counter-selection with medium containing fluoroacetamide should be tested, as there was not sufficient time to perform this experimental procedure.

5 Conclusion and Outlook

I investigated the effect of three specific *P. pastoris* deletion strains in respect to the production of extracellular model proteins. The deleted genes code for proteins involved in cell wall assembly and glycosylation.

The two model proteins IlvE and *Ca*lB were successfully secreted by the wild type *P. pastoris* CBS7435 Mut^S strain. We conceived a strategy to generate three mutants, Δ*cwp1*, Δ*och1* and Δ*scw10* and tested the protein expression in those mutants, and in double mutants combining the different deletions. A knockout of locus *OCH1* is beneficial for extracellular BCAT and, to a lesser extent, for *Ca*lB production. Since Och1 is involved in glycosylation, an inactivation of *OCH1* may cause proteins to be less or differently glycosylated, which may also lead to changes in specific activity. Testing specific activity of total protein preparations of both, *Ca*lB and BCAT, resulted in little or no decrease for BCAT and *Ca*lB compared to the control, confirming either little changes in specific activities or even an increase in view of a higher content of other proteins released from the leaky deletion strains.

In strains MutS Δ*cwp1* and MutS Δ*scw10*, no improvement could be achieved for volumetric or specific activity of BCAT. In MutS Δ*scw10*, there could not be detected an improved *Ca*lB activity as well, whereas an improved volumetric activity of *Ca*lB was determined in MutS Δ*cwp1*. Hence, a deletion of *SCW10* may cause changes in cell wall behaviour, but does not influence protein activity significantly. In contrast, in MutS Δ*cwp1* an improved activity was detected only for *Ca*lB, confirming that, each gene deletion has a different effect on the respective recombinant proteins.

Additionally, *Ca*lB was tested in both double knockout strains as well, MutS Δ*och1*/Δ*scw10* and MutS Δ*och1*/Δ*cwp1*, leading to the result of an improved normalised (by OD_{600}) volumetric activity of *Ca*lB and, furthermore, to an increased specific activity in MutS Δ*och1*/Δ*scw10*. It seems that the combination of Δ*och1* and Δ *scw10* leads to a syngeristic effect relating to *Ca*lB activity. The same applies to the other double knockout combination, MutS Δ*och1*/Δ*cwp1*, however, not to such a great extent. A qualitative analysis of *Ca*lB on SDS-PAGE did not show any obvious change in *Ca*lB when expressed in the double knockouts, although several bands, specifically present in the supernatant of cultivation with MutS Δ*och1*/Δ*scw10*, could be observed. Further investigation will be required for characterising precisely the biologic effect of the mutation on protein expression.

In summary, I improved the expression of both, *Ca*lB and BCAT; the deletions of *CWP1*, *OCH1*, *OCH1*/*SCW10* and *OCH1*/*CWP1* proved to be beneficial for the expression and secretion of these model enzymes.

Due to financial reasons as well as reason of time the copy number determination of either *il*vE or *ca*lB in the diverse knockout strains was performed in this thesis quite late. Therefore,

the knockout strains harboured different copy numbers of the certain expression cassette. In order to make the results comparable with each other, the experiments should be repeated starting with the transformation and activity screening in deep well plates followed by the determination of the copy number of the certain expression cassette present in each strain and the verification of the particular knockout strains by colony PCR. Moreover, it would be interesting, if the ~130 kDa band appears again in MutS Δ*och1* containing either the *ilv*E or *ca*lB expression cassette, in the empty knockout strain MutS Δ*och1*/Δ*scw10* and in MutS Δ*och1*/Δ*scw10* containing an expression cassette in order to confirm this surprising effect.

As an additional activity, a new selection marker for *P. pastoris* was explored. For each modification a selection marker is required, and therefore, it was of interest to investigate another selection marker in order to enlarge the toolbox for engineering *P. pastoris*. Since the construct pUG-amdSYM did not work in *P. pastoris*, further experiments were performed with pPpT4 containing *amd*S. In *P. pastoris* the strong and constitutive *GAP* promoter was essential for a successful selection on acetamide, whereas the promoter *ILV5* was insufficient and, therefore, only background growth was detectable: a higher amount of acetamidase is necessary to convert sufficient acetamide to ammonia in order to enable cells to grow in colonies. At the end I succeeded in having the cassette for selection on acetamide working in *Pichia*.

Although further development would be required, this selection marker appears to be suitable, when applied to a flipper cassette. A flipper cassette enables the removal of a certain DNA sequence at specific sites by recombinase (e. g. Flp or Cre): DNA sequence is flanked by short target sequences (e. g. *FRT* or *lox*P), which are recognised by recombinase and allow excision of DNA. An advantage to use *amdS* in a flipper cassette is that counter-selection can be easily performed. Cells, which contain the selection marker, can be selected by acetamide, whereas, if cells have lost the selection marker by recombinase excision, those can be counter-selected by fluoroacetamide - negative selection. This is due to the fact that these cells are not able anymore to convert fluoroacetamide to the toxic component fluoroacetate and therefore, these cells will survive.

Good results could be obtained by MutS Δ*och1* as well as by both double knockout strains. Since in all of these strains a deletion of *OCH1* was applied, it would be interesting to explore which effect this deletion has relating to the protein activity. Hence, further experiments should be performed. As it is already known, Och1 is a mannosyltransferase, which avoids high levels of mannosylation. Therefore, it would be expected that BCAT, *Ca*lB or other proteins are less glycosylated. So, the attention would be placed on the glycosylation sites. In order to verify that, it would be interesting to deglycosylate BCAT or *Ca*lB for example with Endo H. Afterwards, diverse screenings should be performed with the deglycosylated

proteins again, in order to determine if an improvement or a deterioration has taken place. The molecular weight should be detected for example by western blot analysis or SDS-PAGE followed by silver staining. In addition, it would be interesting to find out whether the protein folding is affected by the glycosylation and this leads to an increased activity. For that, possibly a structural biologist must be consulted in order to determine how the folding differs.

Furthermore, in MutS Δ*och1* and MutS Δ*och1*/Δ*scw10* a protein band of ~130 kDa could be detected by SDS-PAGE with subsequent silver staining, which was not identified so far. In order to determine this band, it would be interesting to cut out this band from a coomassie stained gel and send it for sequencing (e. g. Edman sequencing). Afterwards, with the help of BLAST it can be searched for homologs in order to define its possible function and possible correlations with the other deleted or overexpressed genes.

Another experiment can be performed relating to *Ca*lB activity. According to Vadhana et al. an improved secretion and further improved activity can be achieved by using the native signal sequence of *Ca*lB instead of using the secretion signal sequence from *S. cerevisiae*. Therefore, all procedures should be repeated in order to identify if the native secretion signal sequence leads to an improved secretion and activity in the certain knockout strains as well or with other weaker promoters or in PDI coexpressing strains.

Moreover, the BCAT assay should be optimised in order to receive more reliable data for the BCAT activity. Slight differences in the initial concentration of HRP in the assay solution would already have a great impact. An improvement could be achieved by performing an absorption spectrum of HRP dissolved in buffer [1 mg/mL] in order to determine an accurate wavelength as well as an OD. Buffer will be used as blank. The obtained OD value at the certain wavelength for 1 mg/mL of HRP will be used for every assay solution.

Additionally, it was not possible to obtain the double knockout strain MutS Δ*cwp1*/Δ*scw10*, we suspect such deletion combination might be lethal, but until this is proven it might be interesting to produce such a double mutant and furthermore, to get to know how this combination of gene deletions influences the activity and secretion of BCAT and *Ca*lB.

6 Abbreviations

α-KG	α-ketoglutarate
ABTS	2,2´-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt
Amp^R	ampicillin resistance
BCAT	branched chain aminotransferase
BSA	bovine serum albumin
CFE	cell free extract
ddH_2O	double-distilled water
DMSO	dimetyhl sulphoxide
DTT	1,4-dithiothreitol
DWP	deep well plate
EDTA	ethylenediaminetetraacetic acid
Endo H	endoglycosidase H
EtOH	ethanol
Gen^R	geneticin resistance
GlcNAc	N-acetylglucosamine
HCl	hydrochloric acid
HRP	horse radish peroxidase
Kan^R	kanamycin resistance
MeOH	methanol
MS/MS	tandem mass spectrometry
MUT	methanol utilisation
NAD	nicotinamide adenine dinucleotide
ONC	overnight culture
PCR	polymerase chain reaction
PLP	pyridoxal-5-phosphate
PP	polypropylene
PPB	potassium phosphate buffer
PPCO	polypropylene copolymer
PS	polystyrene
qPCR	real-time PCR
SN	supernatant
SPB	sodium phosphate buffer
TBG	L-tert-butylglycine
YNB	Difco™ yeast nitrogen base with ammoniumsulphate w/o amino acids
Zeo^R	zeocin resistance

7 Supplementary Materials

ACCACCAAGAAGGCCGACTACATCTGGTTCAACGGTGAGATGGTCAGATGGGAGGAC
GCCAAGGTCCACGTCATGTCCCACGCCTTGCACTACGGAACCTCCGTCTTCGAGGGA
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S 1: Nucleotide sequence from the codon-optimised *ilv*E gene.

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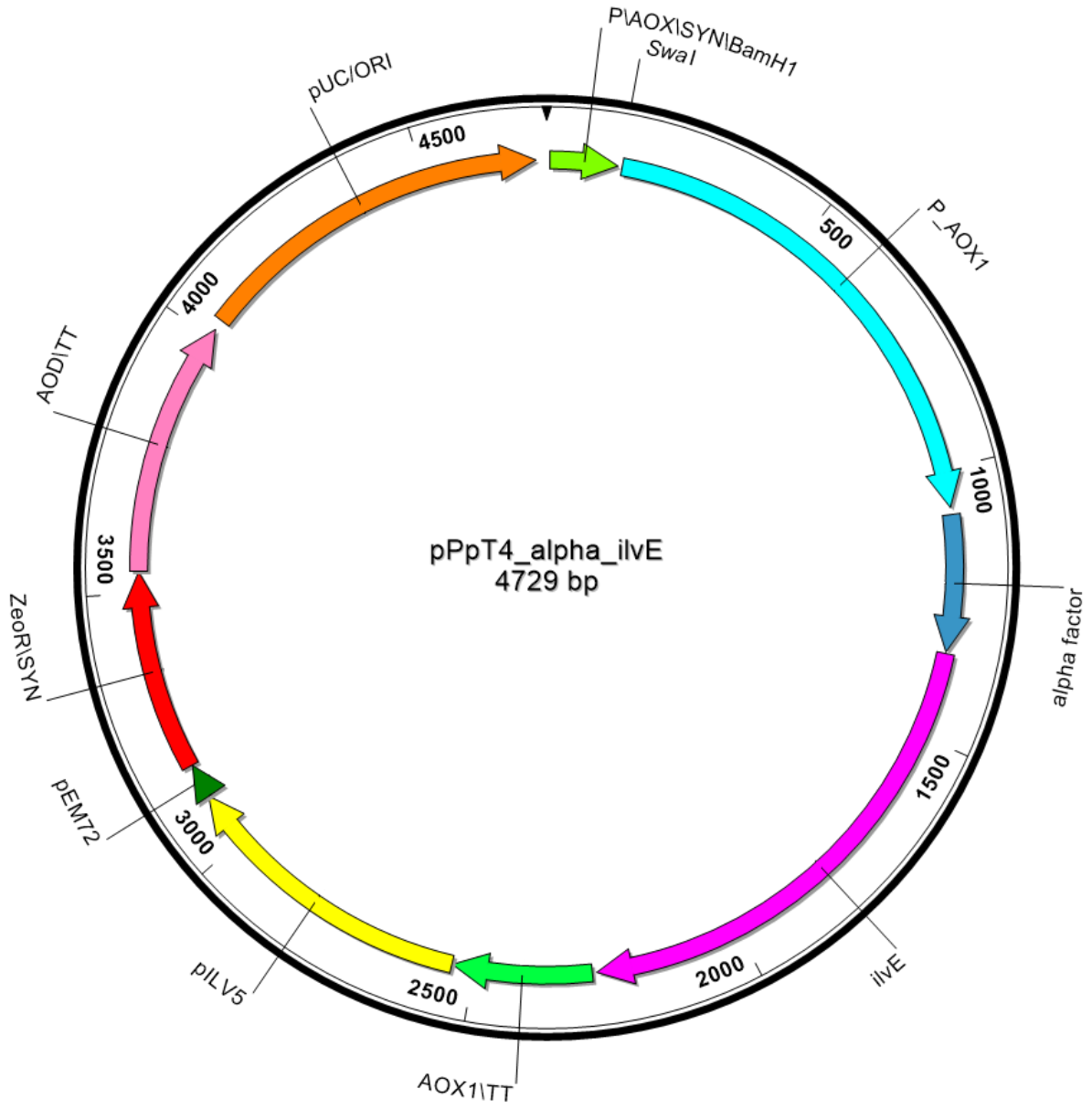
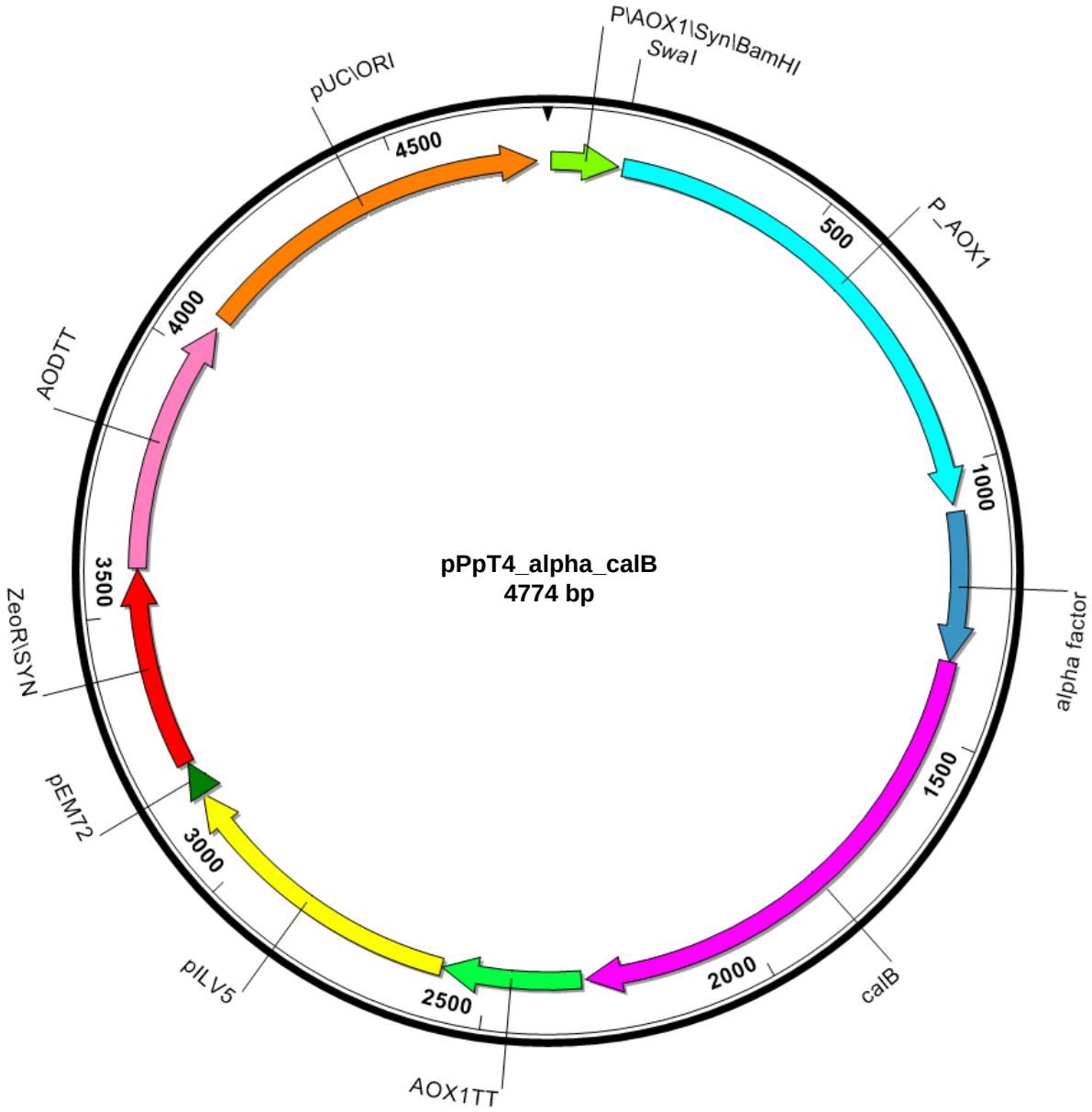
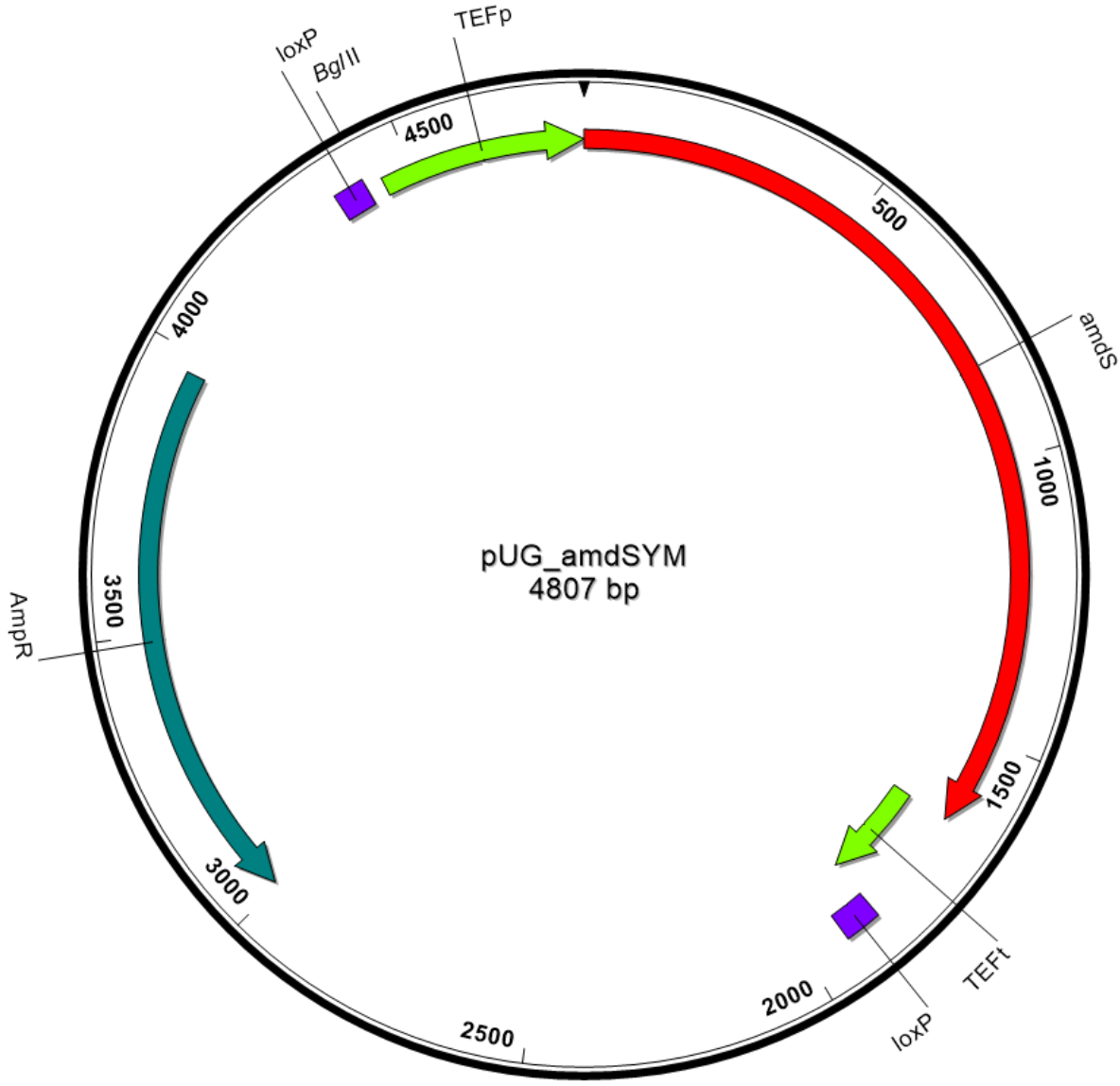
S 2: Nucleotide sequence from the codon-optimised *ca*lB gene.



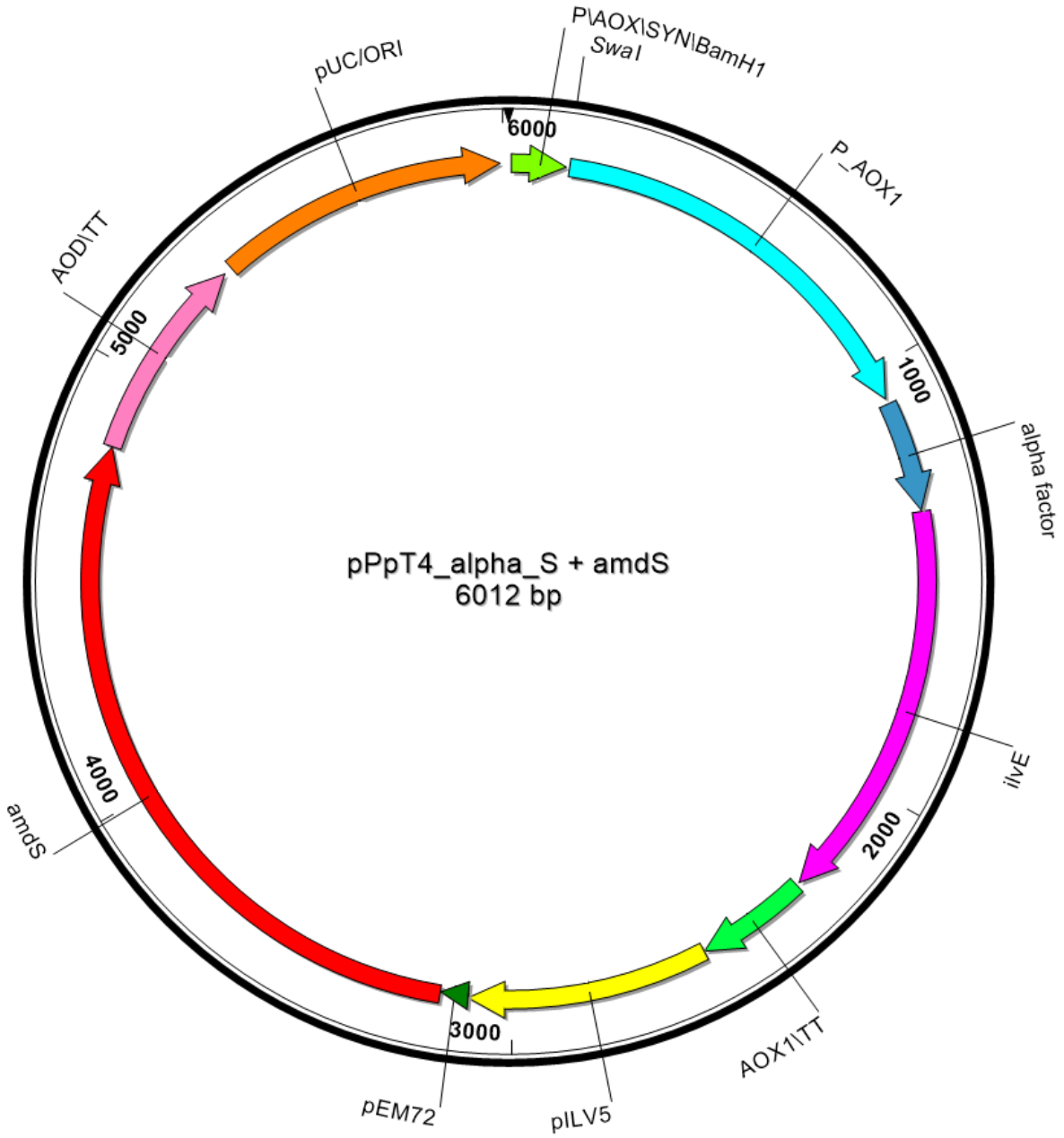
S 3. Map of pPpT4 α S containing the *ilv*E gene. The plasmid was linearised with *Smi*I and afterwards transformed into *P. pastoris* strains. The plasmid contains a zeocin resistance (red) and an *ILV5* promoter (yellow). Furthermore, the plasmid contains an origin of replication (orange) and an α factor (blue) derived from *S. cerevisiae.*



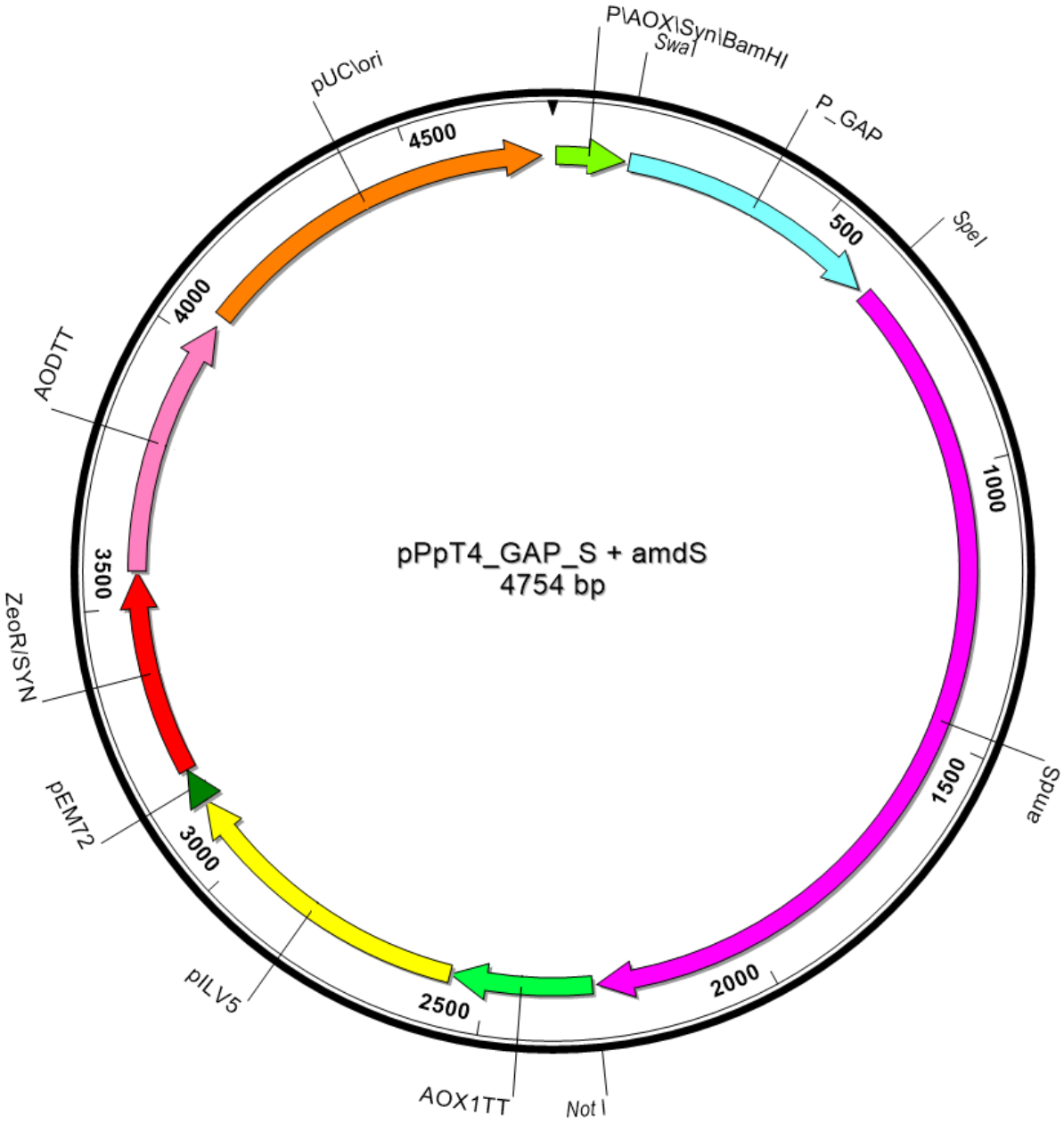
S 4: Map of pPpT4 α S containing the *cal*B gene. The plasmid was linearised with *Smi*I and afterwards transformed into *P. pastoris* strains. The plasmid contains a zeocin resistance (red) and an *ILV5* promoter (yellow). Furthermore, the plasmid contains an origin of replication (orange) and an α factor (blue) derived from *S. cerevisiae.*



S 5: Vector map of pUG-amdSYM. This graph shows the restriction site *Bgl*II, where it was linearised. It also contains the necessary features, these are the *amd*S gene coloured in red, the *TEF* promoter and terminator coloured in green and the gene encoding ampicillin resistance coloured in teal.



S 6: Map of pPpT4 α S containing *amd*S. The *amd*S gene (red) was cloned into pPpT4 α S by Gibson cloning. The plasmid was linearised with *Smi*I and afterwards transformed into MutS. The plasmid contains an acetamide resistance, shown in red and an *ILV5* promoter, coloured in yellow. Furthermore, the plasmid contains an origin of replication (orange) and an α factor (blue) derived from *S. cerevisiae.*



S 7: Map of pPpT4 containing *GAP* promoter. The *amd*S gene (pink) was cloned into the site between *Spe*I and *Not*I. The plasmid was linearised with *Smi*I. The plasmid contains a zeocin resistance, shown in red and a *GAP* promoter, coloured in turquoise. Furthermore, the plasmid contains an origin of replication (orange) and a promoter to express zeocin, labelled in yellow.

8 Reference List

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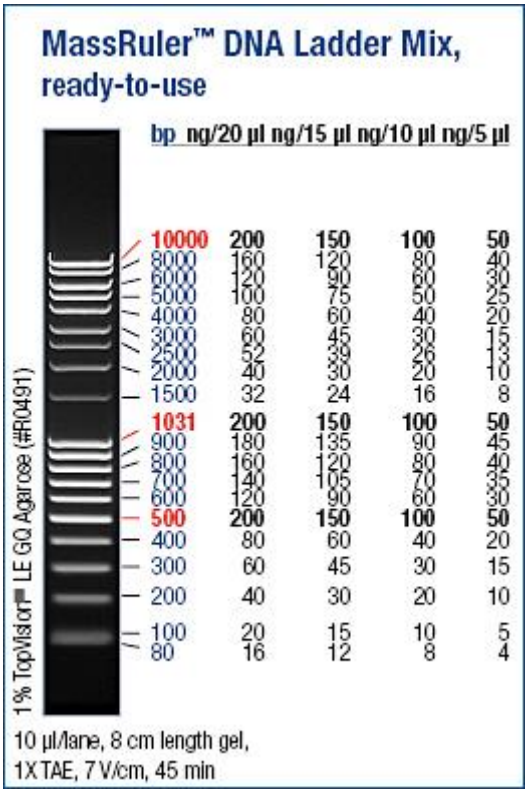
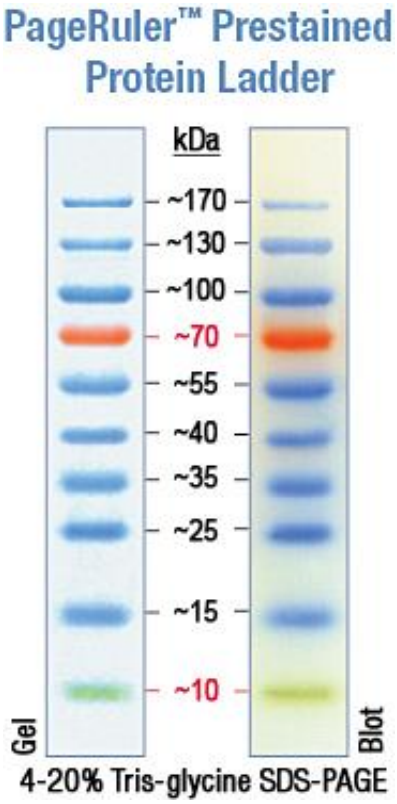
9 Appendix



A 1: MassRuler™ DNA Ladder Mix from Thermo Scientic.
This standard was used for agarose gels.



A 2: PageRuler™ Prestained Protein Ladder from Thermo Scientic.
This standard was used for SDS-PAGE.