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Expression and charactarization of amine and amino acid oxidases in *Pichia pastoris*

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STATUTORY DECLARATION

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Danksagung

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Kurzfassung

Amin- und Aminosäureoxidasen sind wichtige Enzyme für Biokatalyse. Zum Bespiel D-Aminosäureoxidase von *Trigonopsis variabilis* (*Tv*DAO) ist ein gut untersuchtes Enzym, welches für die Umwandlung von Cephalosporin C verwendet wurde um halbsynthetische Antibiotika herzustellen. Weitere Anwendungen von *Tv*DAO sind die analytische Bestimmung von D-Aminosäuren in Lebensmitteln und Produktion von enantiomeren reinen Aminosäuren durch Deracematisierungsprozesse.

Diese Arbeit berichtet von einer neue Strategie zur verbesserten Expression von Amin- und Aminosäureoxidasen. Dazu wurden die Proteinsequenzen von *Tv*DAO, *Rg*DAO (*Rhodotorula gracilis* D-Aminosäureoxidase), *Cr*LAO (*Calloselasma rhodostoma* L-Aminosäureoxidase) and *An*MAO (*Aspergillus niger* Monoaminoxidase) mittels Webtools analysiert. Basierend auf dieser Analyse wurden synthetische, Kodon-optimierte Gene entworfen. *Tv*DAO wurde als Modelenzym für die Expressionsoptimierung ausgewählt.

Heterologe Expression in *Pichia pastoris* hat im Vergleich zur Expression in *E. coli* einige Vorteile. Die methylotrophe Hefe *P. pastoris* zählt zu den Eukaryoten und ist daher in der Lage postranslationelle Veränderungen durchzuführen. Weiters können einfache DNA Veränderungstechniken und Kultivierungsprotokolle verwendet werden. Ein weiterer Vorteil sind die hohen Zelldichten, die während Fermentationen erreicht werden können.

Um die Kosten für diesen wichtigen Biokatalysten zu senken, muss die Expression von *Tv*DAO und anderen Amin-oxidierenden Enzymen in *P. pastoris* optimiert werden.

Zur Erreichung dieses Ziel wurde als erstes die peroxisomale Targeting-Sequenz ausgetauscht, um eine Lokalisierung von *Tv*DAO in den Peroxisomen sicherzustellen, wo auch Katalase vorhanden ist, die während der Oxidase Reaktion entstehendes zellgiftiges Wasserstoffperoxid, durch Umwandlung in Wasser und Sauerstoff, entgiftet. In einem zweiten Schritt wurden Kodon-optimierte synthetische Gene konstruiert, die optimale Expression in *P. pastoris* bei Methanol Induktion gewährleisten. Zusätzlich wurde ein Fusionsprotein von *Tv*DAO mit Citrine hergestellt um die Lokalisierung in den Peroxisomen zu bestätigen. Letztendlich wurden die besten *Tv*DAO Expressionsstämme in 1.5 L Bioreaktoren kuliviert. Die Kultivierungsbedingungen wurden optimiert um die maximale Aktivität und Biomasse-Ausbeute zu erhalten. Die erhaltenen Stämme zeigen die höchste volumetrische *Tv*DAO Aktivität, von der bis jetzt berichtet wurde. Ein neues Fed-Batch Protokoll wurde verwendet, bei dem durch Einsatz einer Mischung aus Methanol und Glyzerin in der Induktionsphase die höchste volumetrische Aktivität durch eine erhöhte Biomasse-Ausbeute erzielt wurde.

Abstract

Amine and amino acid oxidases are important enzymes for biocatalysis. For example *Trigonopsis variabilis* D-amino acid oxidase (*Tv*DAO) is a well characterized enzyme which mainly was used for conversion of cephalosporin C to produce semisynthetic antibiotics as well as for analytical determination of D-amino acids in food and deracemization processes such as the production of enantiopure amino acids.

This work reports a new strategy for enhanced expression of amine and amino acid oxidases. Therefore the protein sequences of *Tv*DAO, *Rg*DAO (*Rhodotorula gracilis* D-amino acid oxidase), *Cr*LAO (*Calloselasma rhodostoma* L-amino acid oxidase) and *An*MAO (*Aspergillus niger* monoamine oxidase) were analyzed with webtools. Based on this analysis synthetic, codon-optimized genes were designed. *Tv*DAO was chosen as model enzyme for expression optimization.

Heterologous expression in *Pichia pastoris* has several advantages as compared to *E. coli*. The methylotrophic yeast *P. pastoris* is a eukaryote and therefore able to perform post translational modifications while simple DNA modification techniques and cultivation protocols can be applied. Furthermore high cell densities can be reached during fermentation. However, to decrease the cost of this important biocatalyst, expression of *Tv*DAO and other amine oxidizing enzymes in *P. pastoris* has to be optimized.

To reach this goal in my thesis firstly an exchange of the peroxisomal targeting sequence was carried out to secure efficient subcellular localization of *Tv*DAO to the peroxisomes where catalase is present to detoxify cell toxic hydrogen peroxide which is generated during oxidase reaction. Secondly a codon-optimized synthetic gene was designed for optimal expression in *P. pastoris* under conditions of methanol induction. In addition, a fusion protein of *Tv*DAO and Citrine (a GFP variant) was generated in order to confirm the peroxisomal targeting. Finally the best *Tv*DAO expression strains were cultivated in 1.5 litre bioreactors and the cultivation conditions were optimized for maximum activity and biomass yield.

The resulting strains showed the highest volumetric *Tv*DAO activity that has been reported so far. A new fed-batch protocol was applied, where a mixture of methanol and glycerol in the induction phase led to highest volumetric activity by an additional increase of biomass yield.

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Abbreviations

Table 1: General abbreviations used in this work

Abbreviation	Description
4-AAP	4-aminoantipyrine
A	activity
AA	Amino acid
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
Amp	ampicillin
*An*MAO	*Aspergillus niger* monoamine oxidase
*AOX*1	alcohol oxidase 1 gene
*AOX*2	alcohol oxidase 2 gene
ATCC	American Type Culture Collection, Manassas, VA, USA
B	Biotin
B1	pPpB1 (E. coli / P. pastoris shuttle vector)
BMD1	buffered minimal dextrose medium1%
BMM10	buffered minimal methanol medium 10%
BMM2	buffered minimal methanol medium 2%
bp	base pair
CBS	Centraalbureau voor Schimmelcultures, Utrecht, Netherlands
CDS	coding sequence
CIAP	calf intestine alkaline phosphatase
*Cr*LAO	*Calloselasma rhodostoma* L-amino acid oxidase
D	dextrose
Da	dalton
ddH_2O	double distilled water "Fresenius"
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
dwp	deep well plate
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
EtBr	ethidiumbromide
EtOH	ethanol
Fd	fast digest
*FLD*1	formaldehyde dehydrogenase 1 gene
fcw	fresh cell weight
fwd	forward
GAP	glyceraldehydes 3-phosphate dehydrogenase gene
HRP	horse radish peroxidase
kb	kilo base pairs
kDa	kilo Dalton
LB	Luria Bertani medium
MeOH	methanol
mRNA	messenger ribonucleic acid
MW	molecular weight
NRRL	Northern Regional Research Laboratories, Peoria, IL, USA
ntc	no template control

OD600	optical density at 600 nm
oePCR	overlap extension PCR
ON	over night
ONC	over night culture
P(AOX1)	promoter of AOX1 gene
P(AOX2)	promoter of AOX2 gene
P(FLD1)	promoter of FLD1 gene
P(GAP)	promoter of GAP gene
PCR	polymerase chain reaction
pI	isoelectric point
PPB	potassium phosphate buffer
qRT-PCR	quantitative real time PCR
rev	reverse
*Rg*DAO	*Rhodotorula gracilis* D-amino acid oxidase
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	revolutions per minute
RT	room temperature
RT-PCR	real time PCR
SD	standard deviation
T2	pPpT2 (E. coli / P. pastoris shuttle vector)
TBHBA	2,4,6-tribromo-3-hydroxybenzoic
*Tv*DAO	*Trigonopsis variabilis* D-amino acid oxidase
wcw	wet cell weight
YNB	Yeast nitrogen base
YPD	Yeast extract / peptone / dextrose medium
Zeo	Zeocin^{TM}

1 Introduction

1.1 The expression system Pichia pastoris

1.1.1 Important features of *Pichia pastoris*

Pichia pastoris is a methylotrophic yeast and therefore able to grow on methanol as sole carbon and energy source [1]. It is nowadays one of the most commonly used expression systems for heterologous expression due to its cultivation in cheap and simple media and accessible high cell densities (130 g/L dry cell weight in continuous cultures) [2]. Furthermore the genome of *Pichia pastoris* is well characterized [3] and common recombinant DNA techniques can be used for modification [4].

Even though *Escherichia coli* is widely used for heterologous expression it exhibits some major drawbacks due to its prokaryotic protein expression which often hinders the expression of correctly folded and processed proteins from eukaryotic genes. In contrast *P. pastoris* is advantageous for heterologous expression of proteins from eukaryotic genes due to its eukaryotic expression machinery [5, 6]. Compared to other eukaryotic expression systems like mammalian and insect cell cultures *Pichia pastoris* displays faster growth characteristics and is cheaper and easier to handle. Nevertheless it is able to perform typical eukaryotic posttranslational modifications like folding, methylation, disulfide bond formation, O- and N-linked glycosylation, proteolytic processing and processing of signal sequences and targeting to subcellular compartments [7].

Furthermore *P. pastoris* is able to carry out intracellular and extracellular protein production due to the possibility to secrete proteins into the media. Secretion of heterologous proteins is often necessary because some post-translational modifications are carried out during the secretory pathway, in the media limitation of space does not occur, proteins which are toxic for *P. pastoris* are diluted. In addition no cell disruption is necessary to get the protein, which can be seen as a first purification step [6].

Moreover there are several strong promoters for expression in *P. pastoris* available. The most commonly used is the promoter of the alcohol oxidase 1 gene P(AOX1). It is tightly regulated and allows strong inducible expression. P(AOX1) is repressed by carbon sources like glucose and glycerol, which is crucial for the expression of for *P. pastoris* toxic proteins because it is possible to repress the promoter during cell growth and induce expression with methanol later on.

Another well-known promoter is the P(GAP), the promoter of glyceraldehydes 3-phosphate dehydrogenase gene, a constitutive promoter of similar strength. It works best with high levels of glucose, but glycerol and methanol can also be used. Special feeding strategies can enhance the productivity of strains employing the GAP promoter system.

Alternatively other promoters such as the FLD1 promoter of the formaldehyde dehydrogenase 1 gene can be used. It is inducible by methanol and methylamine, therefore it provides an alternative for methanol free expression [6, 8].

Since there are no stable plasmids for *Pichia pastoris* available, linearized expression vectors are usually integrated into the genome via homologous recombination [9]. Therefore, generated expression strains show high stability [6].

A basic *P. pastoris* expression system is easily available as commercial kit from Invitrogen Corporation (Carlsbad, CA, USA) providing several strains, vectors, selection markers (auxotrophy, antibiotic resistance) [10] and protocols.

1.1.2 Targeted integration into the genome

Currently there are no stable plasmids available hence transformations are usually done by integration of linearized expression vector DNA into the genome [6]. This integration via homologous recombination occurs between sequences shared by the insert (transformed DNA) and the genome [9]. In contrast to *S. cerevisiae* longer homologous regions are needed for integration in *P. pastoris*.

Linearization of the plasmid can be done in any homologous region (e.g. promoters), but it is important to consider that the integration locus is determined by the site of linearization of the plasmid. Depending on the constructed vector single or double crossover events are favoured nevertheless single crossover events (insertions) are more likely to happen than double crossover event (replacements). Multiple insertion events occur spontaneously at about 1-10 percent of single insertion events [11].

Single crossover events between the free plasmid end and a homologous region of the genome lead to insertions of the expression cassette. Depending on the number of integration events, single and multi copy strains are possible.

Double crossover events occur when both ends of the linear plasmid are homologous to sequences of the genome and lead to gene replacement. Gene replacement within the AOX1 locus results in slow methanol utilization (Mut^s) phenotype strains.

1.1.3 Methanol metabolism

As a methylotrophic yeast *Pichia pastoris* is able to use methanol as sole carbon and energy source. The necessary methanol utilization pathway is partly located in the peroxisomes. Since peroxisomes are important organelles in *Pichia pastoris* it became a model organism for the examination of peroxisome biogenesis and function [12].

Peroxisomes are abundant organelles present in almost any eukaryotic cell. They harbour enzymes to detoxify peroxides (catalase) by conversion into water and oxygen. Therefore peroxisomes harbour many important metabolic reactions such as ß-oxidation of fatty acids and oxidation of methanol [13].

An important feature of *Pichia pastoris* is the proliferation of peroxisomes when cells are shifted from glucose media to methanol medium. In glucose media there are no peroxisomes required for cell growth consequently only a small amount of peroxisomes is present. When cells are shifted to methanol media, the number and size of peroxisomes increase to cope with the new conditions. The increased number of peroxisomes have to be selectively degraded when the cells are shifted to glucose media and not needed anymore. This selective degradation of peroxisomes is carried out by the yeast vacuole and is called pexophagy [14]. In contrast to secretory pathway organelles, which arise from budding from the endoplasmatic reticulum (ER) or de novo synthesis, peroxisomes are thought to be formed by budding from pre-existing peroxisomes [15]. Nevertheless, the exact mechanism is not completely clear.

The peroxisomal proteins are produced on cytosolic or free ribosomes and are posttranslationally transported into the organelle. For this purpose two peroxisomal targeting signals (PTSs) are available [16]. The most commonly used is PTS1, a C-terminal tripeptide sequence serine-lysine-leucine (SKL) and a few conservative variants which are recognised by transport protein Pex5p [17, 18]. In a few cases PTS2 is used, which is an amino-terminal signal sequence and is proteolytically cleaved upon import [19].

The first step of the methanol utilization pathway [6, 20], which is located in the peroxisomes is oxidation of methanol to formaldehyde and hydrogen peroxide by alcohol oxidase (AOX). In *Pichia pastoris* there are two genes that code for equally functional homologous alcohol oxidases AOX1 and AOX2. Even though the similarity is very high, 92% for the nucleotide and 97% for the amino acid level, AOX1 displays 10-20% higher specific activity [21]. In addition the promoter DNA sequence for AOX1 and AOX2 is quite different leading to a much higher production of the more active oxidase AOX1. Alcohol oxidase is expressed in

high amounts (up to 30% of the total cellular protein) because of the slow conversion of methanol caused by the low affinity for oxygen [20].

The by-product of the first step is H_2O_2, this toxic compound is neutralized in the peroxisome by catalase to form oxygen and water. Generated formaldehyde is used in the assimilatory pathway for further energy production and biomass generation. In the dissimilation pathway formaldehyde (which is also toxic) is oxidized in the cytoplasm resulting in CO_2 and energy for cell growth on methanol. This is an oxidative process, where electrons are transported to produce energy.

Formaldehyde reacts in the assimilation pathway with xylulose-5-phosphate (Xu_5P) resulting in the 3-C compounds dihydroxyacetone (DHA) and glyceraldehyde-3-phosphate (GAP), which is catalyzed by the dihydroxyacetone synthase (DHAS) in the peroxisome. Both molecules enter the cytoplasm and regenerate one molecule xylose-5-phosphate and glyceraldehyde-3-phosphate which is used for biomass production (see Figure 1).

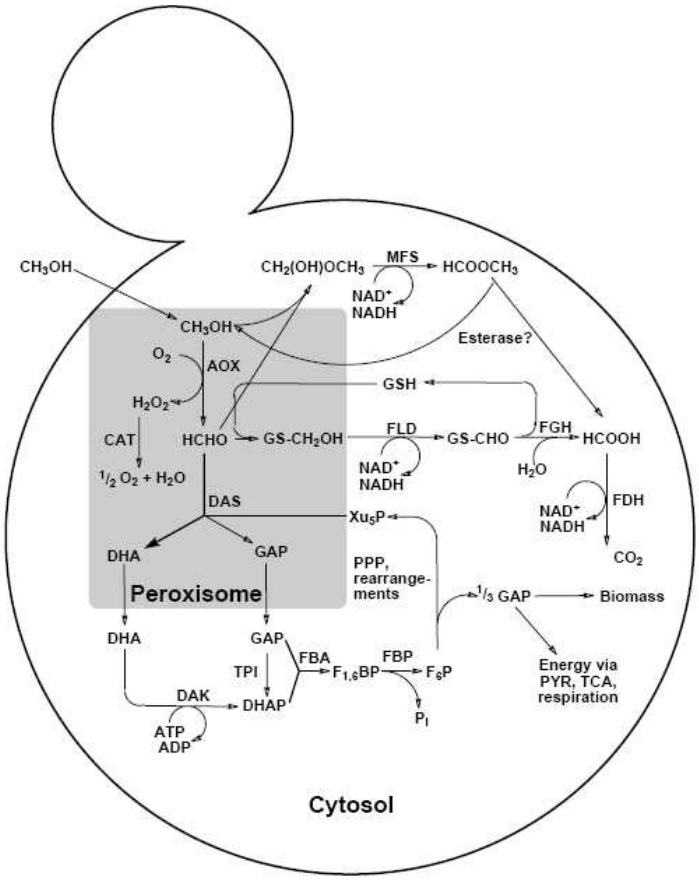

Figure 1: Methanol utilization pathway in methylotrophic yeasts [20]: AOX: alcohol oxidase, CAT: catalase, FLD: formaldehyde dehydrogenase, FGH: S-formylglutathione hydrolase, FDH: formate dehydrogenase, DAS: dihydroxyacetone synthase, TPI: triosephosphate isomerase, DAK: dihydroxy-acetone kinase, FBA: fructose 1,6-bisphosphate aldolase, FBP: fructose 1,6-bisphosphatase, MFS: methylformate synthase; DHA: dihydroxyacetone, GAP: glyceraldehyde 3-phosphate, DHAP: dihydroxyacetone phosphate, F1,6BP: fructose 1,6-bisphosphate, F6P: fructose 6-phosphate, Pi: phosphate, Xu5P: xylulose 5-phosphate, GSH: glutathione, PYR: pyruvate; PPP: pentose phosphate pathway, TCA: tricarboxylic acid cycle

1.1.4 Secretory machinery

Heterologous protein expression in *Pichia pastoris* can be intracellular or extracellular (secretion into the media). *Pichia pastoris* secretes only low levels of native, endogenous proteins. Hence the secreted foreign, heterologous proteins are usually highly pure due to the separation of heterologous from cellular protein [6]. This fact is important for industrial processes as it is easy to separate the target protein by filtration or centrifugation from the (unwanted) biomass.

Unfortunately, secretion is usually limited to proteins that are also secreted by their native host due to protein stability and folding requirements. Additionally a signal sequence that leads the protein into the secretory pathway is required for secretion. Especially the following sequences were frequently used for successful secretion: The protein's own native secretion signal (if existing), the pre-pro leader sequence of the α-mating factor of *Saccharomyces cerevisiae* (α-MF), the acid phosphatase signal sequence of *Pichia pastoris* (PHO1) and the invertase signal sequence (SUC2) [6, 22].

Another important reason for secretion is that some post translational modifications are carried out during the secretory pathway.

1.1.5 Linear expression cassettes for fast enzyme expression

In order to generate reliable expression constructs (e.g. expression strain generation or directed evolution in *Pichia pastoris*) without time-consuming ligation and cloning steps in *E. coli*, a new strategy was developed by Liu *et al* [9]. Besides the shorter experiment time another advantage is the better library quality for eukaryotic proteins, because the common subcloning step in *E. coli* results in loss of diversity and library efficiency.

This new strategy is based on overlap extension PCR (oePCR). Three fragments (promoter, gene of interest and terminator/selection marker) with overlapping ends form an expression cassette which is assembled and amplified by PCR (see Figure 2).

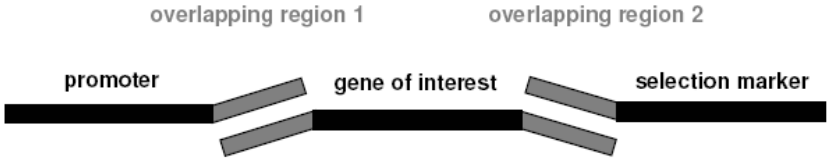

Figure 2: Assembly of linear expression cassettes [9]

The application of universal overlapping regions allows combinations of any promoter and selection marker with the gene of interest without affecting its expression (see Table 2).

Table 2: Universal overlapping regions for the assembly of linear expression cassettes

Overlapping region	Fragments linked		Sequence (5' → 3')
1	promoter	gene of interest	ctaggtacttcgaaacgaggacttcacg
2	gene of interest	selection marker	gtcagatagcgaggtcactcagtc

The fragments are linked and amplified by oePCR in two steps: For the first step a PCR mix without primers and equimolar ratios of fragments with overlapping ends is produced. During the first PCR cycles the fragments are linked by their overlapping ends and act therefore themselves as primers. In the second step, primers that bind to both ends of the generated construct are added and the expression cassette is amplified to produce a sufficient amount of DNA. This generated expression cassette can be directly used for *Pichia pastoris* transformation without any amplification in *E. coli*.

1.2 Enzymes used during this work

Amine and amino acid oxidases are well charactarized enzymes with significant value for industrial purposes. Therefore D-amino acid oxidase from the fungus *Trigonopsis variabilis*, L-amino acid oxidase from the snake *Calloselasma rhodostoma* and monoamine oxidase from the fungus *Aspergillus niger* were chosen for this study.

1.2.1 D - Amino Acid Oxidases

1.2.1.1 General information

D-Amino acid oxidases (DAO, E.C. 1.4.3.3) are flavine adenine dinucleotide (FAD) containing enzymes [23]. These putative peroxisomal enzymes catalyze the oxidative deamination of D-amino acids (D-AA) to give the corresponding imino acid as an intermediate, which then hydrolyzes spontaneously to the α-keto acid, ammonia and hydrogen peroxide.
DAO activity is found in few bacterial and many eukaryotic organisms including yeasts, fungi, insects, amphibians, reptiles, birds, mammals and humans [24].

D-amino acid oxidases play an important physiological role in many organisms. The prominent role of D-amino acids (D-AA) as components of the peptidoglycan cell wall of bacteria is well investigated [25]. The presence of DAO in yeasts is related to their ability to use D-AA for growth [26]. In animals high levels of expression of peroxisomal DAO were found in certain tissues (kidney and liver) [27]. D-amino acids are oxidised in vivo in animals and humans in various tissues, particularly kidney and liver, resulting in the same compounds as in vitro (α-keto acid, ammonia and hydrogen peroxide) [28]. Furthermore there is evidence, that D-amino acid oxidase plays a regulatory role in the human brain. It is involved in controlling the levels of the neuromodulator D-serine [29].

1.2.1.2 Properties

All DAOs contain a non covalently bound FAD subunit as prosthetic group. As FAD-dependant flavooxidase, DAO belongs to the subgroup of the dehydrogenase and oxidase class of flavoproteins which share some interesting features: All members of this family react rapidly with molecular oxygen in the reduced form and stabilize the red anionic flavin radical via one-electron reduction.

D-amino acid oxidases from microorganisms show properties that make them suitable for many biotechnological applications [30]. Such characteristics include high level of protein expression (native and recombinant), high activity, high turnover number, tight binding of the non-covalent FAD cofactor, broad substrate specificity and a strict stereoselectivity.

DAO activity has been mostly reported in tissues of mammals and eukaryotes. The DAO from pig kidney (pkDAO) was the first to be isolated in 1973 [31]. More than a decade later the DAOs from the yeasts *Trigonopsis variabilis* (*Tv*DAO) [32] and *Rhodotorula gracilis* (*Rg*DAO) [33] became available. Nowadays DAO activity has been found in a number of eukaryotic microorganisms such as *Aspergillus niger*, *Candida utilis, Hansenula polymorpha, Fusarium solani*. Surprisingly only one DAO of bacterial origin has been characterized till now: *Actinobacteria protophormiae* (*Ap*DAO) [34].

Known DAOs share high primary sequence similarity, consequently six highly conserved regions were identified (see Figure 3) [35]. Regions I and III are involved in coenzyme binding, whereas the regions II, IV and V contain highly conserved residues of the active site. As peroxisomal enzymes most DAOs share a conserved c-terminal sequence motif (Ser-Lys/His-Leu or a similar sequence), representing the type 1 peroxisomal targeting signal [36]. Mammalian DAOs (human, pig, mouse) show 63% sequence identity, in contrast to DAOs from microorganisms (*T. variabilis, R. gracilis, F. solani*), which share a much lower degree of identity (18%).

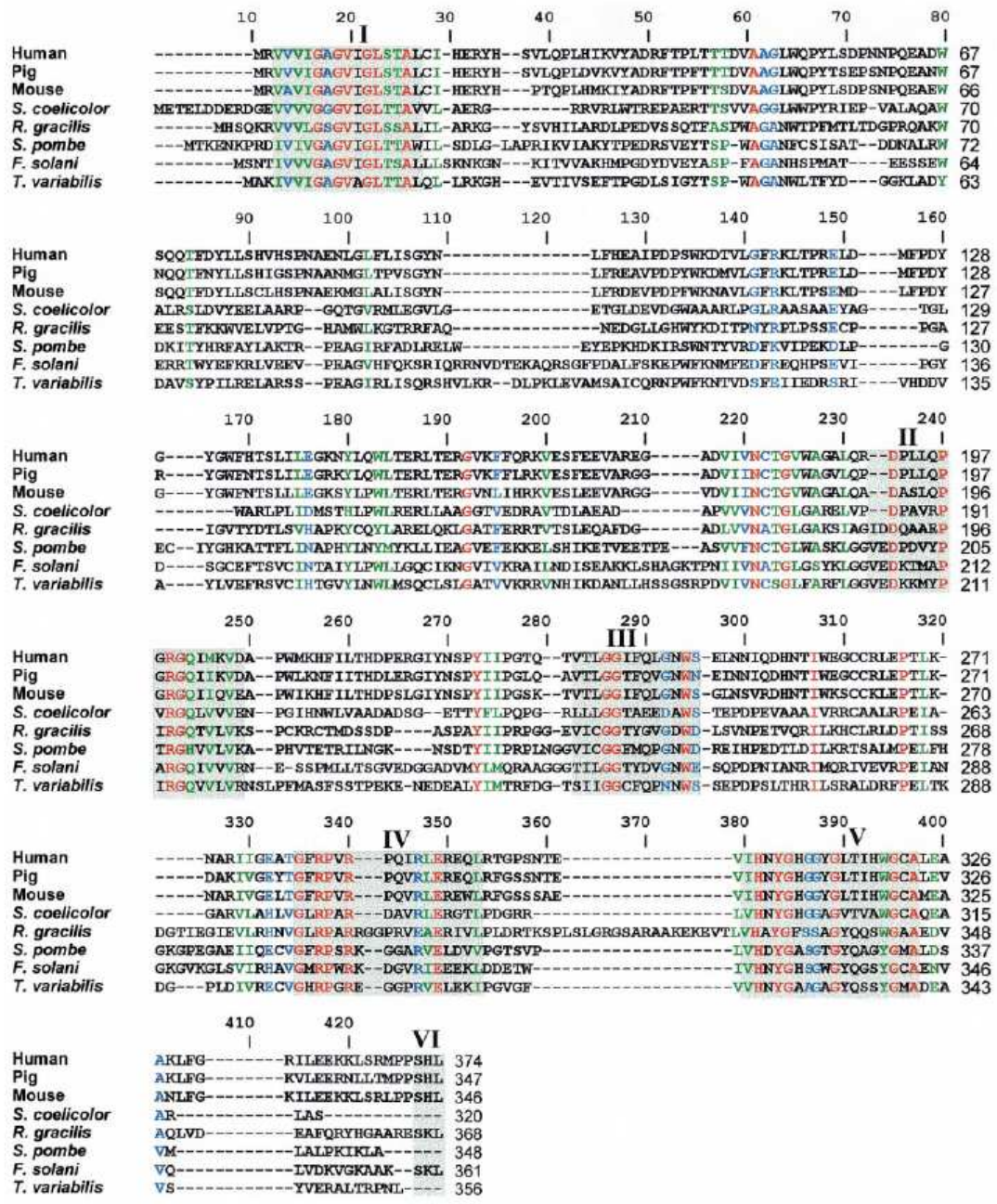

Figure 3: Comparison of the primary structure of DAOs from significative sources [23]
Red: identity, blue: high similarity, green: low similarity, black: differnence. Total alignment length: 429 amino acids (accession numbers: Human P14920, Pig P00371, Mouse P18894, *Streptomyces coelicor* T35265, *Rhodotorula gracilis* P80324, *Schizosaccharomyes pombe* T40989, *Fusarium solani* P24552, *Trigonopsis variabilis* Q99042). Region I and III: coenzyme binding sites. Region II, IV and V: acitive site

1.2.1.3 Oxidation of D-amino acids

As mentioned before DAO catalyzes the oxidative deamination of D-AA to give α-keto acids and ammonia. The reaction scheme can be seen in Figure 4.

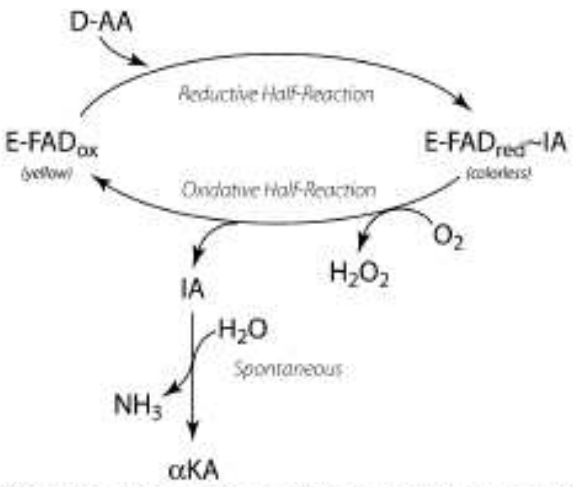

Figure 4: Catalytic reaction of D-amino acid oxidase [37]. The redox state of the FAD cofactor is reported as subscript: ox oxidized; red reduced. E-FAD$_{red}$~IA is the non-covalent complex between the reduced enzyme form and the imino acid product. IA Imino acid. α-KA α-keto acid.

The enzymatic step, the reductive half–reaction, dehydrogenates the amino acid to the corresponding iminoacid. This step is coupled with the reduction of FAD. $FADH_2$ reoxidizes spontaneously in the presence of molecular oxygen, producing hydrogen peroxide. The imino acid hydrolyzes to the α-keto acid and ammonia non-enzymatically.

The redox reaction, catalyzed by DAO, can be employed in a variety of biotechnological processes due to its absolute stereoselectivity and the broad substrate specificity.
The reaction is restricted to D-isomers when both D- and L-amino acids are supplied as substrate. L-amino acids are neither substrates nor inhibitors. DAO in vitro displays a broad substrate specificity even though amino acids with hydrophobic side chains tend to be the most efficient substrates.

1.2.1.4 Applications / DAO in biotechnological processes

DAOs from microorganisms show characteristics which facilitate their application in biotechnological processes. As mentioned above, high level of expression, high turnover number, tight coenzyme binding as well as strict stereoselectivity and broad substrate specificity are essential for successful employment as biocatalyst in industrial applications.

1.2.1.4.1 7-ACA production

The most important biotechnological application of DAO is the first step of the chemo-enzymatic conversion of cephalosporin C (ceph C) to 7-aminocephalosporanic acid (7-ACA). This large scale (> 1000 tons/year) process is of great interest to antibiotic manufacturers as 7-ACA is necessary for the production of semi-synthetic cephalosporins (world market ~200 million US dollars/year).

The traditional method to generate 7-ACA involves chemical deacylation in organic solvents and usage of toxic chemicals which can be replaced by a two-step enzymatic transformation. First oxidative deamination of ceph C to 7-(5-oxadipoamido) cephalosporanic acid is catalyzed by DAO. In presence of H_2O_2 spontanous decarboxylation occurs to yield glutaryl-7-ACA, which is hydrolyzed in a second enzymatic step by glutaryl amidase to produce 7-ACA (see Figure 6).

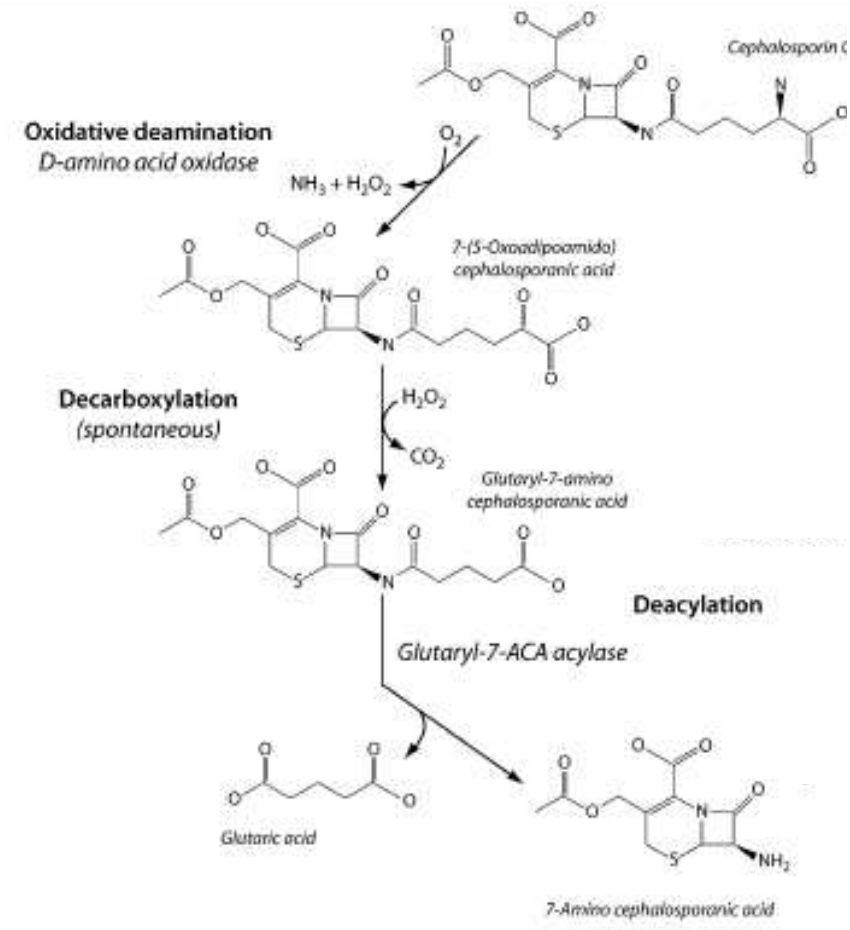

Figure 5: Conversion of cephalosporin C into 7-aminocephalosporanic acid (7-ACA) [30]

1.2.1.4.2 Production of pure L-amino acids and α-keto acids

The importance to produce pure L-amino acids for usage as feed additives, components of infusion solutions and starting materials for pharmaceuticals is undoubted. Optimization of L-amino acid production has been achieved by application of a deracemization strategy by Beard and Turner in 2002 and this process has already been scaled up for industrial purposes. Deracemization has been accomplished by conversion of a racemic mixture into pure L-amino acids by combination of an enantioselective enzyme catalysed oxidation reaction (catalysed by DAO) with a non enzymatic reduction reaction (see Figure 6) [38].

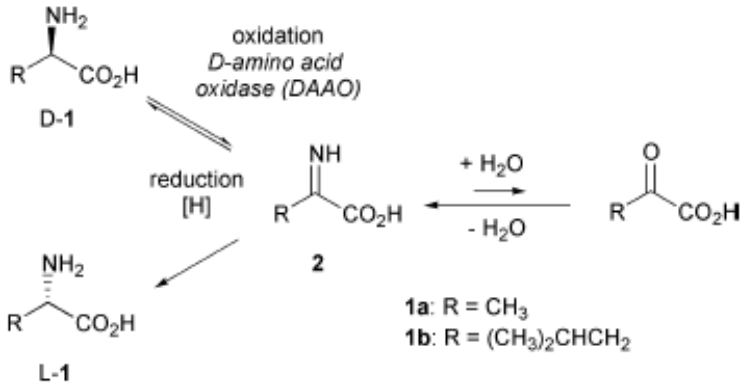

Figure 6: Stereoinversion of D- to L-amino acids [38]

Another interesting industrial application of DAOs is the conversion of D-amino acids into their corresponding α-keto acids which are valuable and useful compounds[30].

1.2.1.4.3 Analytical determination of D-AA

The traditional D-amino acid determination by gas chromatography or reversed-phase liquid chromatography is reliable and very sensitive, however these methods have a serious drawback: they are too slow for the food industry. Therefore the development of fast biosensors, which are able to detect D-AAs in solution is a main advantage for industrial purposes.

The absolute stereoselectivity of the DAO reaction explains the usage as biosensor for the detection and quantification of D-AA in biological samples.

These biosensor are relevant to indicate the presence of bacterial contamination in milk [24], which is possible due to the bacterial peptidoglycan cell walls which consist of D-amino acid components. Furthermore D-amino acids or racemization in food products can decrease the nutritional value of food products and raise concerns about safety because of the possible toxicity of D-AA.

Another important application is the clinical determination of L- and D-amino acids in biological samples. For example the content of D-amino acids in white and grey matter in the brains of Alzheimer patients is 1-4 times higher than in normal patient brain [39].
Another example is the control of D-Serine and its metabolism. It is relevant for NMDA (N-methyl-D-aspartate) receptors whose altered function is associated with schizophrenia, epilepsy and neurogegenerative disorders [40, 41].

1.2.1.5 D-Amino acid oxidase from *Trigonopsis variabilis* (*Tv*DAO)

1.2.1.5.1 General properties

Due to the relevance of *Tv*DAO in industrial processes its pH and temperature stability has been extensively studied. *Tv*DAO is stable from pH 6.0 to 8.2, above this point slight but continuous decrease in protein stability has been observed [42]. Of all DAOs *Tv*DAO is the thermally most stable enzyme. It is fully stable up to 45°C, which means that after 30 min incubation 100% of residual activity can be determined [42]. Similar to other DAOs from yeast, *Tv*DAO can be inactivated by H_2O_2 which is a product of the reaction catalysed by the enzyme [42]. In general it can be said that the apoprotein of *Tv*DAO is far less stable than the corresponding holoenzyme [43].

In addition the broad substrate specificity of *Tv*DAO is important for its application in biotechnological processes. DAOs are able to oxidise amino acids with side chains of different size and polarity. Nevertheless, *Tv*DAO shows highest activity on D-AAs possessing large hydrophobic side chains (D-Trp, D-Met, D-Val, D-Phe) [44] and shows high specific activity (higher than *Rg*DAO [42]) towards modified D-AAs like ceph C, which is highly relevant for industrial purposes. Furthermore the strict stereoselectivity of *Tv*DAO which only uses D-amino acids as substrates and does not interact with the corresponding L-amino acid is essential.

Also kinetic parameters like the turnover number have been determined. The turnover number of *Tv*DAO is very high (52.5 s^{-1}) [45] compared to the turnover number (12.7 s^{-1}) of pig DAO [46]. This leads to the conclusion that yeast DAOs like *Tv*DAO more suitable for biotechnological applications than mammalian DAOs.

Generally *Tv*DAO combines a series of features which are important for biotechnological processes. As a result *Tv*DAO has a long history in the production of 7-ACA from the conversion of ceph C [47].

1.2.1.5.2 Expression of TvDAO in the native host Trigonopsis variabilis

The native host, the yeast *T. variabilis* is a good DAO producer. It shows higher productivity than other natural DAO producers [48]. Another benefit is the possibility of high cell density fermentation where high volumetric DAO yields can be reached.

DAO activity of up to 225U/g cell has been achieved with the *T. variabilis* strains CBS 4095[1] and NRRL Y7770[2] using D-Ala as inducer [32, 49] Various D,L-amino acids with nonpolar or polar side chains, as well as with negatively or postitively charged side groups have been tested for induction of *Tv*DAO. All tested D,L-amino acids induce *Tv*DAO, however D,L-Met, D,L-Ala, D,L-Leu and D,L-Val gave the highest enzyme production. Even D-amino acid analogues that cannot be metabolised induce *Tv*DAO expression. The best results gave N-carbamoyl-D,L-alanine as sole carbon source yielding 650 U/g cell [48].

Furthermore high level of biomass can be produced with *T. variabilis* cells. For example fermentation of the *T. variabilis* CBS 4095 strain using a fed-batch fermentor gave 62 g cell/L fermentation broth and reached a value of DAO production of ~ 35 U/g cell [50].
An other example gave a twofold lower yield of biomass with the mutant *T. variabilis* strain ATCC 20931[3], but in this case DAO production was increased (~ 350 U/g cell) [51].
A summary of *Tv*DAO production in the native host *Trigonopsis variabilis* can be found in Table 3.

1.2.1.5.3 Heterologous expression of TvDAO

*Tv*DAO was expressed in a mutant of the *Trigonopsis variabilis* strain ATCC 10679 and gave 175 U/g cell [52].
Heterologous expression in *E. coli* is well studied [53, 54] and results in enzyme preparations with better stability [55, 56], higher activity [57, 58] and better solubility [59]. Additionally *Tv*DAO has been expressed in various yeasts including *Kluveromyces lactis* [60], *Saccharomyces cerevisiae* [60], a catalase deficient *Schizosaccharamyces pombe* strain [61] and *Pichia pastoris* [62].

[1] CBS: Centraalbureau voor Schimmelcultures, Utrecht, Netherlands

[2] NRRL: Northern Regional Research Laboratories, Peoria, IL, USA

[3] ATCC: American Type Culture Collection, Manassas, VA, USA

*Tv*DAO was also expressed as engineered recombinant protein as His-tagged *Tv*DAO in *E. coli* [54, 63]. Additionally His-tagged protein has been expressed in *Pichia pastoris* [64], but the enzyme expression was threefold lower than in the absence of the tag [62].
A summary of the results of previous studies of heterologous *Tv*DAO expression is shown in Table 3.

Table 3: Level of production of native and recombinant *Tv*DAO [37]

DAO source	Host	Inducer	Fermenation yield (U/g cell)	Reference
T. variabilis CBS 4095	native	D-alanine	175	[32]
NRRL Y7770	native	D,L-alanine	~225	[49]
DSM 70714	native	N-carbamoyl-D-alanine	650	[48]
ATCC20931	native	D,L-methionine	440	[51]
T. variabilis ATCC 20931	*T. variabilis*	D,L-methionine	175	[52]
T. variabilis CBS 4095	*Kluyveromyes lactis*	Galactose	~40	[60]
T. variabilis CBS 4095	*Pichia pastoris*	Methanol	~240	[62]
T. variabilis CBS 4095	*E. coli*	IPTG	n.d. (~9,700 U/L)	[59]
T. variabilis CBS 4095	*E. coli*	Lactose	n.d. (11,700 U/L)	[54]

1.2.1.5.4 TvDAO in biotechnological processes

As already mentioned *Tv*DAO is an important biocatalyst for industrial processes. Even though it has some striking features for biotechnological applications, stability and stabilization of the enzyme is still a major research topic [65]. Some strategies to improve the stability of *Tv*DAO include enzyme immobilization [66], fusion to a pull-down domain [56], entrapment of the enzyme [55], binding of the enzyme to a carrier [67].

However it has to be said that the specifications for *Tv*DAO depend on the field of application, the process which uses the enzyme as biocatalyst. For example the conversion of ceph C relies on hydrogen peroxide, which is generated as a byproduct of the reaction with molecular oxygen, even though *Tv*DAO is inactivated by H_2O_2. This is possible because hydrogen peroxide is necessary for the oxidative decarboxilation to produce the valuable product 7-ACA [66]. In contrast the production of chiral amino acids requires low levels of H_2O_2 by-product [68].

1.2.2 L-Amino Acid Oxidases

1.2.2.1 General information

L-Amino acid oxidases (LAO) are FAD dependent enzymes which catalyse the reaction of L-amino acids with water and oxygen to the corresponding α-keto acid, ammonia and hydrogen peroxide. They are found in many different organisms like bacteria (*Corynebacterium* [69], *Proteus* [70]), cyanobacteria (*Synechococcus* [71]), fungi (*Neurospora crassa* [72]), green algae (*Chlamydomonas rheinhardtii* [73]) and most prominently in venomous snakes (such as crotalids, elapids and viperids) [74].

LAO found in snake venom is considered to contribute to its toxicity [75] through generation of hydrogen peroxide formed as a result of reoxidation of the transiently reduced flavin cofactor by molecular oxygen (see Figure 7). However the exact role of LAO in snake venom is not yet fully understood [74].

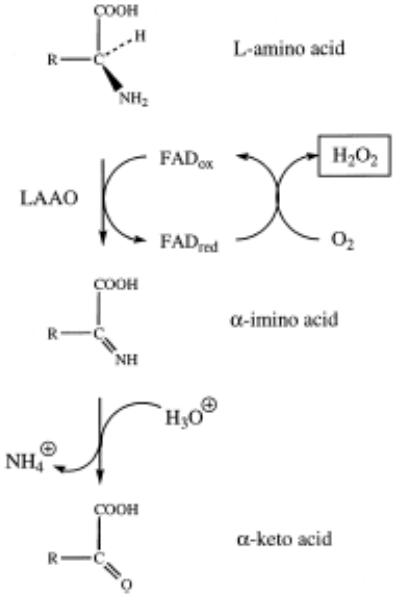

Figure 7: Sterospecific oxidative deamination of an L-amino acid substrate to an alpha-keto acid along with the production of ammonia and hydrogen peroxide via an imino acid intermediate [76]

*Cr*LAO, LAO from *Calloselasma rhodostoma* (Malayan pit viper), has been extensively studied. It was purified from snake venom and the first crystal structure of a LAO [77] was solved in the year 2000. Biochemical studies showed an interesting feature of *Cr*LAO and other ophidian LAOs: freeze and pH-induced reverible inactivation process [76].

Two glycosylation sites (Asn172 and Asn361) have been found in *Cr*LAO [78]. It is thought that glycosylation is essential for the active enzyme secretion and induction of apoptosis [79].

1.2.2.2 Heterologous expression

Reports of ophidian LAO expression are rare because LAOs are predicted to be toxic to the cell due to depletion of L-aminos acids and production of hydrogen peroxide. However a common observation is that the levels of expression are generally very low. Expression of LAOs in the absence of secondary modifications could lead to insoluble protein (native ophidian *Cr*LAO carries two glycans that are assumed to play a role in solubility) [78]. In addition, LAOs exists in two forms, an active and an inactive one. The interconversion is dependent on pH, temperature and the presence of specific ions [80]. All these factors contribute to a very difficult heterologous expression of LAO.

Expression of *Cr*LAO in *Pichia pastoris* [81], using the alpha-mating factor-signal sequence in an expression vector to secrete the protein into the culture, yielded in a protein concentration of 0.4 mg/L culture supernatant. Additionally, attempts were made to express *Cr*LAO in *E. coli*, mammalian cell lines or the baculovirus system but the recombinant protein was either found in insoluble fractions or had no activity.

1.2.2.3 Applications

Ophidian LAOs are used to induce apoptosis [82] in biochemical experiments, have antimicrobial activity [83] and anti-HIV activity [84].

1.2.3 Monoamine Oxidases

1.2.3.1 General information

Monoamine oxidases (MAO) catalyze oxidative deamination of monoamines and belong to the protein family of flavin-containing amine oxidoreductases. In mammals they are found bound to the outer membrane of mitochondria in most cell types in the body.

Monoamine oxidases catalyze the deamination of monoamines therefore oxygen is used to remove an amine group from a molecule, resulting in the corresponding aldehyde and ammonia.

In humans two forms of MAO are known. MAO-A and MAO-B are particularly important in the catabolism of monoamines ingested in food. MAOs are also vital to the inactivation of monoaminergic neurotransmitters, for which they display different specificities.
In lower eukaryotes and bacteria the main biological role of amine oxidases is to provide the organisms with ammonium. Monoamine oxidase in *Aspergillus niger* (*An*MAO-N) is formed when the fungus is cultured in the presence of monoamines as sole nitrogen source [85].
In this study variant of MAO-N with 5 mutations (I246M, N336S, M348K, T384N, D385S), *An*MAO 5M, was used. It is a variant mutated for specificity towards α-methylbenzylamine [86], provided by Ingenza.

1.2.3.2 Heterologous expression

*An*MAO has been expressed in *E. coli* [87]. The activity was determined with or without IPTG induction. The results show, that the addition of the inducer leads to a soluble but inactive protein. It was discussed, that it is likely that the recombinant protein is not correctly folded.

2 Aims, Objectives and Experimental Strategy

During this thesis new expression strategies were developed on the basis of the model enzyme D-amino acid oxidase from *Trigonopsis variablis* (*Tv*DAO). Therefore a multi-level approach was considered. Codon-optimization of the *Tv*DAO gene according to a special *Pichia pastoris* high level expression codon usage served as basis for expression optimization. In addition specific targeting to the peroxisome by usage of the well recognized peroxisomal targeting sequence (PTS1) –SKL gave further improvements. This is especially benefical as expression under the control of the *alcohol oxidase 1* promoter (*AOX1*) in the host organism *P. pastoris* leads to proliferation of peroxisomes which can take over 80% of the cytoplasmic space [10]. Furthermore peroxisomes also harbour catalase, an enzyme which catalyzes is able to neutralize the cell toxic reaction byproduct hydrogen peroxide. Moreover, the fusion of *Tv*DAO to the fluorescence protein citrine was used to confirm the correct cellular location of the target.

Linear expression cassettes were assembled using two different strategies based on two different plasmids pPp-B1 and pPp-T2, which differ in the expression cassette of the Zeocin resistance marker. The *P. pastoris* strain Mut^S7 was transformed with the assembled expression cassettes to generate multi copy production strains and single copy strains for evaluation of the expression strategies. Generated *P. pastoris* strains, harbouring *Tv*DAO were screened for oxidase activity with a high throughput enzyme-coupled colorimetric assay. Several transformants, representing the activity landscape, were chosen for an additional rescreening to confirm initial activity measurements. Copy numbers of interesting transformants were determined using RT-PCR. Finally, the best strains were selected for large scale cultivation in 1.5 L bioreactors and analyzed in more detail.

3 Materials and Methods

3.1 Instruments and devices

3.1.1 Centrifuges

Centrifuge 5810 R: Eppendorf AG, Hamburg, DE

Centrifuge 5415 R: Eppendorf AG, Hamburg, DE

Avanti J-20 XP: Beckman Coulter Inc, Fullerton, CA, USA

3.1.2 Shaker

Thermomixer comfort: Eppendorf AG, Hamburg, DE (3mm)

Titramax 1000: Heidolph Instruments, Schwabisch, DE (1,5mm)

Multitron II: Infors AG, Bottmingen-Basel, CH (25mm)

RS 306: Infors AG, Bottmingen-Basel, CH (50mm)

Certomat® BS1: Sartorius AG, Göttingen, DE (25 mm)

3.1.3 Thermocycler

GeneAmp® PCR System 2700: Applied Biosystems, Foster City, CA, USA

GeneAmp® PCR System 2400: Perkin Elmer Inc, Wellesly, USA

3.1.4 Platereader and photometer

Spectramax Plus 384: Molecular Devices, Ismaning/München, DE

DU 800 Spectrophotometer: Beckman Coulter Inc, Fullerton, CA, USA

Specord 205 Double Beam Spectrophotometer: Analytik Jena AG, Jena, DE

BioPhotometer: Eppendorf AG, Hamburg, DE

3.1.5 Microtiter and deep well plates

PS-Microplate 96-well, flat bottom: Greiner Bio.One GmbH, Frickenhausen, DE

PS-Microplate 384 well, flat bottom: Greiner Bio.One GmbH, Frickenhausen, DE

UV-Star, Plate 96-well, flat bottom: Greiner Bio.One GmbH, Frickenhausen, DE

PS-Microplate 96-well, V-shape: Greiner Bio.One GmbH, Frickenhausen, DE

96-well footprint deep well plate: Bel-Art Products, Pequannock, NJ, USA

3.1.6 Pipettes and devices

Denville XL 3000i (XL2, XL20, XL200, XL1000): Denville Scientific Inc, Westbourne, UK

Biohit Proline® single-channel electronic pipettor 0.2-10 µl: Biohit Plc., Helsinki, FI

Biohit Proline® multichannel electronic pipettor, 8 ch 5-100 µl, 8 ch 50-1200 µl, 12 ch 50-1200 µl: Biohit Plc., Helsinki, FI

Matrix Multichannel Electronic Pipette 15-1250 µl: Thermo Scientifc Inc, Waltham, USA

Easypet Pipetting Aid: Eppendorf AG, Hamburg, DE

Pipette tips 200: Greiner Bio-One GmbH, Frickenhausen, DE

Pipette tips 1000: Greiner Bio-One GmbH, Frickenhausen, DE

Pipette tips, micro P10: Greiner Bio-One GmbH, Frickenhausen, DE,

Biohit®Tips 300 µl: Biohit Plc., Helsinki, FI

Biohit®Tips 1200 µl: Biohit Plc., Helsinki, FI

3.1.7 Electrophoresis

PowerPacTM Basic power supply: Bio-Rad Laboratories, Vienna, AT

Sub-cell GT: Bio-Rad Laboratories, Vienna, AT

PowerEase 500 power supply: Invitrogen Corporation, Carlsbad, CA, USA

XCell SureLock Mini-Cell: Invitrogen Corporation, Carlsbad, CA, USA

NuPAGE® Novex 4-12 % BisTris Gel 1.0 mm, 15 well: Invitrogen Corporation, Carlsbad, CA, USA

3.1.8 Fermentation

FermProbe® pH Electrode: Broadley James Corporation, Irvine, CA, USA

OxyProbe® D100 Series Oxygen Sensor: Broadley James Corporation, Irvine, CA, USA

1.5-L febatch-pro® bioreactor system: DASGIP AG, Juelich, DE

3.1.9 Additional Instruments and devises

MicroPulser™: Bio-Rad Laboratories, Vienna, AT

Vortex-Genie 2: Scientific Industries Inc, Bohemia, NY, USA

InoLab® pH720 pH-meter: WTW, Weilheim, DE

Half-micro cuvettes: Greiner Bio-One GmbH, Frickenhausen, DE

Electroporation cuvettes 2mm, blue cap: Cell Projects, Kent, UK

GP3202 Precision Balance: Sartorius AG, Göttingen, DE

ABS 220-4 analytical balance: Kern & Sohn GmbH, Balingen, DE

3.2 Strains, plasmids and primers

3.2.1 *Escherichia coli* strains

DH5α-T1^{R}: F- φ80*lac*ZDM15, Δ(*lac*Z|YA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk^{-}, mk^{+}) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A

Invitrogen Corporation, Carlsbad, CA, US: BT4689

Top10F´: F'(lacI^{q}, Tn10(Tet^{R})) *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) f80 *lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ (*ara-leu*)7679 *gal*U *gal*K *rps*L(Str^{R}) *end*A1 *nup*G

Invitrogen Corporation, Carlsbad, CA, US : BT1482

3.2.2 *Pichia pastoris* strains

Mut^{S}7: Mut^{S} strain derived from *Pichia pastoris* CBS 7435 (BT3132)

CBS 7435: Wildtype Mut^{+}, BT3132

WT1: *Pichia pastoris* strain containing native *Tv*DAO, kindly provided by Ingenza Ltd. (Roslin, UK)

3.2.3 Plasmids

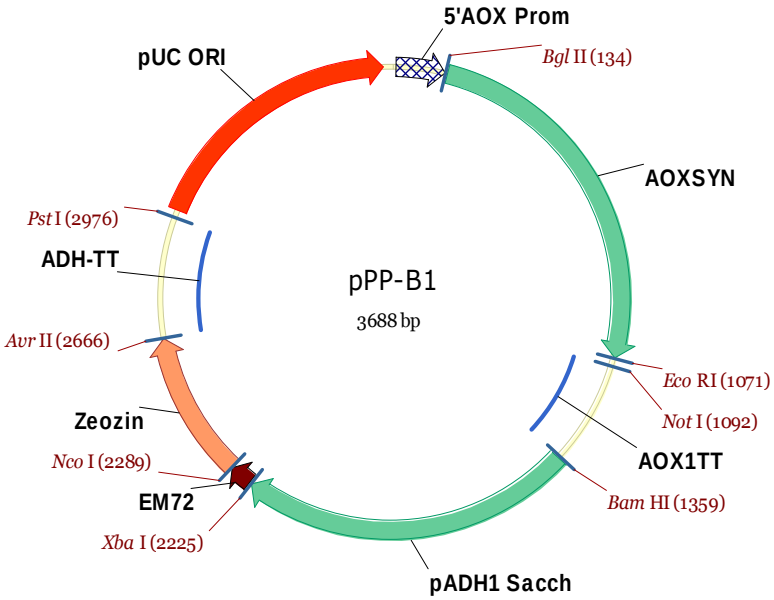

Figure 8: B1 (pPpB1) - *E. coli* / *P. pastoris* shuttle vector kindly provided by Claudia Ruth (IMBT): P_AOX1_Syn_dBamHI: 5'-extension of the standard *AOX1* promoter, P_AOX1_Syn: synthetic *AOX1* promoter, AOX1TT_Syn: synthetic *AOX1* transcription termination sequence, P_ADH1 Sacch: *Saccharomyces. cerevisiae ADH1* (alcohol dehydrogenase) promoter, P_EM72_Syn: synthetic bacterial promoter EM72, Zeocin_Syn: synthetic ZeocinTM resistance gene, ADHTT: *Saccharomyces. cerevisiae ADH1* (alcohol dehydrogenase) transcription termination sequence, pUC ORI: origin of replication for *E. coli*

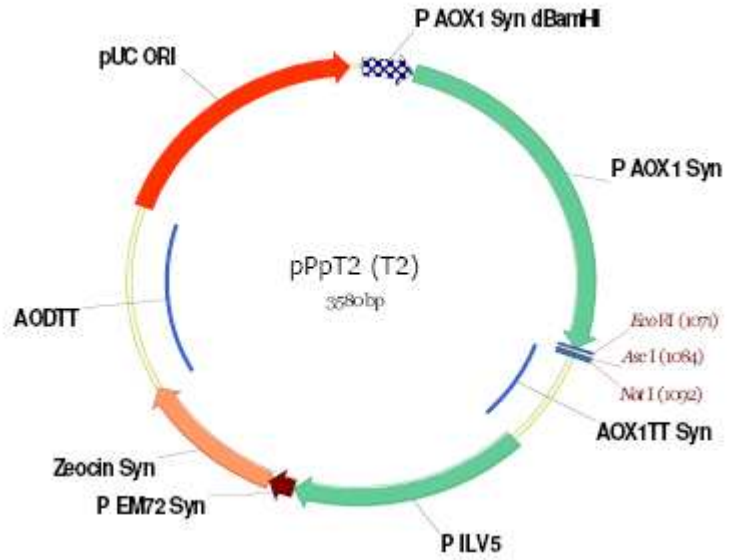

Figure 9: T2 (pPpT2, BT 5709) - *E. coli* / *P. pastoris* shuttle vector kindly provided by Claudia Ruth (IMBT): P_AOX1_Syn_dBamHI: 5'-extension of the standard *AOX1* promoter, P_AOX1_Syn: synthetic *AOX1* promoter, AOX1TT_Syn: synthetic *AOX1* transcription termination sequence, P_ILV5: *P. pastoris ILV5* (acetohydroxyacid reductoisomerase) promoter, P_EM72_Syn: synthetic bacterial promoter EM72, Zeocin_Syn: synthetic Zeocin^{TM} resistance gene, AODTT: *P. pastoris AOD* (alternative oxidase) transcription termination sequence, pUC ORI: origin of replication for *E. coli*

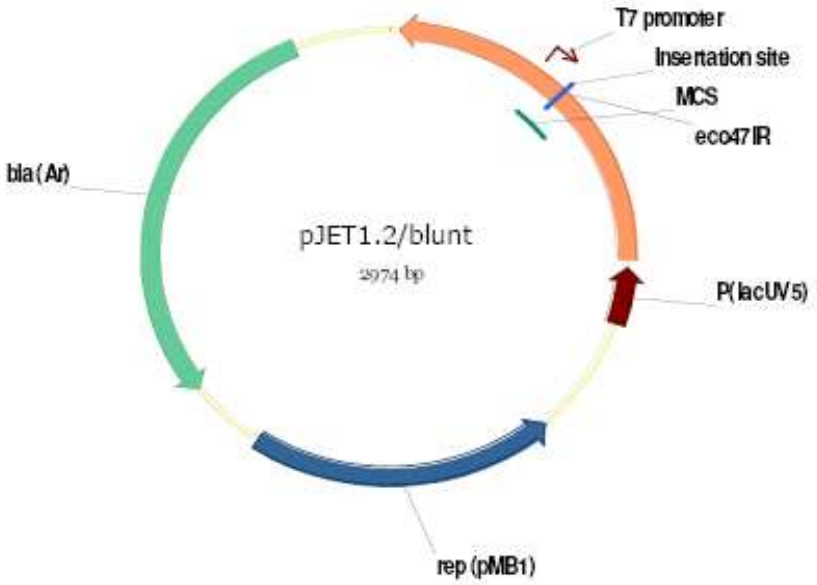

Figure 10: pJET1.2/blunt cloning vector (Fermentas Inc., Glen Burnie MA, USA): *bla*(Ar): β-lactamase gene conferring Ampicillin resistance; rep(pMB1): replicon for plasmid replication in *E. coli*; P(lacUV5): modified Lac promoter for IPTG independent *eco*47*IR* gene expression; *eco*47*IR*: lethal gene for positive selection of transformants bearing an insert; MCS: multiple cloning site containing different restriction sites; T7 promoter: T7 RNA polymerase promoter for *in vitro* transcription of the cloned insert

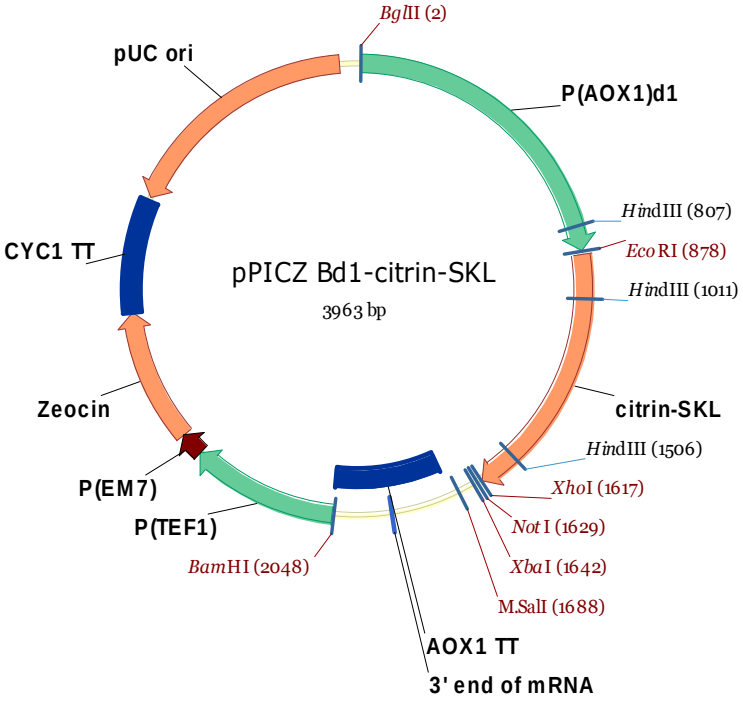

Figure 11 pPICZ-B-Δ1 (BT5038), vector kindly provided by Andrea Mellitzer: P(AOX1)d1: P(*AOX1*)d1 promoter region (deleted basepairs: 166-231), citrine: fluorescence protein, *AOX1* TT: transcription termination region, *S. cerevisiae TEF1* (transcription elongation factor 1) promoter, P(EM7): synthetic prokaryotic promoter region EM7, Zeocin resistance, *CYC1* TT: *t*ranscription termination *S. cerevisiae*, ColE1: replication origin for *E. coli*

3.2.4 Primers

Primers were purchased from Invitrogen Corporation (Carlsbad, CA, USA) or IDT Integrated DNA Technologies BVBA (Leuven, Belgium).

Table 4: Primers used during this work

No.	Name	Sequence (5' --> 3')
P06-117	P(AOX1)-F	agatctaacatccaaagacgaaagg
P07-659	P(AOX1)-Linker1-R	cgtgaagtcctcgtttcgaagtacctag-ttagttgtttttgatcttctcaag
P07-667	Linker1-alpha-F	ctaggtacttcgaaacgaggacttcacg-atgagattcccatctattttcaccg
P08-295	Linker2-AOX1TT-F	gtcagatagcgaggtcactcagtctcaagaggatgtcagaatgcc
P08-296	ADHTT-R	ctgcagttgtcctctgaggacataaaatac
P08-297	TEV-Cit-F	gaaaatttatattttcaaggtcaatttatggctagcaaaggagaagaac
P08-298	Cit-Linker2-R	gactgagtgacctcgctatctgacttacagcttggacttgtacaattc
P08-299	Linker1-Cit-F	ctaggtacttcgaaacgaggacttcacgatggctagcaaaggagaagaac
P08-300	Cit-TEV-R	aaattgaccttgaaaatataaattttccttgtacaattcatccatgccatg
P08-301	Linker1-synTvDAO-F	ctaggtacttcgaaacgaggacttcacgatggctaagattgtcgttatc
P08-302	synTvDAOper-Linker2-R	gactgagtgacctcgctatctgacttacagcttggatctggtcaaag
P08-303	Linker1-natTvDAO-F	ctaggtacttcgaaacgaggacttcacgatggctaaaatcgttgttattg
P08-304	natTvDAOper-Linker2-R	gactgagtgacctcgctatctgacctacagcttggaacgagtaagag
P08-305	synTvDAO-TEV-R	aaattgaccttgaaaatataaattttccaagtttggtctggtcaaagc
P08-306	TEV-synTvDAO-F	gaaaatttatattttcaaggtcaatttgctaagattgtcgttatcggtg
P08-307	alpha-synTvDAO-F	gagaagagagaggccgaagctgctaagattgtcgttatcg
P08-308	alpha-synTvDAO-R	ccgataacgacaatcttagcagcttcggcctctctcttct
P08-309	synTvDAOsec-Linker2-R	gactgagtgacctcgctatctgacttatctggtcaaagctctctc
P08-310	alpha-natTvDAO-F	gagaagagagaggccgaagctgctaaaatcgttgttattg
P08-311	alpha-natTvDAO-R	ccaataacaacgattttagcagcttcggcctctctcttc
P08-312	natTvDAOsec-Linker2-R	gactgagtgacctcgctatctgacctaacgagtaagagctctttc
P08-313	Linker1-synRgDAO-F	ctaggtacttcgaaacgaggacttcacgatgcactctcagaaacgtgtc
P08-314	synRgDAO-Linker2-R	gactgagtgacctcgctatctgacttacagcttggactctctagcg
P08-315	alpha-synCrLAO-F	gagaagagagaggccgaagctgctgacgatagaaaccctc
P08-316	alpha-synCrLAO-R	agagggtttctatcgtcagcagcttcggcctctctcttc
P08-317	synCrLAO-Linker2-R	gactgagtgacctcgctatctgacttaaagttcgttgtcgttag
P08-318	Linker1-synCrLAO-F	ctaggtacttcgaaacgaggacttcacgatgaatgttttctttatgttc
P08-319	Linker1-natAnMAO-F	ctaggtacttcgaaacgaggacttcacgatgacctcccgtgacggttac
P08-320	natAnMAOper-Linker2-R	gactgagtgacctcgctatctgactcacagcttggacttcacctcc
P08-321	Linker1-synAnMAO-F	ctaggtacttcgaaacgaggacttcacgatgacttctagagatggttatc
P08-322	synAnMAO-TEV-R	aaattgaccttgaaaatataaattttccaatcttgctttaacttctctc
P08-323	TEV-synAnMAO-F	gaaaatttatattttcaaggtcaatttcttctagagatggttatcaatg
P08-324	synAnMAOper-Linker2-R	gactgagtgacctcgctatctgacttacagcttggatttaacttctc
P08-325	alpha-synAnMAO-F	gagaagagagaggccgaagctacttctagagatggttatc
P08-326	alpha-synAnMAO-R	tgataaccatctctagaagtagcttcggcctctctcttctc
P08-327	synAnMAOsec-Linker2-R	gactgagtgacctcgctatctgacttatttaacttctctcttggtac
P08-328	alpha-natAnMAO-F	gaagagagaggccgaagctatgacctcccgtgacggttac
P08-329	alpha-natAnMAO-R	gtaaccgtcacgggaggtcatagcttcggcctctctcttc
P08-330	natAnMAOsec-Linker2-R	gactgagtgacctcgctatctgactcacttcacctccctcttcgttccc
P08-360	Strep-synTvDAO-F	atgtggagccacccgcagttcgaaaaaatcgaagggcgcgctaagattgtcgttatc
P08-361	Linker1-Strep-R	gcgcccttcgatttttcgaactgcgggtggctccacatcgtgaagtcctcgtttcgaag

P08-362	Strep-natTvDAO-F	atgtggagccacccgcagttcgaaaaaatcgaagggcgcgctaaaatcgttgttattg
P08-363	NdeI-Strep-F	gatgtgcatatgtggagccacccgcagttc
P08-364	synTvDAO-BamHI-R	ttggatcccagcttggatctggtcaaagc
P08-365	natTvDAO-BamHI-R	ttggatccacgagtaagagctctttcgac
P08-366	Strep-synRgDAO-F	atgtggagccacccgcagttcgaaaaaatcgaagggcgccactctcagaaacgtgtc
P08-367	synRgDAO-BamHI-R	ttggatcccagcttggactctctagcggc
P08-368	Strep-Linker2-F	tggagccacccgcagttcgaaaaaatcgaagggcgctaagtcagatagcgaggtcactc
P08-369	synRgDAO-Strep-R	gcgcccttcgatttttcgaactgcgggtggctccacagcttggactctctagcggcac
P08-370	NdeI-synRgDAO-F	gatgtgcatatgatgcactctcagaaacg
P08-371	Strep-BamHI-R	ttggatccgcgcccttcgatttttcgaac
P08-441	Seq-Lcit-rv	tccgtatgtagcatcaccttcaccctctc
P08-442	Seq-CitL-fw	gccctgtccttttaccagacaaccattac
P08-503	Alpha-natTvDAO-XhoI-F	aactcgagaagagagaggccgaagc
P08-504	Alpha-natTvDAO-SpeI-R	aaactagtctaacgagtaagagctctttc
P08-507	SpeI-natTvDAO-F	actagtgaaacgatggctaaaatcgttg
P08-508	natTvDAOper-AscI-R	ggcgcgccctacagcttggaacgagtaag
P08-509	EcoRI-synTvDAO-F	gaattcgaaacgatggctaagattgtcg
P08-510	Cit-NotI-R	gcggccgcttacagcttggacttg
P08-511	EcoRI-Cit-F	gaattcgaaacgatggctagcaaaggag
P08-512	synTvDAOper-NotI-R	gcggccgcttacagcttggatctgg
P08-513	EcoRI-alpha-F	gaattcgaaacgatgagattcccatctattttc
P08-514	synTvDAOsec-NotI-R	gcggccgcttatctggtcaaagctctctc
P08-515	EcoRI-synRgDAO-F	gaattcgaaacgatgcactctcagaaacg
P08-516	synRgDAO-NotI-R	gcggccgcttacagcttggactctctagc
P08-551	natTvDAO PNL-AscI-R	ggcgcgccctaaaggtttggacgagtaag
P08-552	synTvDAO PNL-NotI-R	gcggccgcttaaaggtttggtctggtc

3.3 Chemicals and media

Unless otherwise stated all media were autoclaved at 121°C for 20 minutes. All antibiotic stock solutions were filter sterilized.

3.3.1 *E. coli* media

LB (Low Salt Luria Bertani) Agar: 35 g/L LB-agar (Lennox) (Roth) were dissolved in distilled H2O and autoclaved. If needed, antibiotics were added after autoclaving and cooling down to ~50°C to a final concentration of 100 µg/mL Ampicillin and 50 µg/mL Zeocin™ respectively.

SOC: 20 g/L BactoTM Tryptone, 0.58 g/L NaCl, 5 g/L BactoTM Yeast Extract, 2 g/L, $MgCl_2$, 0.18 g/L KCl, 2.46 g/L $MgSO_4$, 3.81 g/L α-D(+)-Glucose monohydrate were dissolved in distilled H_2O and autoclaved.

3.3.2 *P. pastoris* media

YPD: 10 g Bacto™ Yeast Extract, 20 g Bacto™ Pepton, 900 mL dH_2O. After autoclaving and cooling down to ~50°C 100 mL/L 10xD were added. If needed, antibiotic was added to final concentrations of 100 µg/mL Zeocin™.

YPD Agar: YPD medium was prepared as described above but 15 g/L Bacto™ Agar was added before autoclaving.

BMD1 (1 % D-Glucose): 200 mL 10xPPB, 100 mL 10xYNB, 50 mL 10xD, 2 mL 500xB, 650 mL dH_2O

BMM2 (1 % MeOH): 200 mL 10xPPB, 100 mL 10xYNB, 10 mL Methanol, 2 mL 500xB, 700 mL dH_2O

BMM10 (5 % MeOH): 200 mL 10xPPB, 100 mL 10xYNB, 50 mL Methanol, 2 mL 500xB, 640 mL dH_2O

BMGY: 10 g/L yeast expract, 20 g/L peptone, 100 mM PPB (pH 6.0), 13.4 g/L YNB, 10 g/L glycerol

Modified basal salt medium: 40 g/L glycerol, 0.17 g/L $CaSO_4$, 2.32 g/L $MgSO_4 \cdot 7H_2O$, 2.86 g/L K_2SO_4, 17 g/L 85 % H_3PO_4, 2.56 g/L KOH, 0.22 g/L NaCl, 0.6 g/L EDTA. After autoclaving and cooling down 0.8 mL/L antifoam and 4.35 mL/L PTM1 trace element solution were added filter sterilized.

PTM1: 0.2 g/L Biotin, 6 g/L $CuSO_4 \cdot 5H_2O$, 93.33 mg/L KI, 3.067 g/L $MnSO_4 \cdot H_2O$, 0.2 g/L $Na_2MoO_4 \cdot 2H_2O$, 20 mg/L H_3BO_3, 0.916 g/L $CoCl_2 \cdot 6H_2O$, 42.2 g/L $ZnSO_4 \cdot 7H_2O$, 65 g/L $FeSO_4 \cdot 7H_2O$, 3.75 mL/L H_2SO_4

Antifoam: ACEPOL 83 E (Lubrizol GmbH, DE)

3.3.3 Buffers and solutions

Sterile water: purchased from Freseinus Kabi, Graz, Austria

1 M DTT: 1.54 g dithiothreitol/10 mL dH2O, filter sterilized

BEDS solution: 10 mM bicine-NaOH, pH 8.3, 3 % (v/v) ethylene glycol, 5 % (v/v) DMSO, 1 M sorbitol, filter sterilized

500xB: 0.02 % (w/v) d-biotin, filter sterilized

10xD: 220 g/L a-D(+)-glucose monohydrate were dissolved in distilled H_2O and autoclaved.

10xG: 100 g/L glycerol were dissolved in distilled H_2O and autoclaved.

10xYNB: 134 g/L DifcoTM Yeast Nitrogen Base w/o Amino Acids were dissolved in distilled H_2O and autoclaved.

10xS: 200 g/L D-sorbitol were dissolved in distilled H_2O and autoclaved.

10xPPB: 1M K2HPO4 and 1 M KH_2PO_4 were mixed to pH 6.0 and autoclaved.

Y-Per™: Yeast Protein Extraction Reagent # 78990, Pierce Biotechnology Inc., Rockford, IL, USA

YeastBuster™: Protein Extraction Reagent # 71186-3, Novagen, Merck, Darmstadt, DE

dNTP mix: dATP, dTTP, dCTP, dGTP were mixed to a concentration of 10 mM each.

3.4 Enzymes

3.4.1 Restriction enzymes

Restriction enzymes were purchased either as conventional or FastDigest™ enzymes from MBI Fermentas. Digestion with conventional enzymes was performed as recommended by the producer using the unique five buffer system with color coded tubes and digestion with FastDigest™ enzymes was performed in 1x FastDigest™ buffer.

The concentration of the conventional enzymes was 10 U/µL and those of the FastDigest™ enzymes was 1 FDU/µL. 1 unit (U) was defined as the amount of enzyme required to digest 1 µg of λ-DNA in 1 hour at 37°C in 50 µL of recommended reaction buffer and 1 FastDigest™ Unit (FDU) was the amount of enzyme required to digest 1 µg of λ-DNA in 5 min at 37°C in 50 µL FastDigest™ buffer. A list of the used restriction enzymes and their recognition sites can be found in Table 5.

Table 5: Restriction enzymes and their recognition site

Enzyme	Recognition site
*Eco*RI	5´-G^AATTC-3´
*Not*I	5´-GC^GGCCGC-3´
*Bgl*II	5´-A^GATCT-3´
*Spe*I (*Bcu*I)	5'-A^CTAGT-3'
*Asc*I (*Sgs*I)	5'-GG^CGCGCC-3'
*Xho*I	5'-C^TCGAG-3'

3.4.2 Polymerases

The following polymerases were used in PCR according to the supplied instructor manuals:

Phusion™ High-Fidelity DNA Polymerase, 2 U/µl: Finnzymes Oy, Espoo, FI

GoTaq^{R} Polymerase, 5 U/µL: Promega Corporation, Madison, WI, USA

DreamTaq, 5 U/µL: Fermentas Inc., Glen Burnie, MA, USA

PfuUltra, 2.5 U/µL: Stratagene Corporation, La Jolla, CA, USA

3.4.3 Other enzymes

T4 DNA Ligase, 5 U/µl: Fermentas Inc, Glen Burnie, MA, USA

Calf Intestine Alkaline Phosphatase (CIAP), 1 U/µL: Fermentas Inc, Glen Burnie, MA, USA

Power SYBR^{TM} Greeen Master Mix, 2x: Applied Biosystems, Foster City, CA, USA

3.5 Software and webtools

3.5.1 Software

EditSeq 5.02: DNASTAR Inc., Madison, WI, USA

GeneDesigner 1.1.2: DNA2.0, Menlo Park, CA, USA

Leto 1.0: Entelechon GmbH, Regensburg, DE

PyMOL^{TM}: DeLano Scientific LLC, Palo Alto, CA, USA

SeqMan 5.01: DNASTAR Inc., Madison, WI, USA

Simple Primer Tool V0.1: ©Günther Gruber

SoftMax Pro 4.8: Molecular Devices, Ismaning/München, DE

Vector NTI Advance 10.0: Invitrogen Corporation, Carlsbad, CA, USA

3.5.2 Webtools

ClustalW - multiple sequence alignment: www.ebi.ac.uk/Tools/clustalw2/index.html

Compute pI/Mw – Computation of theoretical pI/Mw: www.expasy.org/tools/pi_tool.html

GeneBee - Secondary RNA structure: www.genebee.msu.su/services/rna2_reduced.html

MultAlin - multiple sequence alignment: prodes.toulouse.inra.fr/multalin/multalin.html

NetNGlyc 1.0 - Prediction of N-glycosylation sites: www.cbs.dtu.dk/services/NetNGlyc/

NetOGlyc 3.1 - Prediction of O-glycosylation sites: www.cbs.dtu.dk/services/NetOGlyc/

Phyre - Protein homology modeling: www.sbg.bio.ic.ac.uk/~phyre/

Prosite 20.33 - Database of protein domains, families and functional sites: expasy.ch/prosite/

PSIPRED 2.6- protein secondary structure prediction: bioinf.cs.ucl.ac.uk/psipred/

PSORTII - Prediction of protein subcellular localization: www.psort.org/

PTS1 - Prediction of peroxisomal targeting signal 1 containing proteins: mendel.imp.ac.at/mendeljsp/sat/pts1/PTS1predictor.jsp

SignalP 3.0 –Signal peptide cleavage sites prediction: www.cbs.dtu.dk/services/SignalP/

TargetP 1.1 – Prediction of subcellular location: www.psort.org/

3.6 Kits and protocols

3.6.1 PCR and overlap extension PCR

The components and the temperature program of a standard PCR can be found in Table 6 and Table 7. The total volume of the PCR mixtures was 50 µL.

Table 6: Components of a standard PCR mixture

Component	Volume [µL]
5x Phusion HF Buffer	10
dNTP (2 mM)	5
forward primer (5 pmol/µL)	2
reverse primer (5 pmol/µL)	2
template DNA (10-30 ng/µL)	1-3
Phusion™ Polymerase (2 U/µL)	0.3
ddH_2O	add to 50 µL

Table 7: Standard PCR temperature program used during this work

Temperature [°C]	Time	
98	30 s	
98	10 s	35 cycles
58	15 s	
72	<2 kb: 1 min 30 s >2 kb: 30 s per kb	
72	7 min 00 s	
4	∞	

The length of the overlapping regions of two fragments linked in an oePCR was chosen to match a melting temperature of 70°C which was calculated by the Simple Primer Tool V0.1 but they were at least 20 bp long. In oePCRs, the fragments were employed in equimolar amounts. 1-10 ng of the smallest fragment were applied. First 15-20 PCR cycles were

performed without primers and the described temperature program. Then another 20 µL of a PCR mixture (Table 8) without template but with primers were added. The temperature program of the second step was the same as for the first one but the number of cycles was varied between 20 and 30 depending on the amount of DNA needed.

Table 8: Mixture added for second step of oePCR

Component	Volume [µL]
5x Phusion HF Buffer	4
dNTP (2 mM)	2
forward primer (5 pmol/µL)	2
reverse primer (5 pmol/µL)	2
Phusion™ Polymerase (2 U/µL)	0.3
ddH_2O	10

3.6.2 Plasmid isolation and DNA purification

GeneJET™ Plasmid Miniprep Kit (Fermentas Inc, Glen Burnie, MA, USA)

An *E. coli* colony was streaked out evenly with a sterile toothpick on a selective media plate which was incubated overnight at 37°C. Cell material from a quarter to half of an agar-plate was abraded with a sterile toothpick and resuspended in resuspension buffer. The DNA was eluted with 50 µL of distilled water.

QIAquick Gel extraction kit (Qiagen N.V., Venlo, NL)

The Spin Protocol was performed strictly as described in the manual. The amounts of distilled water for elution were varied from 15 to 30 µL according to the desired end volume.

QIAquick PCR-purification kit (Qiagen N.V., Venlo, NL)

The Spin Protocol was performed strictly as described in the manual. The amounts of distilled water for elution were varied between 15 and 30 µL according to the desired end volume.

3.6.3 Molecular coloning

CloneJET™ PCR Cloning Kit (Fermentas Inc, Glen Burnie, MA, USA)

The Blunt-End Cloning Protocol was performed according to the manual of the provider. The amount of purified PCR product was varied from 1 to 8 µL depending on concentration and availability. The ligation mixture was incubated at RT for 30 min and then all of it was directly used for *E. coli* transformation.

Dephosphorylation

After linearization of vector DNA, restriction enzymes were inactivated by heating as recommended by the distributor. Subsequent dephosphorylation was done for 30 min at 37°C by adding 1 µL of CIAP. The CIAP was inactivated by heating up the mixture to 85°C for 20 min. For ligations of inserts into a dephosphorylated vector, molar ratios from 1:1 to 3:1 of insert to vector were used.

Standard Ligation

Ligation was done with 1 µL T4 DNA ligase and 1 µL 10x Ligation Buffer from MBI Fermentas in a total volume of 10 µL either over night at 16°C or at RT for 2h. A molar ratio vector/insert of 1:2 was used for rapid ligation (Formula 1).

Formula 1: Calculation of the amount of insert DNA necessary for ligation into a certain vector

$$ng\ of\ insert = \frac{kb\ size\ of\ insert}{kb\ size\ of\ vector} \cdot ng\ of\ vector$$

ng nano grams

kb kilo bases

3.6.4 *E. coli* transformation

Chemical competent cells

Transformation of *E. coli* by heat shock was done using chemical competent *E. coli* DH5α-T1^R cells. Therefore, 80 µL competent cells were incubated with either 10 µL ligation mixture or 1 µL plasmid for retransformations on ice for approximately 30 min. Afterwards cells were heat shocked for 40 sec at 42°C and regenerated in 500 µL SOC medium at 37°C and 600 rpm

for 60 min. They were plated on LB plates containing the appropriate antibiotic and incubated overnight at 37°C.

Electro competent cells

Transformation of *E. coli* by electroporation was done using electro competent *E. coli* TOP10F' cells. Therefore, 80 µL competent cells were gently mixed with 1-2 µL plasmid and transferred into pre-cooled 0,2cm electroporation cuvettes and 5 minutes incubated on ice.
Electroporation was carried out with 2.5kV (program: Ec:2). 500 µL ice cold SOC medium were added immediately as recovery medium and regeneration was accomplished at 37°C and 600 rpm for 1 hour. They were plated on LB plates containing the appropriate antibiotic and incubated overnight at 37°C.

3.6.5 *P. pastoris* transformation

Competent cell preparation and transformation of *P. pastoris* were prepared as described in the condensed protocol [88]. A single colony was inoculated in 50 mL YPD medium and grown over night in a 300 mL baffled wide-mouthed flask at 30°C and 100 rpm (Certomat® BS1, Sartorius AG). Then a 50 mL YPD main culture was inoculated to an OD_{600} of 0.25 with the appropriate amount of preculture. Cells were harvested at an OD_{600} between 0.9 and 1.3 and centrifuged for 5 min at 4000 rpm at room temperature. The pellet was resuspended in a mixture of 9 mL BEDS and 1 mL 1 M DTT and incubated for 5 min at 30°C and 100 rpm. Then the cells were centrifuged again at 4000 rpm for 5 min and after resuspension in 1 mL BEDS they were ready for transformation.
For each transformation, 80 µL of competent cells were mixed with up to 1-3 µg of linearized DNA and incubated on ice for at least 2 min. The DNA used for transformation was either a vector linearized with *Bgl*II or *Sac*I or a linear expression cassette created by oePCR. Before transformation, DNA was either purified by gel electrophoresis or by the PCR-purification kit. The total amount of DNA was varied between 0.3 and 5 µg depending on the total amount and concentration of DNA as well as on the aim to either create mainly single or multi copy strains. Electroporation was carried out with 2 kV, 200 W and 25 µF. 500 µL 10xS and 500 µL YPD were added immediately as recovery medium and regeneration was accomplished in a 15 mL Greiner tube at 30°C and 100 rpm for 2 hours. Different aliquots were plated on YPD-agar plates containing an appropriate antibiotic. Single colonies could be seen after about 2 days of incubation at 28°C.

3.6.6 Microscale cultivation in deep well plates

Microscale cultivation and expression in 96-well deep well plates was done according to Weis *et al*. [89] with some modifications. Cells were grown in 250 µL BMD1 at 28°C, 320 rpm and 80 % humidity (Multitron II, 25 mm, Infors, Bottmingern, Switzerland) for approximately 60 hours to reach the stationary growth phase and depletion of glucose. Then 250 µL BMM2 were added, resulting in a final concentration of 0.5 % methanol. About 70 and 82 hours after the start of the cultivation 50 µL BMM10 were added. The cultivation was stopped at approximately 108 hours, resulting in an overall induction period of ~48 hours. For harvesting the deep well plates were centrifuged at 4000 rpm and 4°C for 10 minutes. Cell lysis of the cells of each well was done by usage of 200 µL YeastBuster™ the cell debris were removed by centrifugation and the supernatant was assayed for enzyme activity in microtiter plates (see section 3.6.8). In order to store the clones, they were either replicated from the deep well plate on YPD-agar-plates containing the appropriate antibiotic or glycerol stocks were made in microtiter plates directly after the cultivation.

3.6.7 Shake flask cultivation

Precultures of cells were grown in 50 mL shake flasks. Therefore 5 mL BMD 1 medium was inoculated with one colony and grown for 48 hours at 28°C, 160 rpm and 80% humidity (Multitron II, 25 mm, Infors, Bottmingern, Switzerland) until stationary phase was reached. 50 mL BMD1 was inoculated with the preculture to an OD_{600} of 0.5. Cultures were grown in 250 mL baffled shake flasks at 28°C, 160 rpm and 80% humidity.

Methanol induction started by addition of 5 mL BMM10 which gives a methanol concentration of 1% to start expression. From that point on 500 µL methanol were fed after 10, 24 and 48 hours. Prior to the first methanol induction and prior to the harvest of the cells, aliquots of 1 mL were tahen to measure OD_{600} values. After a centrifugation step at 13,200 rpm for 5 min, cells were broken up with YeastBuster™ and oxidase activity was measured using the photometric assay described in section 3.6.8.

3.6.8 Screening

An enzyme-coupled colorimetric assay was used to determine the amino acid oxidase activity [86]. The enzyme reacts with amine (amino acid) to the corresponding α-keto acid and produces hydrogen peroxide as by product, which develops a pink colour in presence of HRP (horse radish peroxidase).

In the lab, 10 µL of appropriately diluted YeastBuster™ lysate were transferred into a mircrotiter plate. 190 µL assay solution were added and colour development was monitored at 510 nm for 5 minutes at room temperature. The composition of the assay solution was 100 mM potassium phosphate buffer (pH 7.8), 0.5 mM 2,4,6-tribromo-3-hydroxybenzoic acid, 0.75 mM 4-aminoantipyrine, 10 mM D-methionine, and 0.025 mg/mL horseradish peroxidase (type VIa; Sigma-Aldrich catalogue number P6782).

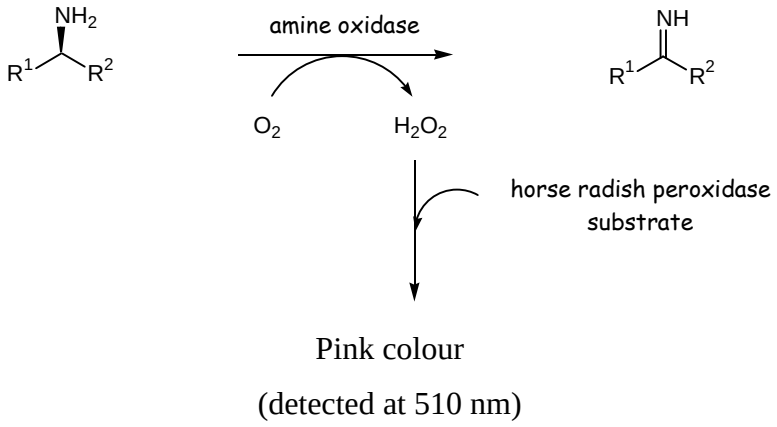

Figure 12: Oxidase activity assay

3.6.9 Protein Assay

Detergent Compatible Protein Assay (Bio-Rad, Hercules, CA, USA)

A special protein assay which is detergent compatible was applied to determine the total protein concentration. The microplate procedure was performed according to the manual of the provider with the supplied BSA as standard. 5 µL of each sample or standard were mixed with 25 µL reagent A' (20 µL reagent S / mL reagent A) and 200 µL reagent B was added. The solution was mixed thoroughly on a plate shaker for about 1 min. Then the plate was covered tightly with a plate sealer and incubated for 15 minutes at room temperature. Absorption was determined at 562 nm on a plate reader.

3.6.10 Fluorescence microscopy

Strains were cultivated as described in chapter 3.6.6. After 24 h of further methanol induction 50 µL samples were taken for fluorescence microscopy. Fluorescence microscopy was performed using an AXIOVERT 35 microscope (Carl Zeiss, Oberkochen, Germany) with filter sets no. 09 and 14 (Carl Zeiss). Hg light and filter set no. 09 was used for green fluorescent protein variants and Xenon light with filter set no. 14 for red fluorescent protein detection. The fluorescent microscop was provided by FWF (Fonds zur Förderung der wissenschaftlichen Forschung) and pictures were taken with kind permission of Ao.Univ.-Prof. Dr.phil. Hermetter A.

3.6.11 Quantitative real-time PCR for copy number determination

Quantitative real-time PCR (qRT-PCR, RT-PCR) was used to determine the number of integration of our target genes into the *P. pastoris* genome. Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used as detection dye which intercalates nonsequence specifically into dsDNA and generates a bright fluorescence. The *ARG4* gene was used as internal reference gene and a reference strain bearing a single copy of the expression cassette of interest was used for the calibration curve. The calibration curves were set using DNA amounts between 33 and 0.04 ng/reaction, calculation of the copy quantity of interest (Qty) occurred by multiplication with 94000, and the cycle threshold values (Ct) are referring to [90].

ABI PRISM 7300 Real Time PCR System (Applied Biosystems) was used for all experiments. The following oligonucleotide primers were applied in a concentration of 250 nM when using 2 ng genomic DNA of *P. pastoris* [91] as template: AOX1-fw-RT (gaagctgccctgtcttaaacctt) / AOX1-rv-RT (caaaagcttgtcaattggaacca) and ARG4-RT-fw (tcctccggtggcagttctt) / ARG4-RT-rv (tccattgactcccgttttgag). The temperature conditions were: 10min at 95°C; 40 cycles for 15 s at 95°C and 60 s at 60°C followed by a dissociation step (15 s at 95°C, 30 s at 60°C, 15 s at 95°C) at the end of the last cycle.

Genomic DNA isolation for real time PCR

Isolation was performed according to the protocol of Hoffman *et al*. [91] the gDNA isolation was performed as follows. A single colony of the desired strain was used to inoculate 50 mL YPD media and grown over night at 30°C, 80 % relative humidity and 110 rpm. 10 mL of the cell culture were harvested at an OD_{600} between 8 and 15 by a centrifugation step at 1,600 rpm for 5 min. The cells were resuspended in 0.5 mL dH_2O and transferred to a microtube. After centrifugation at maximum speed for a few seconds, cells were resuspended in 200 µL YEAST Lysis Buffer. Cell disruption occurred after addition of 200 µL phenol: chloroform: isoamylalcohol (25: 24: 1) and 0.3 g glass beads by vortexing for about 5 min. Extraction of water soluble substances occurred by 200 µL TE Buffer pH 8. After centrifugation for 5 min at maximum speed, aqueous phase was transferred into a new microtube and precipitation of the DNA occurred with 1 mL ice cold 100 % EtOH and centrifugation for 1 min at maximum speed. The resulting pellet was air-dried and than resuspended in 400 µL TE Buffer. RNAse A was added to a final concentration of 10 mg/mL and digestion occurred at 37°C for 30-60 min. To get rid of the proteins, ammonium acetate was added to a final concentration of about 0.1 M. After two washing steps (first with 1 mL 100 % EtOH and centrifugation at maximum speed for 1 min, second with 1 mL 70 % EtOH and centrifugation at maximum speed for 1 min), the pellet was air-dried and resuspended in 50 µL dH_2O.

Estimation of gDNA concentration

gDNA samples for estimation were prepared with the method described above. To determine the quality and the concentration of the DNA, absorption at 230, 260, 280 and 320 nm was measured in 96-well UV Star plates.

4 Results and Discussion

4.1 Sequence analysis and gene design

4.1.1 Strategy

D-Amino acid oxidases catalyse the oxidative deamination of D-amino acids forming hydrogen peroxide as byproduct. Therefore, an efficient targeting to the peroxisomes of *Pichia pastoris* seems promising, because its peroxisomal catalase can neutralize toxic H_2O_2. Nevertheless due to several high level expression examples in *P. pastoris* secretion to the media was set as an alternative. The inducible promoter *AOX1* was used to control expression. In addition a fusion of *Tv*DAO to citrine, a GFP variant, was generated to confirm the correct localization of the target protein.

Since Mut^S strains have shown to be more reliable in DWP and bioreactor cultivation this work focused on Mut^S strains. Furthermore Mut^S strains need less oxygen, which seemed beneficial as the target enzyme *Tv*DAO is not very stable in the presence of oxygen (bubble aeration).

Integration cassettes were generated for targeted integration into the *AOX*1 locus, which stability is widely known. Different copy numbers of integrated expression cassettes can have a dramatic effect on protein expression. Zeocin serves as selection marker, where manipulation of the number of integrated expression cassettes is easy to handle. An HTP-Screening (High Throughput-Screening) was used to identify high expressing transformants, which were further characterized using RT-PCR (real time-PCR) to determine the copy number. A *Pichia pastoris* variant called *Tv*DAO WT1 was kindly provided by Ingenza Ltd. and served as benchmark strain. This strain produced a protein that has the same substrate specificity but was more stable than the wild-type protein.

Although the main focus of this work was set on *Tv*DAO, other amino acid and amine oxidases were considered to be interesting targets as well. Therefore this chapter, the computational sequence analysis and design of synthetic genes, deals with a number of different enzymes.

In addition to the D-amino acid oxidase from *Trigonopsis variabilis* (*Tv*DAO) and another yeast source namely *Rhodotorula gracilis* (*Rg*DAO), a L-amino acid oxidase from *Calloselasma rhodostoma* (*Cr*LAO) and a monoamine oxidase from *Aspergillus niger* (*An*MAO) have been investigated (see Table 9) as well.

4.1.2 Sequence analysis

The native sequence of each gene was used for the sequence analysis. The accession number for the PubMed database can be found in Table 9. Webtools used for the following sequence analysis are listed in Table 10.

Table 9: Informations about investigated proteins

Protein	Accession #	Organism	Enzym	PDB
*Tv*DAO	AAR98816	*Trigonopsis variabilis*	D-Amino acid oxidase	Karl Gruber*
*Rg*DAO	CAA96323	*Rhodotorula gracilis*	D-Amino acid oxidase	1c0i
*Cr*LAO	CAB71136	*Calloselasma rhodostoma*	L-Amino acid oxidase	1f8r / 1f8s
*An*MAO	AAA98490	*Aspergillus niger*	Monoamine oxidase N	2vvl / 2 vvm

* unpublished

Table 10: Webtools used for sequence analysis

Webtool	Homepage
TargetP 1.1	http://www.cbs.dtu.dk/services/TargetP/
PTS1 predictor	http://mendel.imp.ac.at/mendeljsp/sat/pts1/PTS1predictor.jsp
SignalP 3.0	http://www.cbs.dtu.dk/services/SignalP/
NetNGlyc 1.0	http://www.cbs.dtu.dk/services/NetNGlyc/
Prosite (release 20.33)	http://expasy.org/tools/scanprosite/
PSORTII Prediction	http://psort.ims.u-tokyo.ac.jp/form2.html

*Tv*DAO

Although *Tv*DAO is supposed to be localized in the peroxisome, the exact targeting is not completely clear. The prediction with TargetP and PSORT II suggest that it is secreted via the ER (endoplasmic reticulum), which is sustained by the prediction with SignalP indicating that the protein has a signal peptide (between 17 and 18: TTA-LQ), but the cleavage site probability is quite low. Furthermore 2 putative N-glycosylation sites (192 NCSG, 262 NWSS) were found, but with the uncertain targeting prediction glycosylation remains doubtful.

The PTS1 predictor calculated a low prediction score for peroxisomal targeting when standard parameters (e.g. metazoa) are chosen. Whereas the fungi-specific algorithm showed that the

protein is probably not targeted into the peroxisomes. Closer observation of the amino acid sequence reveals that the native C-terminal amino acid triplet of *Tv*DAO is -PNL, which is quite different from the ideal PTS1 sequence -SKL.

*Rg*DAO

*Rg*DAO is targeted to the peroxisome with a high prediction score. But, similar to *Tv*DAO, it seems to be a mixed form, as it also has a predicted signal peptide (cleavage site between 20 and 21: SSA-LI). The predicted glycosylation site is unlikely to be actually glycosylated.
*Rg*DAO shows higher probability scores for peroxisomal targeting as compared to *Tv*DAO.

*Cr*LAO

*Cr*LAO is targeted to the secretory pathway via ER. A putative cleavage site between 18 and 19 (GSC-AD) has been recognized and the protein contains 2 putative N-glycosylation sites (190 NCSY, 379 NFTN), which are likely to be glycosylated.

*An*MAO

*An*MAO is not secreted and has no signal peptide. There is a putative glycosylation site, but because the protein is not exposed to the glycosylation-machinery these sites won't be glycosylated.
According to the PTS1 predictor tool, the protein is targeted to the persoxisome quite clearly. The result of the PSORT II prediction suggested targeting to the cytoplasm. This webtool only offers cytoplasmic, mitochondrial, nuclear, vacuolar and endoplasmic reticulum as location and as the peroxisome is a cytoplasmic organell there is no conflict in these results.

4.1.3 3D Analysis

*Tv*DAO

Unfortunately there is currently no structure available in the database for this protein, but the structure was solved by Karl Gruber's group, who kindly provided the structure and made substantial contribution during expression strategy conception.
In Karl Gruber's *Tv*DAO structure the signal peptide is not cleaved off (probably due to the expression host *E. coli*). "The resulting α-helix is involved in the protein structure and a

truncation of the signal peptide would probably lead to an inactive protein." (Karl Gruber, personal communication)

The C-terminal PNL triplet, the putative peroxisomal targeting sequence, is not visible in the 3D structure, probably due to its mobility. Concerning the fusion of the protein with a fluorescence protein (FP) the C-terminal fusion seems to be more promising, because the C-term is more accessible than the N-term. In case of the N-terminal fusion, a linker region of approximately 10 amino acids would perhaps improve the correct folding of DAO and FP. The sequence analysis showed two putative glycosylation sites (192-194: NCSG and 262-264: NWSS). The first one is located on the inside of the structure and is not likely to be glycosylated, the second one lies on the protein surface and is potentially glycosylated. As mentioned it is unlikely that the native protein is secreted and therefore exposed to the glycosylation machinery. Furthermore the structure of the homologous *Rg*DAO showed no glycosylation.

*Rg*DAO

The protein structure of *Rg*DAO with the PDB number 1c0i was analysed using the program PyMOL. In this structure (see Figure 13) the signal peptide is visible (highlighted in pink) and it is part of the protein structure. This leads to the conclusion that it is probably not cleaved off in the native protein, because a missing signal protein would lead to an inactive protein.

Similar to the Karl Gruber's structure for *Tv*DAO; the C-term consists of an alpha-helix which points to the outside.

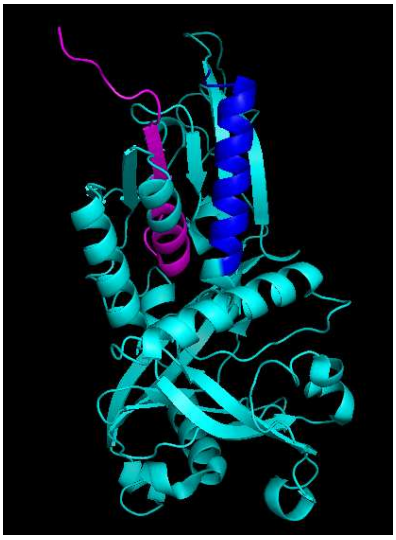

Figure 13: 3D structure of *Rg*DAO (1c0i): N-terminal signal peptide is highlighted in pink, C-term is highlighted in blue.

*Cr*LAO

The 3D structure of the protein *Cr*LAO with the PDB number 1f8r was analysed using the program PyMOL. It was found that the N-terminal signal peptide was missing in the crystallized protein. This leads to the conclusion that the native protein is actually processed and secreted. In Figure 14 the N-term of the protein is highlighted in pink, the C-term is highlighted in blue.

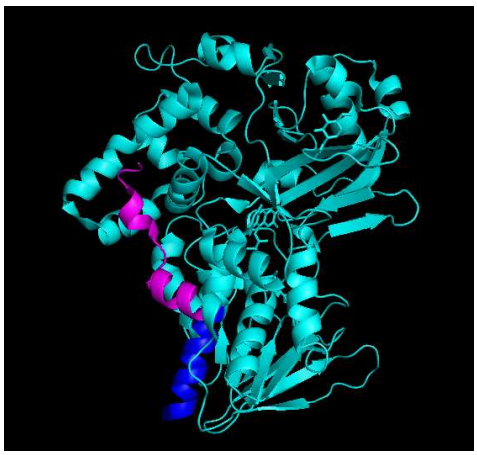

Figure 14: 3D structure of *Cr*LAO (1f8r): N-term is highlighted in pink, C-term is highlighted in blue

*An*MAO

Figure 3 shows the protein structure of *An*MAO (PDB number: 2vvm). The C-term is highlighted in yellow. Interestingly the C-term of this peroxisomal protein looks very similar to the C-term of *Tv*DAO and *Rg*DAO for which the native location is not quite clear.

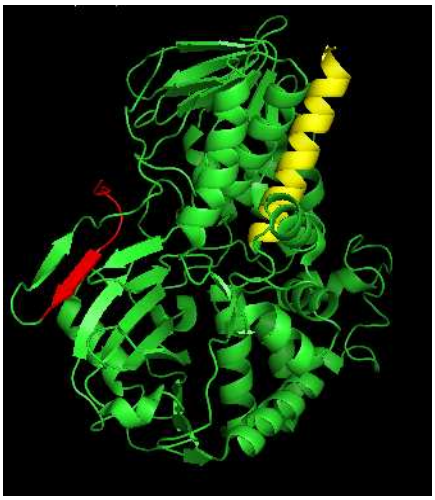

Figure 15: 3D structure of *An*MAO (2vvm): N-term is highlighted in red, C-term is highlighted in yellow

4.1.4 Synthetic gene design

The protein sequence of the codon optimized oxidase genes for expression in *P. pastoris* under conditions of methanol induction can be found at GenBank (Accession numbers see Table 9). For *An*MAO a variant of MAO-N with 5 mutations (*An*MAO 5M: I246M, N336S, M348K, T384N, D385S) has been generated, which has shown a broader substrate spectrum in *E. coli* at studies carried out by Ingenza [86].

Synthetic genes were codon optimized for high expression level in *Pichia pastoris* with the Leto 1.0 (Entelechon, Regensburg, DE) software and parameters summarized in Table 11. Therefore the high methanol-codon usage (cumulative codon usage) was used (see Table 12), which was calculated from three highly transcribed genes encoding enzymes of the *Pichia pastoris* methanol utilization pathway (alcohol oxidase 1 AOX1 [GenBank: U96967]; dihydoxyacetone synthase DAS1 [GenBank: FJ754551]; formaldehyde dehydrogenase FLD [Genbank: XP_002493270]) and from a plant gene (*Hevea brasiliensis* hydroxynitrile lyase *Hb*HNL [Genbank: U40402]) that is efficiently expressed in *P. pastoris*. Additionally internal restriction enzyme recognition sequences were eliminated using the program Gene Designer (DNA 2.0). The resulting sequence was checked with VectorNTI 10.0 (Invitrogen) to avoid internal *EcoR*I, *Not*I, *Bgl*II, *Sac*I, *Avr*II and *BamH*I restriction sites. To prevent strong stem-loop structures, the sequence was checked by GeneBee, loops with < -20 kcal/mol were classified as acceptable. All genes were ordered from GenScript Corporation (Piscataway, NJ, USA) with a 5' Kozak consensus sequence and flanking restriction sites *EcoR*I and *Not*I.

Table 11: Optimized parameters for synthetic gene design

Parameter	Limits	control
Codon Usage	*Pichia pastoris* high methanol usage, threshold 10%	Leto analysis report
Restriction sites	avoidance of following restriction sites *Avr*II (*XmaJ*I), *BamH*I, *Bgl*II, *EcoR*I, *Not*I, *Sac*I	Vector NTI
GC peaks, GC content	no peaks over 60%	Leto analysis report
A/T streches	not more than four same nucletides in a row	Gene Designer
Codon repeat	not 2 same codons near each other	Gene Designer
RNA 2° structures	strongest structure not over -20 kcal/mol and not within the first ~ 50 bp	GeneBee

Table 12: Cumulative codon usage for high expression levels in *P. pastoris*:

codon usage was calculated from the *AOX1* [92], *DAS1* [93], *FLD1* [94] and *Hb*HNL [95] genes, AA: amino-acid, N: quantity of the codon, RSCU: relative synonymous codon usage

AA	Codon	N	RSCU	AA	Codon	N	RSCU
A-Ala	GCU	82	2.17	N-Asn	AAU	16	0.43
	GCC	35	0.93		AAC	59	1.57
	GCA	31	0.82				
	GCG	3	0.08	P-Pro	CCU	43	1.69
					CCC	6	0.24
C-Cys	UGU	28	1.40		CCA	53	2.08
	UGC	12	0.60		CCG	0	0.00
D-Asp	GAU	39	0.67	Q-Gln	CAA	40	1.33
	GAC	78	1.33		CAG	20	0.67
E-Glu	GAA	61	0.94	R-Arg	CGU	17	1.21
	GAG	69	1.06		CGC	0	0.00
					CGA	1	0.07
F-Phe	UUU	27	0.62		CGG	1	0.07
	UUC	60	1.38		AGA	60	4.29
					AGG	5	0.36
G-Gly	GGU	115	2.57				
	GGC	15	0.34	S-Ser	UCU	55	2.89
	GGA	45	1.01		UCC	31	1.63
	GGG	4	0.09		UCA	13	0.68
					UCG	5	0.26
H-His	CAU	12	0.40		AGU	5	0.26
	CAC	48	1.60		AGC	5	0.26
I-Ile	AUU	61	1.56	T-Thr	ACU	54	1.86
	AUC	51	1.31		ACC	44	1.52
	AUA	5	0.13		ACA	11	0.38
					ACG	7	0.24
K-Lys	AAA	33	0.51				
	AAG	96	1.49	V-Val	GUU	67	1.94
					GUC	41	1.19
L-Leu	UUA	18	0.69		GUA	9	0.26
	UUG	68	2.62		GUG	21	0.61
	CUU	33	1.27				
	CUC	12	0.46	W-Trp	UGG	27	1.00
	CUA	4	0.15				
	CUG	21	0.81	Y-Tyr	UAU	19	0.44
					UAC	68	1.56
M-Met	AUG	37	1.00				
				Stop	UAA	3	2.25
					UAG	0	0.00
					UGA	1	0.75

4.1.5 Designed constructs

Information from the sequence analysis resumed before was used to design interesting expression constructs which were assembled via oePCR.

*Tv*DAO expression constructs

For the expression of *Tv*DAO two strategies were considered. Firstly targeting to the peroxisome and secondly secretion into the media. Secretion has the advantage, that expressed protein is exported from the cell and therefore has a lot of space in the media. In contrast the peroxisomal environment seems beneficial as it harbours catalase which converts emerging, celltoxic hydrogen peroxide. Addtionally fusions with citrine, a codon optimized GFP variant, seem interesting to prove correct targeting of the protein.

Tv1/Tv1a: Peroxisomal targeting, replacement of the native peroxisomal targeting with –SKL. Synthetic and native CDS.
Tv2/Tv3: Fusion with the fluorescent protein citrine to prove the correct targeting of the peroxisomal constructs. Synthetic CDS, C- and N-terminal fused.
Tv4/Tv4a: Targeting into the media, *Pichia pastoris* codon optimized alpha factor leader sequence from *Saccharomyces cerevisiae* has been N-terminal fused to the gene. The native peroxisomal sequence has been removed. Synthetic and native CDS.
Tv5/Tv5a: Peroxisomal targeting, native peroxisomal targeting -PNL. Synthetic and native CDS.

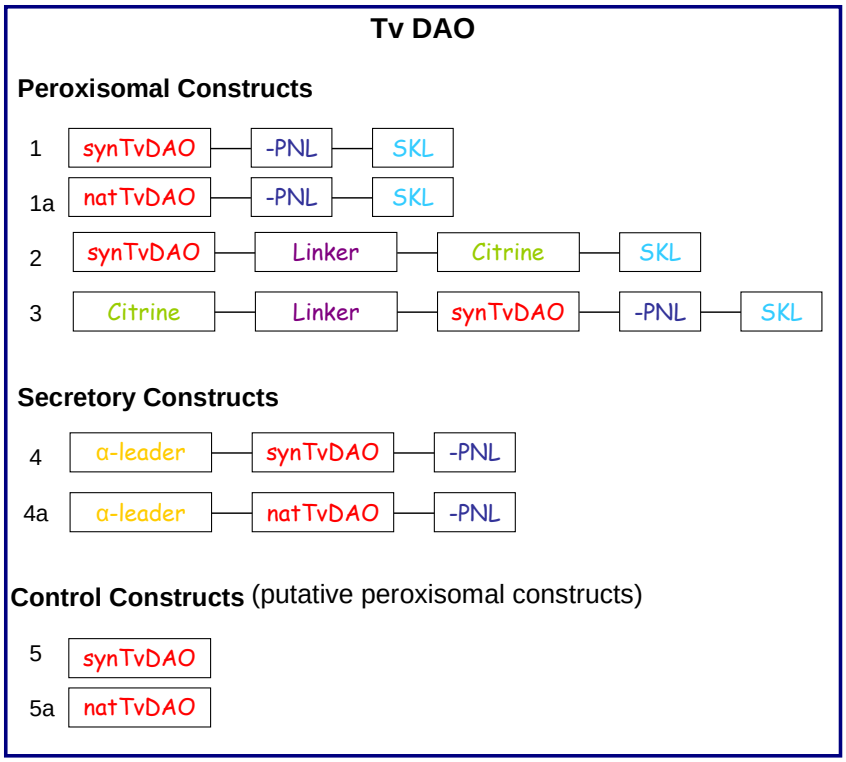

Figure 16: Scheme of the *Tv*DAO expression constructs.

1 Codon optimized synthetic *Tv*DAO gene with exchanged of native peroxisomal targeting sequence, SKL. **1a** Native *Tv*DAO with exchanged of native peroxisomal targeting sequence, SKL. **2** C-terminal citrine fusion with codon optimized synthetic *Tv*DAO and C-terminal addition of engineered peroxisomal targeting sequence, SKL. **3** N-terminal citrine fusion with codon optimized synthetic *Tv*DAO and C-terminal exchanged of native peroxisomal targeting sequence, SKL. **4** N-terminal *S. cerevisiae* α- mating factor leader sequence fusion with codon optimized synthetic *Tv*DAO and removal of native peroxisomal sequence. **4a** N-terminal *S. cerevisiae* α-mating factor leader sequence fusion with native *Tv*DAO and removal of native peroxisomal sequence. **5** Codon optimized synthetic *Tv*DAO gene with native peroxisomal targeting sequence, PNL. **5a** Native *Tv*DAO with native peroxisomal targeting sequence, PNL.

*Rg*DAO expression constructs

For *Rg*DAO the construct of the synthetic gene, which already includes a well recognized PTS1 seemed to be a promising protein expression strategy.

Rg1: Synthetic CDS

Figure 17: Scheme of the *Rg*DAO expression construct.
1 Codon optimized synthetic *Rg*DAO gene.

*Cr*LAO expression constructs

According to the sequence analysis *Cr*LAO is clearly a secreted protein. As the LAO catalyses the oxidative deamination of L-amino acids and generated hydrogen peroxide, consequently it is cell toxic. Two different secretory constructs have been designed which use either the native leader sequence or the native alpha factor leader sequence from *Saccharomyces cerevisiae*.

Cr1: Targeting to the media, exchange of the native leader sequence with the *Pichia pastoris* codon optimized alpha factor leader sequence from *Saccharomyces cerevisiae*. Synthetic CDS.
Cr2: Targeting to the media with the native leader sequence. Synthetic CDS.

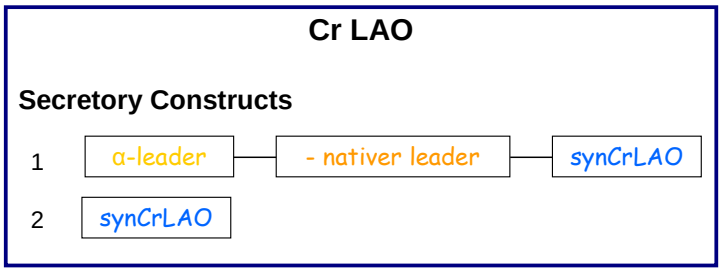

Figure 18: Scheme of the *Cr*LAO expression constructs.
1 N-terminal *S. cerevisiae* α- mating factor leader sequence fusion with codon optimized synthetic *Cr*LAO, removal of the native leader sequence. **2** Codon optimized synthetic *Cr*LAO with native leader sequence.

*An*MAO 5M expression constructs

Sequence analysis showed that *An*MAO has no signal sequence for secretion and is targeted to the peroxisome. Nevertheless expression constructs for secretion into the media were designed as this option has some advantages concerning space limitations and purification.

An1/An1a: Peroxisomal targeting, replacement of the native peroxisomal targeting with –SKL. Synthetic and native CDS.
An2/An3: Fusion with the fluorescent protein citrin to prove the correct targeting of the peroxisomal constructs. Synthetic CDS, C- and N-terminal fused.
An4/An4a: Targeting into the media, *Pichia pastoris* codon optimized alpha factor leader sequence from *Saccharomyces cerevisiae* has been N-terminal fused to the gene. The native peroxisomal sequence has been removed. Synthetic and native CDS

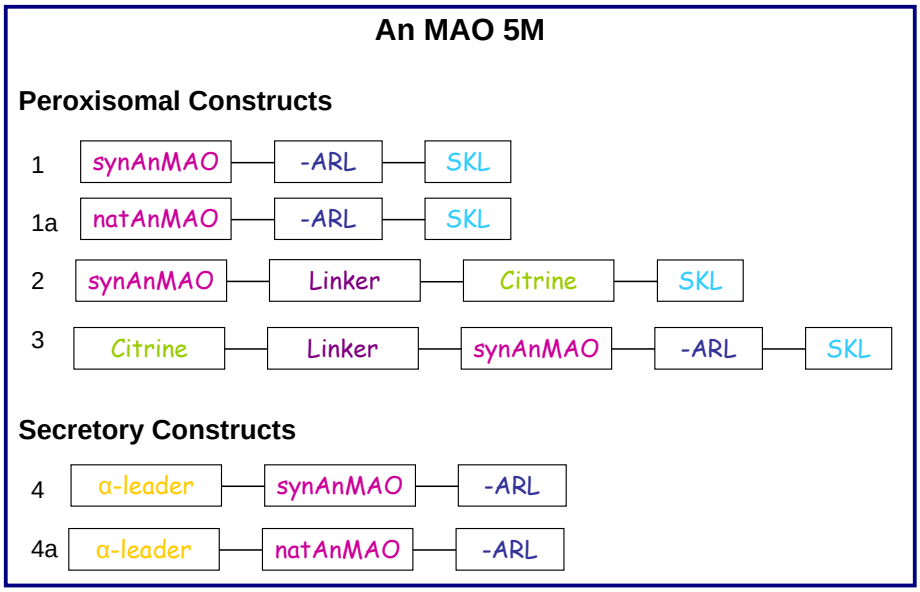

Figure 19: Scheme of the *An*MAO expression constructs.
1 Codon optimized synthetic *An*MAO gene with exchanged of native peroxisomal targeting sequence, SKL. **1a** Native *An*MAO with exchanged of native peroxisomal targeting sequence, SKL. **2** C-terminal citrine fusion with codon optimized synthetic *An*MAO and C-terminal addition of engineered peroxisomal targeting sequence, SKL. **3** N-terminal citrine fusion with codon optimized synthetic *An*MAO and C-terminal exchanged of native peroxisomal targeting sequence, SKL. **4** N-terminal *S. cerevisiae* α- mating factor leader sequence fusion with codon optimized synthetic *An*MAO and removal of native peroxisomal sequence. **4a** N-terminal *S. cerevisiae* α-mating factor leader sequence fusion with native *An*MAO and removal of native peroxisomal sequence.

4.2 TvDAO expression

In order to evaluate the impact of a protein sequence analysis for high level expression in *P. pastoris*, the designed *Tv*DAO expression constructs were generated, cultivated and screened during this thesis. The experimental evaluation of the sequence analysis of the *An*MAO, *Rg*DAO and *Cr*LAO was carried out by Sandra Abad.

In order to generate *Tv*DAO *Pichia pastoris* expression strains two strategies were chosen:
1. assembly of linear expression cassettes by oePCR. The pPP-B1 plasmid served as template for the amplification of the Zeocin resistance.
2. classic cloning approach. The gene of interest was cloned into the pPP-T2 plasmid, which uses another promoter/terminator combination for expression of the Zeocin resistance and forces single copy integrations.

The Mut^S7 strain (*P. pastoris* CBS 7435 wildtype strain displaying Mut^S phenotype) served as host strain for the generation of *P. pastoris Tv*DAO expression strains.

Generated *P. pastoris* strains were cultivated on a microscale level using deep well plates. The enzyme activity was measured with a photometric assay (described in section 3.6.8).
Randomly distributed as well as high expressing strains according to the activity landscape were chosen for rescreen to verify the results. Finally strains, with high activity and a low standard deviation, were selected for large scale cultivation in shake flasks and in a 1.5 L bioreactor.

The *Pichia pastoris* WT1 strain from Ingenza was used as benchmark and reference strain. In contrast to the wildtype *Tv*DAO sequence this strain contains 5 mutations which lead to stability improvement, while activity is not improved.

4.2.1 oePCR strategy

In a first round *Tv*DAO expression constructs were based on assembly of linear expression cassettes by oePCR. The amplified expression cassettes can be used directly for *P. pastoris* transformation. In order to obtain back-ups of the expression cassettes, the oePCR products were cloned into the commercially available pJET1.2blunt vector, which is specially designed for blunt end ligation of PCR products. The plasmids containing the *Tv*DAO expression cassette were transformed into *E. coli* DH5α. The plasmid was isolated with the GeneJET Plasmid Miniprep Kit by Fermentas and then the insert was amplificated via PCR. This linear expression cassette was used for *P. pastoris* transformation. Generated *P. pastoris* strains were cultivated in deep well plates on a microscale level. Best clones were selected with a photometric screening assay. Clones representing the landscape were chosen for a rescreen to verify the initial results. The best clones (high activity, low standard deviation) were selected for large scale cultivation in shake flasks.

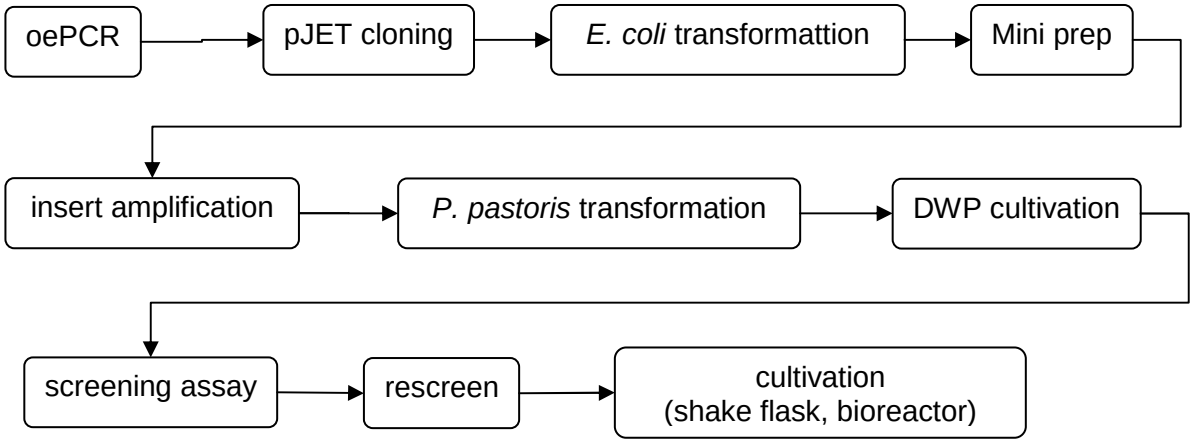

Figure 20: Flow chart of oePCR strategy

Table 13: Overview of oePCR expression constructs

Construct	Gene	Fusion	Peroxisomal targeting sequence
Tv1	synthetic	-	- SKL
Tv1a	native	-	- SKL
Tv2	synthetic	C-terminal citrin	- SKL
Tv3	synthetic	N-terminal citrin	- SKL
Tv4	synthetic	N-terminal α- factor	truncated
Tv4a	native	N-terminal α-factor	truncated

4.2.1.1 Linear expression cassettes by oePCR

Linear expression cassettes were assembled according to the standard procedure described in the introduction (chapter 1.1.5). A promoter and a selection marker fragment were linked with the gene of interest by oePCR.

The fragments needed for the assembly of the linear expression cassettes were amplified with suitable primers listed in Table 14.

Table 14: Primers to amplify fragments for the assembly of the linear expressions cassettes

Fragment		Primer fw	Primer rv	Template	Length / bp
P(AOX1)	P(AOX1)	P06-117	P07-659	pADH-ADHTT-B1[4]	956
Zeo	Zeo	P08-295	P08-296	pADH-ADHTT-B1	1903
CitL	Cit TEV	P08-299	P08-300	pPICZBd1-Citrin-SKL[5]	772
LCit	TEV Cit	P08-297	P08-298	pPICZBd1-Citrin-SKL	780
T1	synTvDAO1	P08-301	P08-302	pUC57-synTvDAO[6]	1123
T1a	natTvDAO1a	P08-303	P08-304	pRES151-DAAOWT[7]	1123
T2	synTvDAO2	P08-301	P08-305	pUC57-synTvDAO	1123
T3	synTvDAO3	P08-306	P08-302	pUC57-synTvDAO	1119
T4	synTvDAO4	P08-307	P08-309	pUC57-synTvDAO	1104
T4a	natTvDAO4a	P08-310	P08-312	pRES151-DAAOWT	1104
αT4	alpha Tv4	P07-667	P08-308	pPICZ-CALBopt[8]	315
αT4a	alpha Tv4a	P07-667	P08-311	pPICZ-CALBopt	315

Between the gene of interest and the other fragments, always the same previously optimized overlapping regions (B. Pscheid, unpublished results) were used. The sequences of the linking regions are listed in Table 15.

[4] kindly provided by Claudia Ruth
[5] kindly provided by Andrea Mellitzer
[6] synthetic gene ordered from Genescript
[7] kindly provided by Ingenza
[8] kindly provided by Beate Pscheid

Table 15: Linker regions used to assemble the linear expression cassettes

Fragments linked		Sequence (5' → 3')
promoter (AOX1)	gene of interest (*Tv*DAO constructs)	ctaggtacttcgaaacgaggacttcacg
gene of interest (*Tv*DAO constructs)	selection marker (Zeo)	gtcagatagcgaggtcactcagtc

All fragments were purified by gel electrophoresis. In the oePCR, usually 1 ng of the *Tv*DAO gene and equimolar amounts of the other fragments were used to link them as shown in Figure 21.

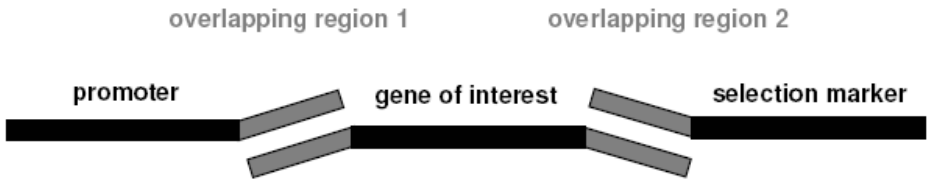

Figure 21: Schematic overview of fragments linked by oePCR to create linear expression cassettes

4.2.1.2 Expression constructs based on vector pPP-B1

The plasmid pPP-B1 served as template for Zeocin resistance in the oePCR strategy. Relevant features and sequence elements of this shuttle vectors include: origin of replication of the *E. coli* plasmid pBR322; the *AOX1* promoter (P_{AOX1}) starting with a *Bgl*II site; a multiple cloning site with unique restriction sites for *Eco*RI, *Spe*I, *Asc*I and *Not*I; the *AOX1* transcription termination sequence; and an antibiotic resistance cassette consisting of a synthetic bacterial promoter called EM72 in tandem with a truncated version of the *P. pastoris ILV5* (*acetohydroxyacid reductoisomerase*) promoter, a synthetic gene coding for the aminoacid sequence of bleomycin (ble) conferring resistance against Zeocin from *Streptoalloteichus hindustanus*. This gene had been codon optimized for function in *E. coli* as well as *P. pastoris*. In the pPpB1 vector the *Saccharomyces cerevisiae* ADH1 (alcohol dehydrogenase 1) promoter and terminator control the transcription of the *ble* gene. However, it seems that expression of the selection marker with this promoter is less efficient than with other promoters and therefore more suitable for multicopy strain generation.

The ADH1 promoter is glucose inducible, therefore directly after *P. pastoris* transformation glucose should be added to the regeneration media and incubated for 2 hours. Noteworthy is that activity landscapes of the generated clones showed several false positive transformants and rather low expression levels.

4.2.1.3 *Tv*DAO Screen

Using the oePCR strategy only three constructs could be generated: Tv1, Tv1a, Tv3. About 90 *P. pastoris* clones of each construct were cultivated on a microscale level in deep well plates. The other constructs were generated via oePCR, but no transformants were obtained. Replicas of the clones were stamped on YPD plates containing Zeocin (YPD-Zeo). In addition glycerol stocks of the clones were produced and stored at -80°C.

Analysis of the clones was carried out with a photometric screening assay to determine enzyme activity. All clones were compared to the *P. pastoris* benchmark strain WT1 from Ingenza and Mut^{S}7 as negative control.

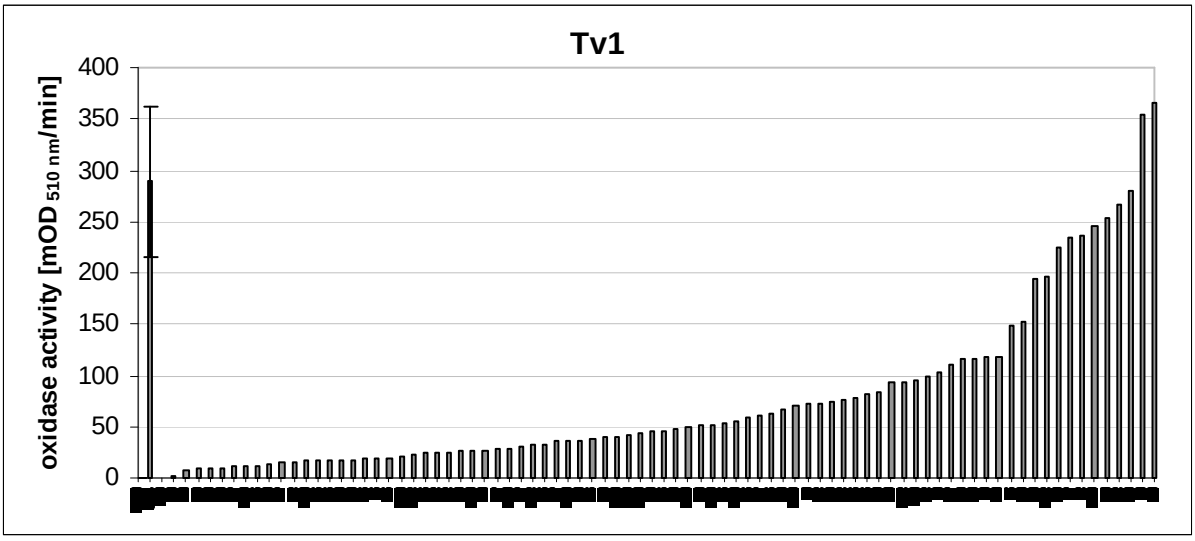

Figure 22: Activity screening landscape of Tv1 transformants

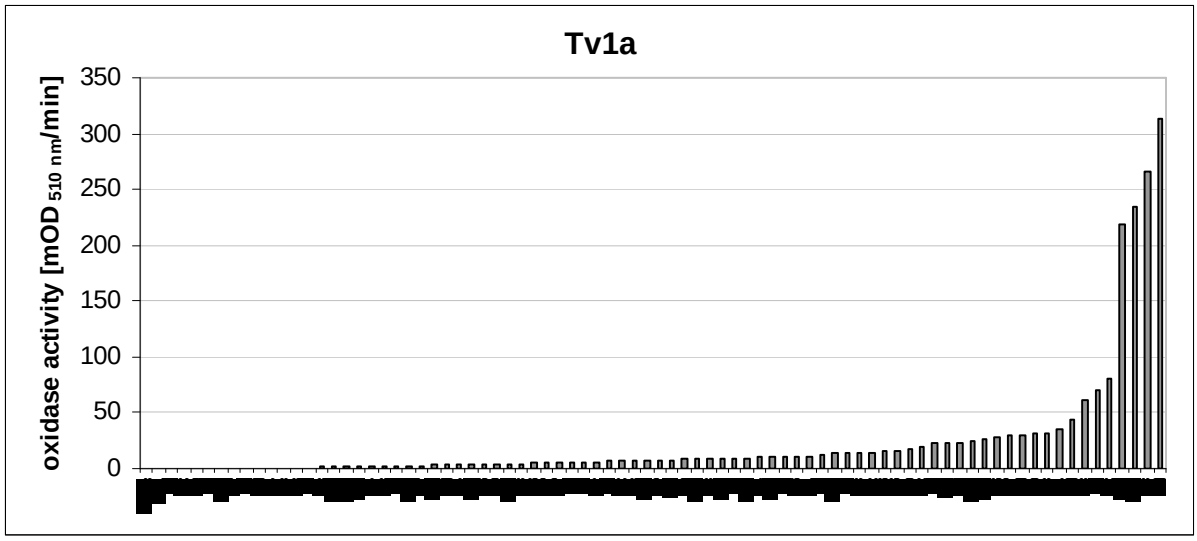

Figure 23: Activity screening landscape of Tv1a transformants

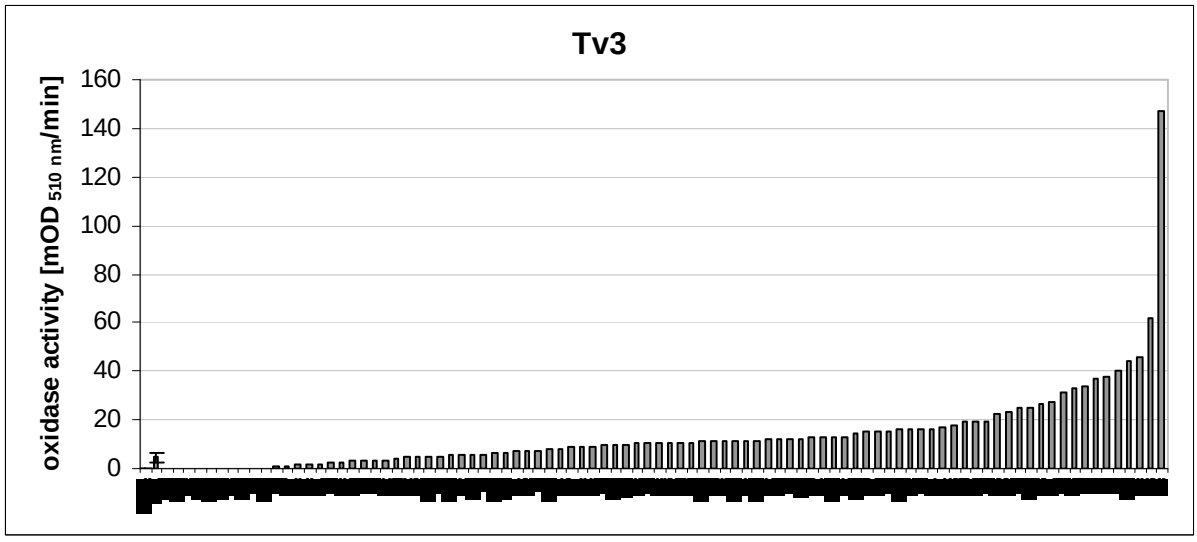

Figure 24: Activity screening landscape of Tv3 transformants

4.2.1.4 *Tv*DAO Rescreen

Several clones representing the landscape were selected for rescreening to verify the initial screening results. Therefore the clones were taken from the replica plate and streaked out on YDP-Zeo (100 mg/L) plates to generate single colonies. Four colonies of each clone were cultivated in deep well plates and analysed with the photometric activity assay.

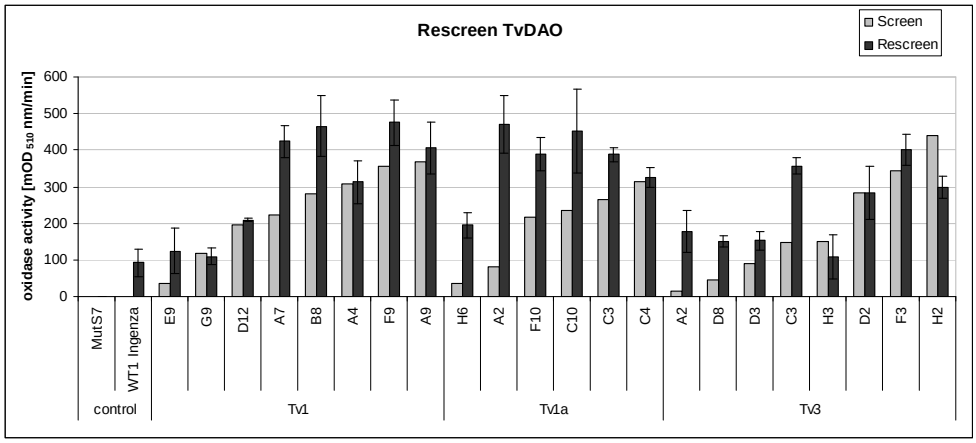

Figure 25: oxidase activity of rescreened *Tv*DAO strains
Strains are ordered according to their activity measured in the initial screening from low to high.
Muts7 *P .pastoris* CBS 7435 wildtype strain displaying MutS phenotype **WT1** Ingenza *P.pastoris* strain created by Ingenza, contains 5 mutations which leads to stability but no activity improvement. **Tv1** Expression strains harbouring codon optimized synthetic *Tv*DAO gene with exchanged native peroxisomal targeting sequence with SKL. **Tv1a** Expression strains harbouring native *Tv*DAO with exchanged native peroxisomal targeting sequence with SKL. **Tv3** Expression strains harbouring N-terminal citrine fusion with codon optimized synthetic *Tv*DAO and C-terminal exchanged native peroxisomal targeting sequence with SKL.
Screen activity during screen, **Rescreen** activity during rescreen

Table 16: Summary of data from the rescreen of oePCR expression constructs:
A: activity, A_{level}: relative activity compared to other transformants, A_{av}: average activity, SD: standard deviation, SD_{rel}: relative standard deviation

Strain		Initial Screen A mOD/min	Initial Screen A_{level}	Rescreen A_{av} mOD/min	Rescreen SD mOD/min	Rescreen SD_{rel} %
MutS7		0		0	0	0
WT1 Ingenza		0		93	37	40
Tv1	**E9**	37	low	125	61	49
Tv1	**G9**	119	medium	110	24	22
Tv1	**D12**	197		209	5	2
Tv1	**A7**	222		424	44	10
Tv1	**B8**	280		465	84	18
Tv1	**A4**	306		312	58	19
Tv1	**F9**	355	high	476	62	13
Tv1	**A9**	366	high	406	70	17
Tv1a	**H6**	35	low	195	34	18
Tv1a	**A2**	81	medium	470	80	17
Tv1a	**F10**	218		389	45	12
Tv1a	**C10**	235		452	114	25
Tv1a	**C3**	265	high	388	20	5
Tv1a	**C4**	313	high	326	28	9
Tv3	**A2**	16		178	57	32
Tv3	**D8**	46	low	150	14	10
Tv3	**D3**	90		153	26	17
Tv3	**C3**	147		357	23	6
Tv3	**H3**	150	medium	109	60	55
Tv3	**D2**	282		282	72	26
Tv3	**F3**	345	high	402	42	11
Tv3	**H2**	441	high	297	30	10

Based on the results of the rescreen, strains were selected for cultivation in shake flasks. Four strains of each construct were chosen, two with high activity, one with medium and one with low activity. If possible strains with low standard deviation were preferred.

4.2.1.5 Fluorescence microscopy

To confirm the *Tv*DAO peroxisomal targeting in combination with the PTS1 sequence –SKL fluorescence microscopy pictures were taken with the kind permission of Ao.Univ.- Prof. Dr.phil. Hermetter A.

The *P. pastoris* strain Tv3 H2, harbouring the N-terminal fused fluorescence protein citrine and the codon-optimized synthetic *Tv*DAO with the exchanged PTS1 sequence –SKL, was cultivated in deep well plates according to standard procedure. After 24 h of further methanol induction 50 µL samples were taken for fluorescence microscopy.

Figure 26: Fluorescence microscopy pictures of Tv3 H2 (Citrine-*Tv*DAO fusion protein) expressing *Pichia pastoris* cells after methanol induction.

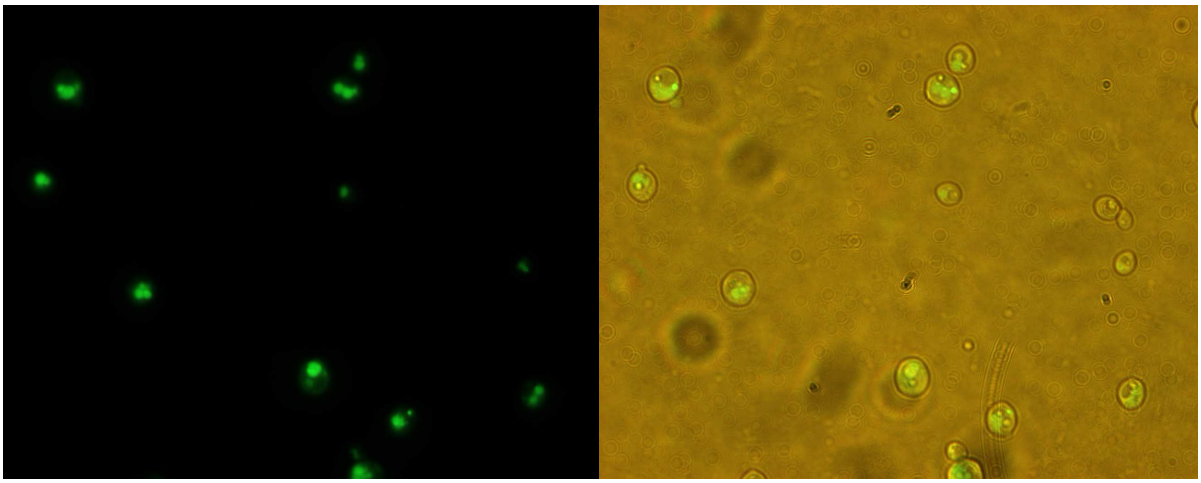

Figure 26 shows the location of citrine and *Tv*DAO in *Pichia pastoris* cells. The protein is not located in the cytoplasm, in which case fluorescence would be seen equally distributed in the cells. Instead fluorescence occurs only in certain parts of the cell, where it is highly concentrated. The fluorescent protein is accumulated in small but numerous compartments of the cell. This leads to the conclusion that the specific targeting to the peroxisomes via the C-terminal PTS1 –SKL sequence is working.

4.2.2 Cloning strategy

We recognized that the oePCR strategy with the pPP-B1 plasmid as template for the Zeocin resistance was not reproducible in terms of transformation efficiency, probably because it forces high multi copy integration, which is a rare event. As DNA preparation for transformations is time consuming, only few transformants could be generated in each round of transformation and many transformants turned out to be false positives (growth on agar plate with antibiotica, but no activity measurable).

In order to generate missing constructs a new strategy was established. It is based on a classical cloning approach using the pPP-T2 plasmid as Zeocin template.

To compare the results of the cloning strategy to the previously obtained results the existing constructs Tv1 and Tv1a were cloned again as controls. Additionally the missing constructs harbouring synthetic and native *Tv*DAO, both with native peroxisomal targeting sequence – PNL (Tv5 and Tv5a), were transformed into *P. pastoris*. Of the also targeted secretion expression constructs Tv4 and Tv4a no transformants were obtained. As the intracellular, peroxisomal constructs were promising, the focus of this work was on these constructs.

In the first step restriction sites were added via PCR to the exciting *Tv*DAO constructs. Digested PCR product was ligated with equally digested and dephosphorylated plasmid (pPP-T2) and transformed into *E. coli* (TOP10F'). The plasmid was isolated with the GeneJET Plasmid Miniprep Kit by Fermentas and linearized by digestion with *Bgl*II. The linear plasmid was then transformed into *P. pastoris* Mut^{S}7.

This strategy has one advantage: once the correct *E. coli* clone is identified the amplification to large DNA yields for *P. pastoris* transformation is much faster and easier to handle.

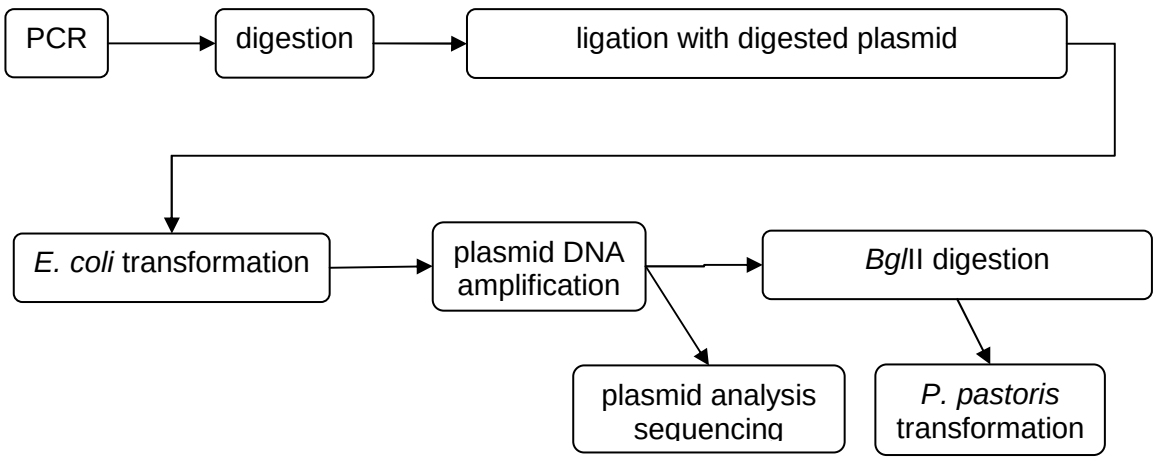

Table 17: Flow chart of the cloning strategy

Table 18: Overview of cloning expression constructs

Construct	Gene	Peroxisomal targeting sequence	Cloning sites		Linearization
cTv1	synthetic	SKL	*EcoR*I	*Not*I	*Bgl*II
cTv1a	native	SKL	*Spe*I	*Asc*I	*Bgl*II
cTv5	synthetic	PNL	*EcoR*I	*Not*I	*Bgl*II
cTv5a	native	PNL	*Spe*I	*Asc*I	*Bgl*II

4.2.2.1 Cloning

The existing linear expression cassettes of Tv1 and Tv1a were used as templates for PCR to add restriction sites to the target gene in order to produce inserts to clone into pPP-T2. Figure 27 shows the procedure in detail.

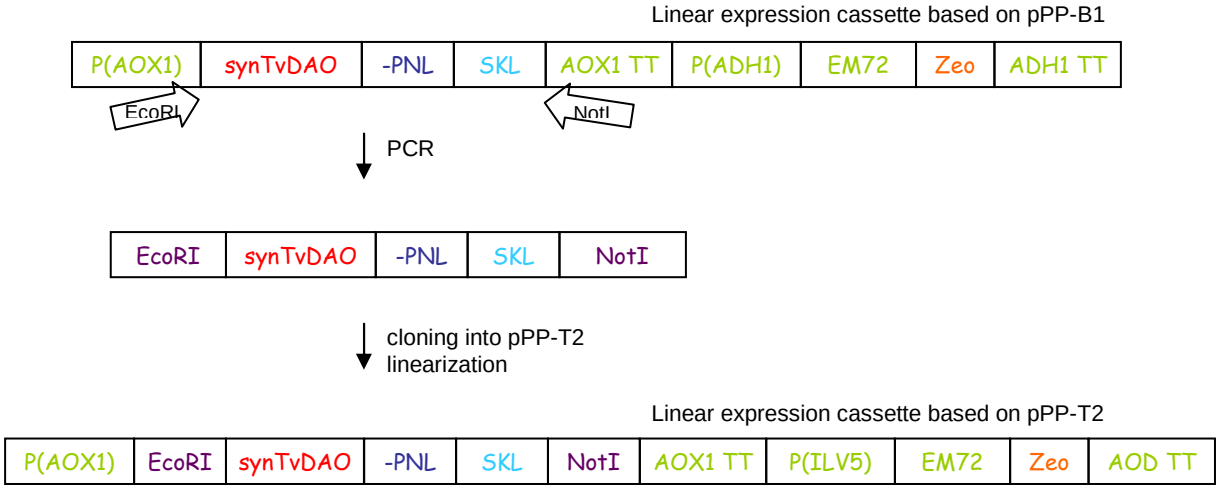

Figure 27: Tv1 Linear expression cassette as template for PCR to clone insert into vector pPP-T2

The primers needed for the addition of restriction sites and the amplification of the insert are shown in Table 19. All generated inserts were purified by gel electrophoresis prior to digestion.

Table 19: Primers to add restriction sites to *Tv*DAO

Construct	Produkt	Primer forward	Primer reverse	Template	Length /bp
cTv1	EcoRI-T1-NotI	P08-509	P08-512	pJET-Tv1	1200
cTv1a	SpeI-T1a-AscI	P08-507	P08-508	pJET-Tv1a	1200
cTv5	EcoRI-T1 PNL-NotI	P08-509	P08-552	pJET-Tv1	1200
cTv5a	SpeI-T1a PNL-AscI	P08-507	P08-551	pJET-Tv1a	1200

Purified inserts were digested with Fast digest™ enzymes and ligated with digested, dephosphorylated pPP-T2 plasmid under standard conditions (16°C, over night).

4.2.2.2 Vector pPP-T2

The plasmid pPP-T2 was used for cloning and provided the antibiotica resistance. This plasmid is similar to pPP-B1, which was used in the oePCR strategy. However they differ in the transcription control features of the *ble* gene for transcription of the antibiotic resistance.
In pPP-T2 the antibiotic resitance cassette consists of a synthetic bacterial promoter called EM72 in tandem with a truncated version of the *P. pastoris ILV5* (*acetohydroxyacid reductoisomerase*) promoter, the synthetic *ble* gene and the *P. pastoris AOD* (*alternative oxidase*) transcription termination sequence.

4.2.2.3 Screening

Using the cloning strategy the constructs cTv1, cTv1a, cTv5 and cTv5a were successfully generated. Clones of each construct were cultivated in deep well plates. Replicas of the clones were stamped on YPD plates containing Zeocin and glycerol stocks of the clones were produced and stored at -80°C.
Analysis of the clones was carried out with a photometric screening assay to determine enzyme activity. All clones were compared to the previously generated *P. pastoris* strain Tv1A9 as positive control and Mut^S as negative control.
With the new cloning approach more transformants with medium to high activity were generated and some transformants are in the range of Tv1A9.

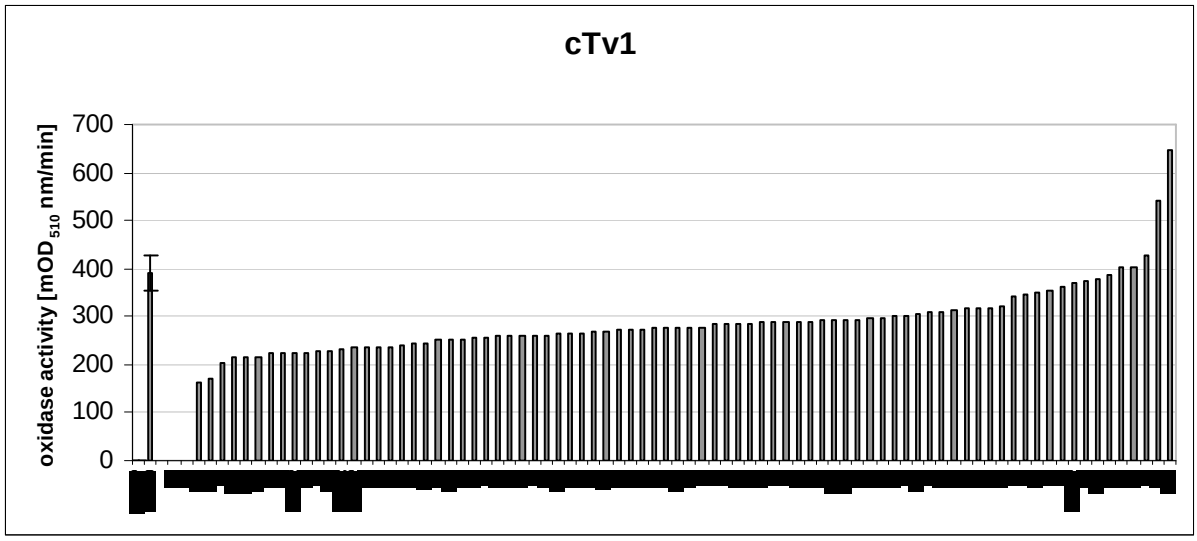

Figure 28: Activity screening landscape of cTv1 transformants

cTv1: Transformants harbouring codon optimized synthetic *Tv*DAO gene with exchanged native peroxisomal targeting sequence with SKL.

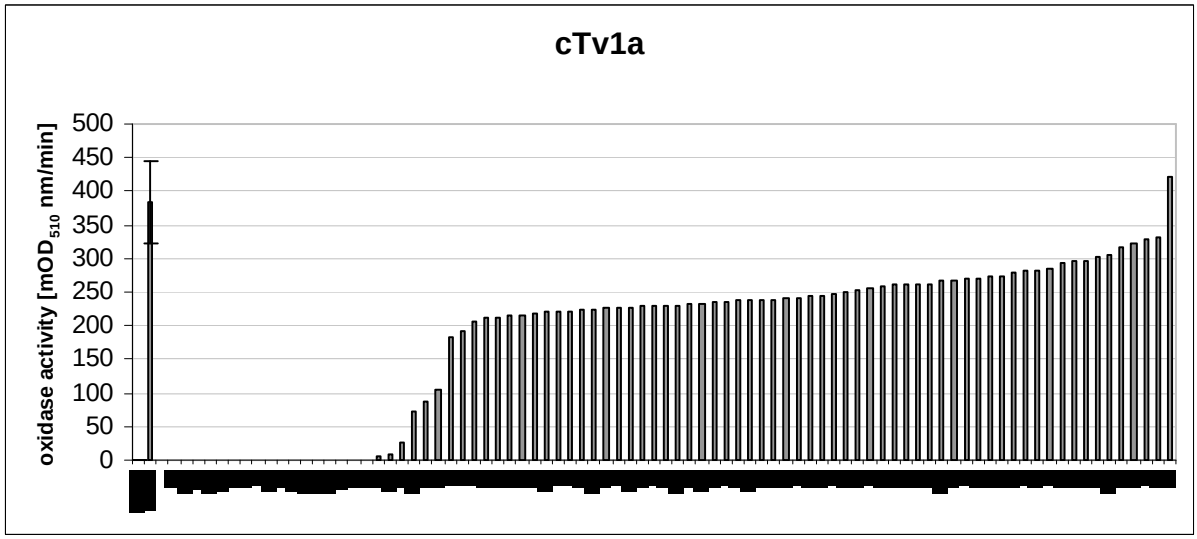

Figure 29: Activity screening landscape of cTv1a transformants

cTv1a: Transformants harbouring native *Tv*DAO with exchanged native peroxisomal targeting sequence with SKL.

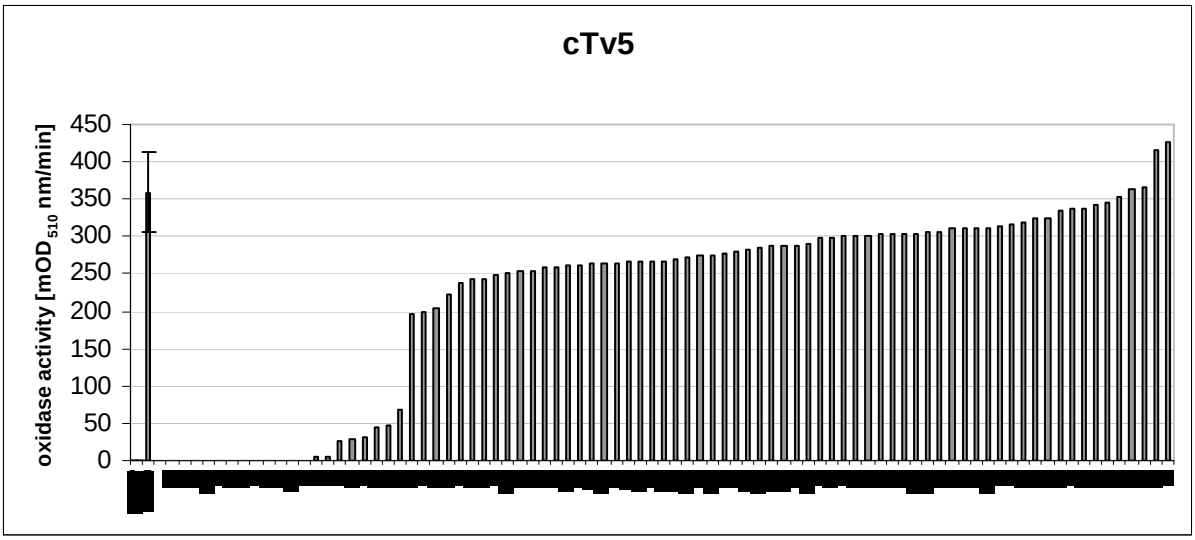

Figure 30: Activity screening landscape of cTv5 transformants

cTv5: Transformants harbouring codon optimized synthetic *Tv*DAO gene with native peroxisomal targeting sequence PNL.

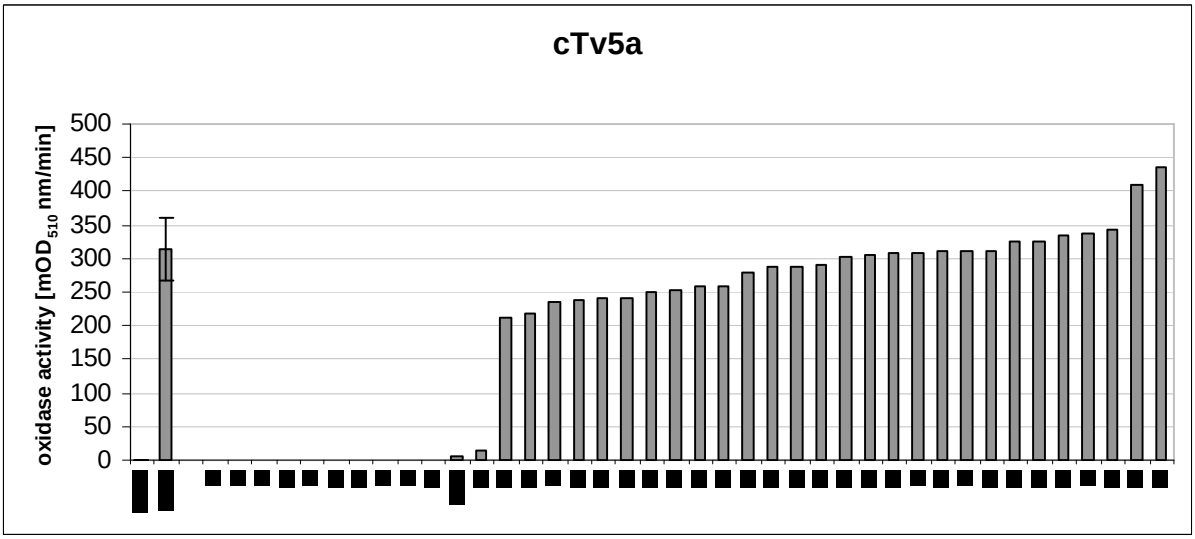

Figure 31: Activity screening landscape of cTv5a transformants

cTv5a: Transformants harbouring native *Tv*DAO with native peroxisomal targeting sequence PNL.

4.2.2.4 Rescreening

Several clones (usually 8-10) representing the landscape were selected for rescreening to verify the initial screening results. Therefore the clones were taken from the replica plate and streaked out on YDP-Zeo plates (100 mg/L) to generate single colonies. Four colonies of each clone were cultivated in deep well plates and analysed with the photometric activity assay.
The results of the most active clones are shown in Figure 32 and Table 20.

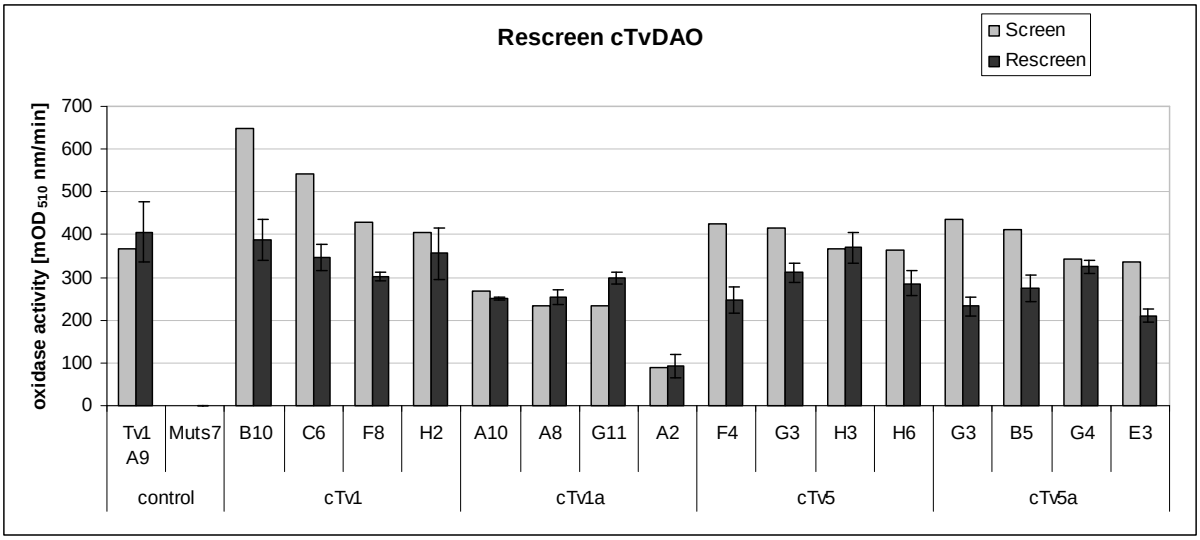

Figure 32: Overview of oxidase activity of rescreened c*Tv*DAO strains
Strains are ordered according to their activity measured in the initial screening from high to low.
Tv1A9 Best expressing *P.pastoris* strain harbouring codon optimized synthetic *Tv*DAO gene with exchanged native peroxisomal targeting sequence with SKL. **Muts7** *P .pastoris* CBS 7435 wildtype strain displaying MutS phenotype **cTv1** Expression strains harbouring codon optimized synthetic *Tv*DAO gene with exchanged native peroxisomal targeting sequence with SKL. **cTv1a** Expression strains harbouring native *Tv*DAO with exchanged native peroxisomal targeting sequence with SKL. **cTv5** Expression strains harbouring codon optimized synthetic *Tv*DAO gene with native peroxisomal targeting sequence PNL. **cTv5a** Expression strains harbouring native *Tv*DAO with native peroxisomal targeting sequence PNL.
Screen activity during screen, **Rescreen** activity during rescreen

Table 20 Summary of data from the rescreen of oePCR expression constructs:
A: activity, A_{av}: average activity, SD: standard deviation, SD_{rel}: relative standard deviation

Strain	Initial Screen A mU/mL	Rescreen A_{av} mU/mL	Rescreen SD mU/mL	Rescreen SD_{rel} %
Tv1 A9	366	406	70	17
Muts7	0	0	0	0
cTv1 B10	647	386	48	12
cTv1 C6	542	346	30	9
cTv1 F8	428	301	10	3
cTv1 H2	405	356	60	17

cTv1a	**A10**	269	251	4	2
cTv1a	**A8**	235	255	17	7
cTv1a	**G11**	234	297	14	5
cTv1a	**A2**	88	92	26	29
cTv5	**F4**	426	248	32	13
cTv5	**G3**	416	312	22	7
cTv5	**H3**	366	369	35	9
cTv5	**H6**	363	286	29	10
cTv5a	**G3**	436	232	23	10
cTv5a	**B5**	410	276	30	11
cTv5a	**G4**	342	324	16	5
cTv5a	**E3**	337	210	15	7

Based on the results of the rescreen, strains were selected for cultivation in 1.5 L bioreactors in order to obtain accurate results for interpretation of the results and perform an evaluation of the expression strategy. Strains with high activity and low standard deviation were preferred.

4.3 Activity assay comparison

During this study two different methods of oxidase activity determination were applied and compared. An enzyme-coupled colorimetric assay as described by Alexeeva et al. [86] with slight modifications (see section 3.6.8) was used. As this is a photometric assay it is easy to handle and many samples can be measured in short time.

In order to confirm the results gained by the first assay a second method of activity measurement was performed by Dr. Jozef Nahalka. This oxygen consumption method [55] does not need a coupled reaction for a colorimetric development. It followed directly the oxygen consumption rate in the reaction solution. Consequently there is no need for clear sample solutions and therefore intracellular activity of lysed cells as well as whole cells can be determined by activity measurement of the clear lysate and the cell debris resulting in more accurate results. Nevertheless this method is time consuming and is therefore only applied on selected samples, and can be seen as a complementation of the enzyme-coupled activity assay.

4.4 Copy number determination by qRT-PCR

The number of copies of *Tv*DAO expression cassettes integrated into the *P. pastoris* genome (see Table 21) was determined by quantitative real time PCR (qRT-PCR) as described in the master thesis of Kerstin Kitz employing the endogenous ARG4 gene as internal reference [96, 97]. This method is based on the relationship between the amount of the PCR starting material and the resulting amount of product at any cycle. Prior to qRT-PCR the isolation of high quality genomic DNA (high concentration, very pure) is crucial for the success of the experiment. The pure gDNA is used to amplify the expression cassette, the amount of generated PCR product is correlated to a standard curve of a known single copy strain, which is amplified at the same time. The PCR products are detected using SYBR Green (Applied Biosystems) as detection dye. This dye intercalates into dsDNA and generates a bright fluorescence upon intercalating. More detailed information about the experimental setup can be found in section 3.6.11. As this method is time consuming and cost intensive it is not a high through put method, however it is a faster method than the established southern blot method and information about copy numbers is essential for many studies. Furthermore it has to be mentioned that, similar to other techniques, qRT-PCR is not exact for multicopy strains (more than 6 copies), but a clear trend can be recognized.

Table 21: Copy number determination *Tv*DAO expression strains
Codon usage: native as in the original *Tv*DAO gene, or optimized for expression using the AOX1 promoter. *PTS1* – peroxisomal targeting using the native C-terminal tripeptide PNL or the engineered motif SKL. *CN*: Number of copies of the expression cassette integrated into the *P. pastoris* genome.

Strain	Sequence		CN
	Codon usage	PTS1	
WT1	native	PNL	7-9
Tv1 A9	optimized	SKL	18-19
Tv1a C4	native	SKL	5
Tv3 H2	optimized	SKL	16-17
cTv1 B10	optimized	SKL	1
cTv1a G11	native	SKL	1
cTv5 H3	optimized	PNL	1-2
cTv5a G4	native	PNL	1

The effect of different copy numbers was already described in the previous section. Noteworthy is that only strains with the same copy number level can be compared directly. Comparison of strains harbouring the same construct in different numbers only gives information about different copy number levels.

4.5 Shake flask experiment

In the first round of expression cassette construction via oePCR strategy, three constructs were generated: Tv1, Tv1a and Tv3. *P. pastoris* strains harbouring these constructs were screened for oxidase activity and initial results were confirmed via rescreen experiments. Based on these results two high, one medium and one low activity strain were selected for shake flask cultivation. Selected strains (see Table 22.) harbouring the constructs Tv1, Tv1a, Tv3 were cultivated in duplicates, as described in chapter 3.6.7.

Table 22: Selected strains for large scale cultivation in shake flasks

Construct	Activity level			
	high		medium	low
Tv1	A9	F9	G9	E9
Tv1a	C4	C3	A2	H6
Tv3	H2	F3	H3	D8

4.5.1 Analysis of enzyme activity

Samples for activity measurements were taken before induction and before culture harvest. Cells were spinned down and lysed with YeastBuster. Cell debris were removed by centrifugation and supernatant was used for the photometric enzyme activity assay.

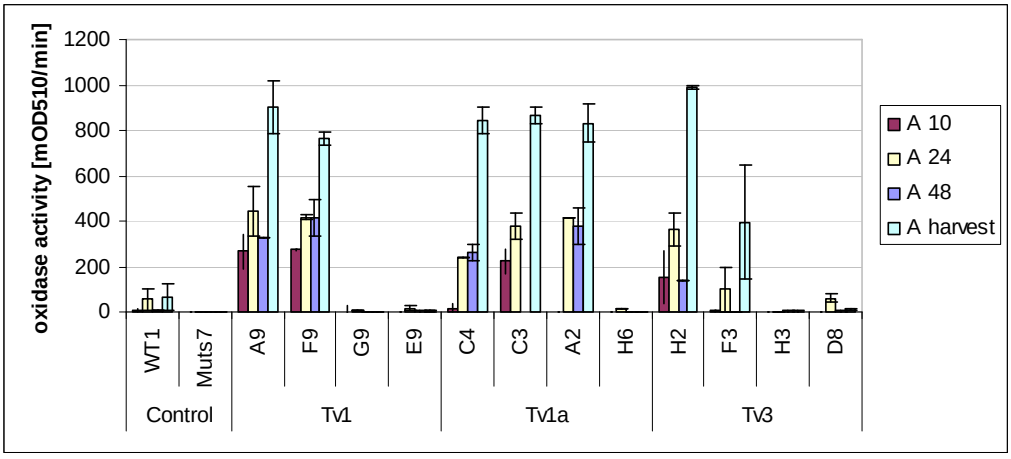

Figure 33: Time course of shake flask experiment
WT1 Ingenza *P.pastoris* strain created by Ingenza, contains 5 mutations which leads to stability but no activity improvement. **Muts7** *P .pastoris* CBS 7435 wildtype strain displaying MutS phenotype **Tv1** Expression strains harbouring codon optimized synthetic *Tv*DAO gene with exchanged native peroxisomal targeting sequence with SKL. **Tv1a** Expression strains harbouring native *Tv*DAO with exchanged native peroxisomal targeting sequence with SKL. **Tv3** Expression strains harbouring N-terminal citrine fusion with codon optimized synthetic *Tv*DAO and C-terminal exchanged native peroxisomal targeting sequence with SKL. **A 10** activity after 10 hours, **A 24** activity after 24 hours, **A 48** activity after 48 hours, $\mathbf{A_{harvest}}$ activity after cell harvest

Figure 33 shows a continuing activity increase over time. This effect is independent from population growth. Optical density at 600 nm (OD_{600}) was measured and activity is even increasing when OD_{600} data are considered.

Some strains show high standard deviation (e.g. Tv3 F3), which suggests that the strains suffered some contamination during isolation or cultivation or that the integration locus of the expression cassette is not stable. Nevertheless, most strains show good standard deviation and therefore experiment reproducibility.

Table 23: Summary of the data of the shake flask experiment
A 10: activity after 10 hours, A 24: activity after 24 hours, A 48: activity after 48 hours, $A_{harvest}$: activity after cell harvest

Constructs		Shake flask							
		A 10 mOD/min	SD mOD/min	A 24 mOD/min	SD mOD/min	A 48 mOD/min	SD mOD/min	$A_{harvest}$ mOD/min	SD mOD/min
	WT1	7	6	56	48	10	0	65	59
	Muts7	0	0	1	0	0	0	0	0
Tv1	**A9**	268	76	445	107	330	0	903	116
	F9	273	1	417	9	417	81	763	29
	G9	-2	32	4	0	2	1	1	1
	E9	-2	0	15	11	3	2	5	2
Tv1a	**C4**	17	17	237	0	263	35	843	56
	C3	223	53	375	59		380	866	34
	A2	0	0	413	0	378	81	833	82
	H6	0	0	16	1	1	0	2	1
Tv3	**H2**	151	117	362	73	137	0	990	9
	F3	4	3	99	99	2	0	396	249
	H3	3	0	0	0	3	1	3	2
	D8	0	0	62	19	3	3	11	6

The final results of the shake flask cultivation were compared to primary results of screen and rescreen. Generally speaking the shake flask cultivation showed to be beneficial for high activity strains, because under shake flask cultivation conditions higher activities could be obtained. Interestingly the medium activity strains (e.g. Tv1 G9) showed better performance during DWP cultivation (see Figure 34).

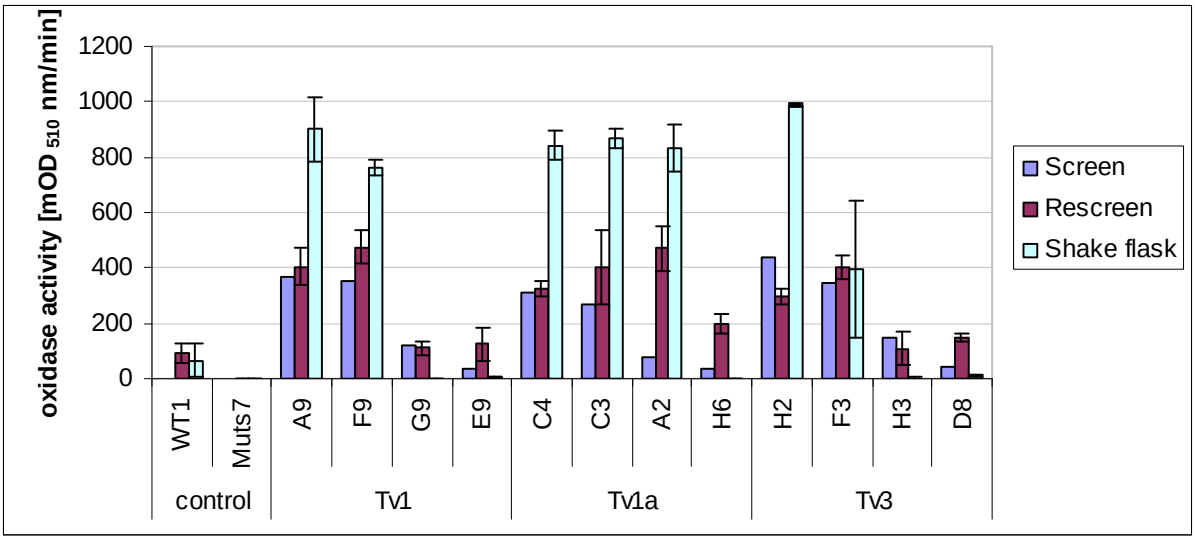

Figure 34: Comparison of shake flask results to primary results of screen and rescreen
WT1 Ingenza *P.pastoris* strain created by Ingenza, contains 5 mutations which leads to stability but no activity improvement. **Muts7** *P .pastoris* CBS 7435 wildtype strain displaying MutS phenotype **Tv1** Expression strains harbouring codon optimized synthetic *Tv*DAO gene with exchanged native peroxisomal targeting sequence with SKL. **Tv1a** Expression strains harbouring native *Tv*DAO with exchanged native peroxisomal targeting sequence with SKL. **Tv3** Expression strains harbouring N-terminal citrine fusion with codon optimized synthetic *Tv*DAO and C-terminal exchanged native peroxisomal targeting sequence with SKL. **Screen** activity during screen, **Rescreen** activity during rescreen, **Shake flask** activity in shake flasks

4.6 Bioreactor cultivation

4.6.1 Motivation

Initial results of screening and rescreening have to be confirmed by a large scale cultivation under reproducible conditions. Bioreactor cultivation has several advantages, selected strains can be cultivated under highly reproducible conditions where pH, temperature and dissolved oxygen can be monitored and controlled over time. In addition methanol feeding is continuous and even mix-feeding of different C-sources for special experimental set-ups is easy to handle. In order to compare the results to literature reports cell material growth with controlled parameters is beneficial. Moreover Dr. Jozef Nahalka conducted activity and stability measurements via oxygen consumption of *Tv*DAO expressed by generated *P. pastoris* strains.

In that manner four different bioreactor cultivation rounds were conducted. The primarily generated constructs Tv1, Tv1a and Tv3 were cultivated in the first round. Therefore the best strain after screening and rescreening was chosen of each construct. Additionally WT1 and Mut^{S}7 were selected as control strains. *Pichia pastoris* high cell density fermentation with standard induction feed (MeOH 3 mL/h) has been run.

The cultivation was controlled via OD_{600}, wet cell weight (wcw) and activity measurements, which suggested that the standard *P. pastoris* fermentation protocol was unfavourable and therefore *Tv*DAO productivity was lower than assumed. For a first optimization during the induction phase a new feeding profile as well as a mix feed strategy (MeOH/glycerin and MeOH/glucose) were established. Furthermore the results of the first fermentation indicated that the highest whole cell activity could be reached with the fusion variant Tv3. Therefore the mix feed strategy was tested in a second fermentation round with Tv3. The positive results of the mix feed strategy encouraged to a third fermentation where the mix feeds were tested on the construct Tv1 which is industrially relevant since no IP issues like for Tv3 (GFP variant fusion) are known.

Finally the constructs generated with the cloning strategy were tested in a high cell density fermentation with standard 3 mL/h MeOH feeding profile. The best results were achieved by the construct Tv1 which harbours the codon-optimized synthetic gene in combination with the improved peroxisomal targeting sequence SKL.

4.6.2 Experimental

Detailed information about the fermentation with the DAS GIP multi-fermentation equipment can be found in the Appendix in section 7.2 “DAS GIP Protocol”.

4.6.3 Fermentation I (oePCR / B1 constructs)

4.6.3.1 Strains and culture conditions

The first bioreactor cultivation was conducted with the primarily generated oePCR constructs Tv1, Tv1a and Tv3. From each construct, the *P. pastoris* strain which gave the best activities in screening and rescreening experiments was selected for large scale cultivation.

Table 24: Strains used for fermentation I

Fermenter	Strain	*Tv*DAO gene	Fusion	Peroxisomal targeting	ZeoR (Plasmid)	Biomass [g wcw/Batch]
F1	Tv1A9	synthetic	-	SKL	B1	122
F2	Tv1A9	synthetic	-	SKL	B1	117
F3	WT1	native	-	PNL	-	210
F4	Tv1a C4	native	-	SKL	B1	240
F5	Muts7	-	-	-	pPICK°	63*
F6	Tv1a C4	native	-	SKL	B1	205
F7	Tv3 H2	synthetic	N-terminal citrine	SKL	B1	131
F8	Tv3 H2	synthetic	N-terminal citrine	SKL	B1	155

° plasmid commercially available by Invitrogen Corp.
* Mut^S7 preculture showed a slow growth behaviour, which could not be supported during bioreactor cultivation. Therefore this biomass yield should not be considered as standard.

For this first fermentation the standard *P. pastoris* fermentation protocol was used with a standard methanol feeding rate of 3 mL/h and an induction phase of 90 hours.

4.6.3.2 Analysis of oxidase activity

During fermentation samples were taken before induction and than every 24 hours until cell harvest. After 90 hours of induction cells were harvested via centrifugation. The cell material was washed in phosphate buffer and total biomass was weight (= fcw, fresh cell weight or

wcw, wet cell weight). The biomass was frozen and transferred for oxygen consumption measurements [55].

Of each sample taken OD_{600}, wcw and activity via photometric assay was determined. Results of the activity measurements (activity per wcw) can be found in Figure 35.

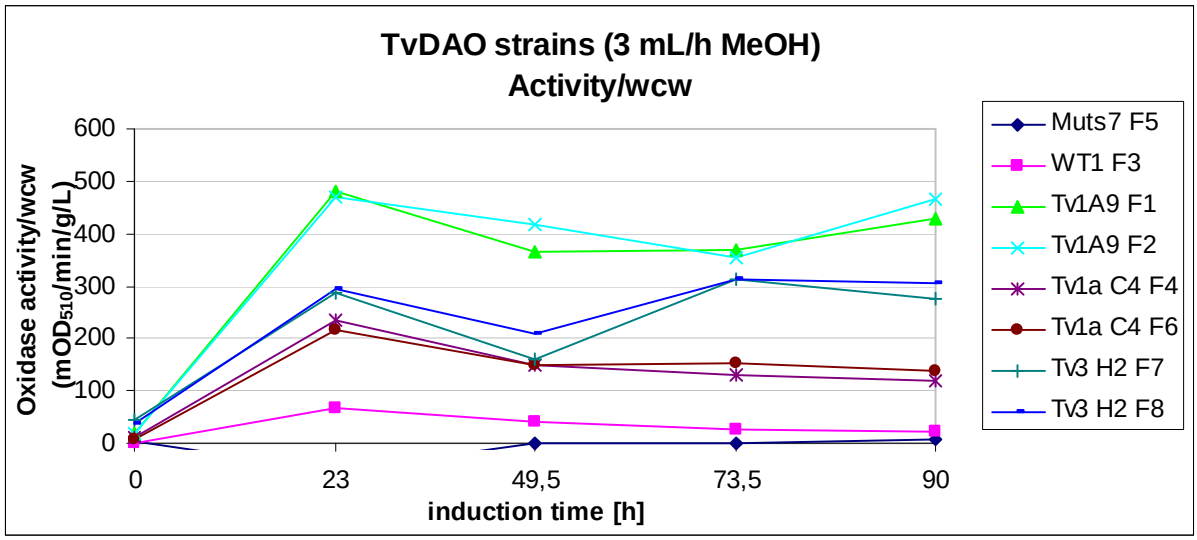

Figure 35: *P.pastoris* high cell density fermentation with standard 3mL/h MeOH induction feed. **Muts7 F5** *P.pastoris* CBS 7435 wildtype strain displaying Mut^S phenotype (fermenter 5) **WT1 F3** Ingenza *P.pastoris* strain created by Ingenza, contains 5 mutations which leads to stability but no activity improvement (fermenter 3). **Tv1 A9 F1/F2** Best expressing strain harbouring codon optimized synthetic *Tv*DAO gene with exchanged native peroxisomal targeting sequence with SKL (fermenter 1and 2). **Tv1a C4 F4/F6** Best expressing strain harbouring native *Tv*DAO with exchanged native peroxisomal targeting sequence with SKL (fermenter 4 and 6). **Tv3 H2 F7/F8** Best expressing strain harbouring N-terminal citrine fusion with codon optimized synthetic *Tv*DAO and C-terminal exchanged native peroxisomal targeting sequence with SKL (fermenter 7 and 8).

From the results of activity determination it can be seen, that the negative control strain (host strain) Mut^S7 displays no activity at all. Furthermore the activity improvement of all generated strains as compared to *P. pastoris* WT1 strain (provided by Ingenza) was confirmed in the large scale bioreactor cultivation. The strain Tv1 A9 harbouring the codon optimized synthetic *Tv*DAO gene with exchanged peroxisomal targeting sequence –SKL, showed the highest activity. Followed by Tv3 H2, the expression strain harbouring N-terminal fused fluorescence protein citrine with codon optimized synthetic *Tv*DAO and peroxisomal targeting sequence -SKL.

The strain Tv1a C4, harbouring the native *Tv*DAO gene with peroxisomal targeting sequence –SKL, showed the lowest activity of the optimized strains. Noteworthy is the high reproducibility of the strain cultivated in duplicates.

Unfortunately the biomass yield for the best expression strain, Tv1 A9, was quite low, especially after 24 hours methanol induction growth was impaired (data not shown). This

situation indicates that the standard *P. pastoris* fermentation protocol overstressed the cells and consequently low *Tv*DAO productivity after 24 hours induction was obtained. To overcome cell stress during the induction phase, a new feeding profile as well as a mix feed strategy (MeOH/glycerin and MeOH/glucose) has been performed in the second fermentation.

Intracellular activity measurements via oxygen consumption method were conducted by Dr. Jozef Nahalka. The intracellular activity was determined by activity measurement of the "soluble" and "insoluble" fraction of french press lysed cells. Compared to the benchmark strain WT1 from Ingenza engineering of the peroxisomal targeting (Tv1a) increased intracellular activity about 8-fold, the codon optimization together with the engineered peroxisomal targeting sequence increased activity about 20-fold and the N-terminal citrine fusion increased about 22.5-fold (see Figure 36).
It should be mentioned, that measurement of so called "soluble" and "insoluble" fraction is one big advantage of the oxygen consumption method compared to the photometric assay were only the "soluble" fraction can be measured.

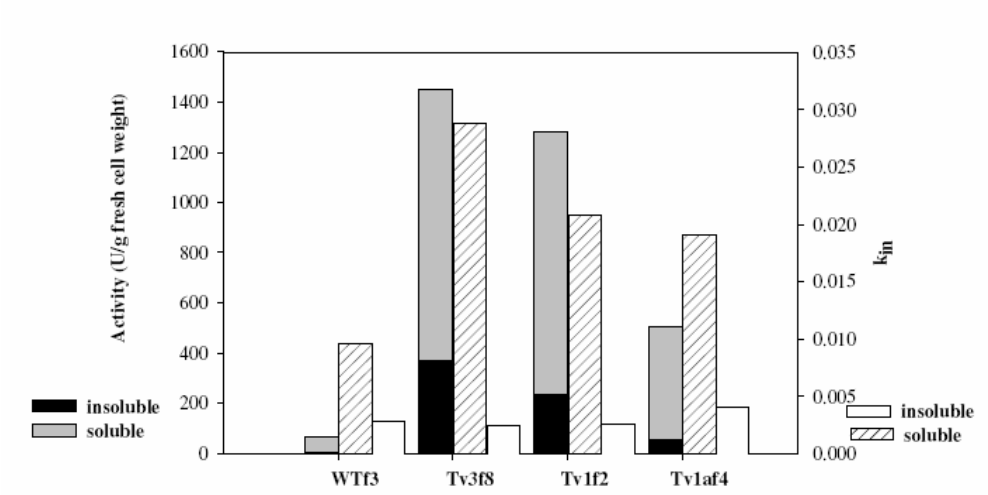

Figure 36: Activity and stability determination after french press biomass disruption.
Best *Tv*DAO expressing strains were cultivated according to high cell density fermentation protocol (3mL/h MeOH). Measurements were performed in a mini-reactor via oxygen consumption method. **WTf3** *P.p.* WT1 *Tv*DAO strain from Ingenza, harbours 5 mutations for stability improvement (fermenter 3). **Tv3f8** *P.p.* Tv3 H2, harbours a codon optimized *Tv*DAO gene, which is N-term. fussed with citrine and C-term. SKL (fermenter 8) **Tv1f2** *P.p.* Tv1 A9, harbours a codon optimized *Tv*DAO gene with a engineered peroxisomal targeting sequence (fermenter 2) **Tv1af4** *P.p.* Tv1a C4, harbours a native *Tv*DAO gene with engineered peroxisomal targeting sequence (fermenter 4).

It is also important to keep in mind, that activity measurements via oxygen consumption method were conducted with french press lysed cells but the photometric activity assay were

based on detergent lysed cells. Comparison between the two methods to lyse cells showed different activity distribution between soluble and insoluble fraction (see Figure 37).

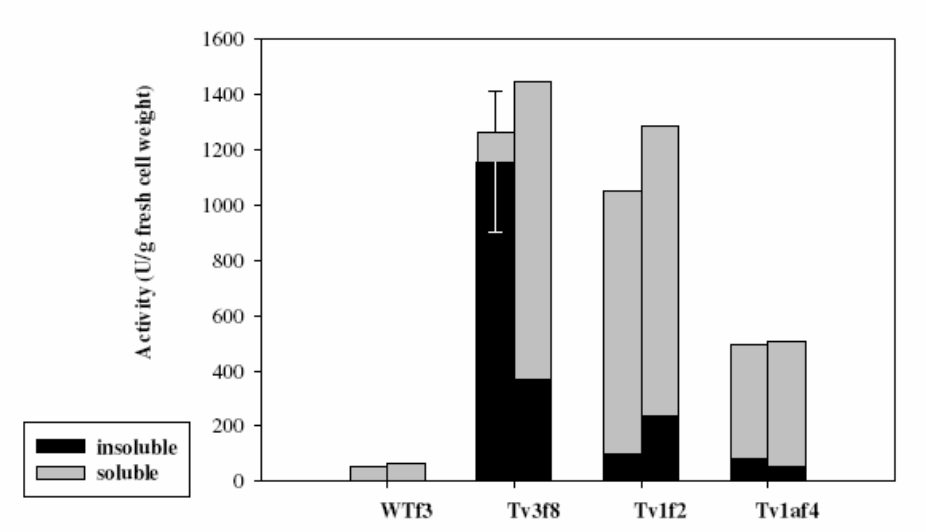

Figure 37: *Tv*DAO activity comparison between detergent (first bar) and French press (second bar) lysed cells.
Best *Tv*DAO expressing strains were cultivated according to high cell density fermentation protocol (3mL/h MeOH). Measurements were performed in a mini-reactor via oxygen consumption method. **WTf3** *P.p.* WT1 *Tv*DAO strain from Ingenza, which harbours 5 mutations for stability improvement (fermenter 3). **Tv3f8** *P.p.* Tv3 H2, which harbours a codon optimized *Tv*DAO gene, which is N-term. fussed with citrine and exchanged C-term. with SKL (fermenter 8) **Tv1f2** *P.p.* Tv1 A9, which harbours a codon optimized *Tv*DAO gene with a engineered peroxisomal targeting sequence (fermenter 2) **Tv1af4** *P.p.* Tv1a C4, which harbours a native *Tv*DAO gene with engineered peroxisomal targeting sequence (fermenter 4) **insoluble** activity measured with cell debris **soluble** activity measured with clear cell lysate.

As mentioned above, conditions for cell growth need to be adapted. Strains with no codon optimization (Tv1a and WT1) grew to high cell densities (200-240 g/L). In contrast Tv1 and Tv3, which harbour the codon optimized synthetic gene, did not grow well (120-150 g/L). This indicates that these strains suffer from metabolic problems and cultivation conditions especially during induction phase should be optimized. Therefore a mix feed fermentation with Tv3, which is the highest *Tv*DAO expression strain after oxygen consumption measurements was performed. In this case glycerine or glucose serves as a carbon source for all growth and energy production and methanol is only used as inducor for expression.

4.6.4 Fermentation II (Tv3, mix feed strategy)

4.6.4.1 Strains and culture conditions

Results of the first fermentation round suggest that optimization of the cultivation conditions is necessary to generate more biomass and therefore more enzyme. The strain Tv3 H2 was chosen since during activity measurements via oxygen consumption method showed that this strain displayed the highest *Tv*DAO activity.

To find the best culture conditions three different strategies were tested and compared to the initial conditions (MeOH feed 3 mL/h). It is known, that a mixed substrate feed in the induction phase can support *P. pastoris* growth and productivity at the same time [98]. Therefore in addition to a methanol induction feed of 1.5 mL/h two mix-feeds with MeOH/glycerin and MeOH/glucose were established.

Table 25: Conditions of fermentation II

Fermenter	Strain	Feed	Biomass [g wcw/Batch]
F1	Tv3 H2	MeOH/Glucose	185
F3	Tv3 H2	MeOH/Glycerin	209
F4	Tv3 H2	MeOH/Glycerin	202
F5	Tv3 H2	MeOH 3 mL/h	123
F7	Tv3 H2	MeOH 1.5 mL/h	115
F8	Tv3 H2	MeOH 1.5 mL/h	125

Beside the change of the induction feed the standard *P. pastoris* fermentation protocol was followed strictly. Samples were taken every 12 hours starting prior to induction phase (90 hours).

4.6.4.2 Analysis of oxidase activity

Samples were taken every 12 hours during induction phase of fermentation until cell harvest. OD_{600}, wcw and activity via photometric assay was determined of each sample (see Figure 38).

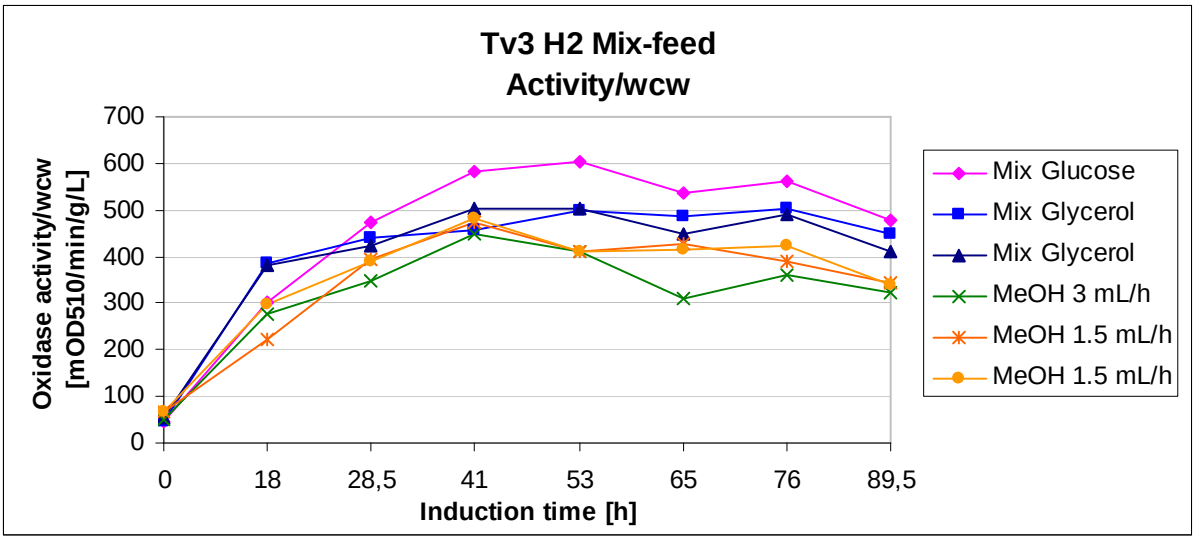

Figure 38: *P.pastoris* Tv3 H2 high cell density fermentation with 1.5 and 3mL/h MeOH, as well as mix-feed glucose/MeOH and glycerin/MeOH.
Cells were lysed with detergent, and supernatant was measured photometrically. **MixGlucose F1** Tv3 H2 induced with glucose/MeOH feed mix with ratio 0.33/0.66 (fermenter 1). **MixGlycerin F3/F4** Tv3 H2 induced with glycerin/MeOH feed mix with ratio 0.33/0.66 (fermenter 3 + 4). **MeOH 3mL/h F5** Tv3 H2 induced with 3mL/h MeOH feed (fermenter 5). **MeOH 1.5mL/h F7/F8** Tv3 H2 induced with 1.5mL/h MeOH feed (fermenter 7 + 8) wcw, wet cell weight [g].

Higher activity was observed in case of both mix feeds. The best result was obtained with glucose/MeOH mix feed, followed by glycerin/MeOH mix feed. Since the activity profile during the cultivation remained unchanged but showing higher activity values it is very likely that the activity improvement is based on a better cell growth due to availability of a C-source which can be metabolized faster than MeOH.
Even though the highest activity was reached with glucose/MeOH mix feed, the addition of glycerin in the methanol feed improved the biomass yield of Tv3 from 123 g/L (F5) to 205 g/L (F3 and F4).

Harvested biomass was washed in phosphate buffer, frozen and transferred to Jozef Nahalka for activity measurements via oxygen consumption method. Activity determination via oxygen consumption method confirmed the trend that mix feed induction increased the *Tv*DAO activity. It was also shown that glucose/MeOH mix feed gave higher specific activity (U/g fcw) than glycerine/MeOH.

4.6.5 Fermentation III (Tv1, mix feed strategy)

4.6.5.1 Strains and culture conditions

Tv1 A9, the best *P. pastoris* expression strain, harbouring the codon optimized synthetic *Tv*DAO gene and exchanged peroxisomal targeting sequence –SKL, was an interesting target, because it did not harbour the fusion protein citrine and therefore is more applicable for industrial processes. Furthermore the reproducibility of the mix feeding strategy could be analyzed. As the final goal is a stable, highly active biocatalyst for usage in a biotechnological process the optimization of culture conditions is necessary.

Based on the mix feed strategy for Tv3 (fermentation II) a similar bioreactor cultivation was conducted with the strain Tv1 A9. Again the standard methanol feed rate of 3 mL/h served as benchmark for evaluation of the different fermentation conditions.

Table 26: Conditions of fermentation III

Fermenter	Strain	Feed	Biomass [g wcw/Batch]
F1	Tv1 A9	MeOH/Glucose	207
F3	Tv1 A9	MeOH/Glycerin	197
F4	Tv1 A9	MeOH/Glycerin	249
F5	Tv1 A9	MeOH 1.5 mL/h	148
F6	Tv1 A9	MeOH 1.5 mL/h	144
F8	Tv1 A9	MeOH 3 mL/h	131

Beside the adaptation of the induction feed the standard *P. pastoris* fermentation protocol was followed strictly. During the induction phase (90 hours) samples were taken every 12 hours to determine OD_{600}, wcw and activity.

4.6.5.2 Analysis of oxidase activity

Results of the photometric activity determination of fermentation samples can be found in Figure 39.

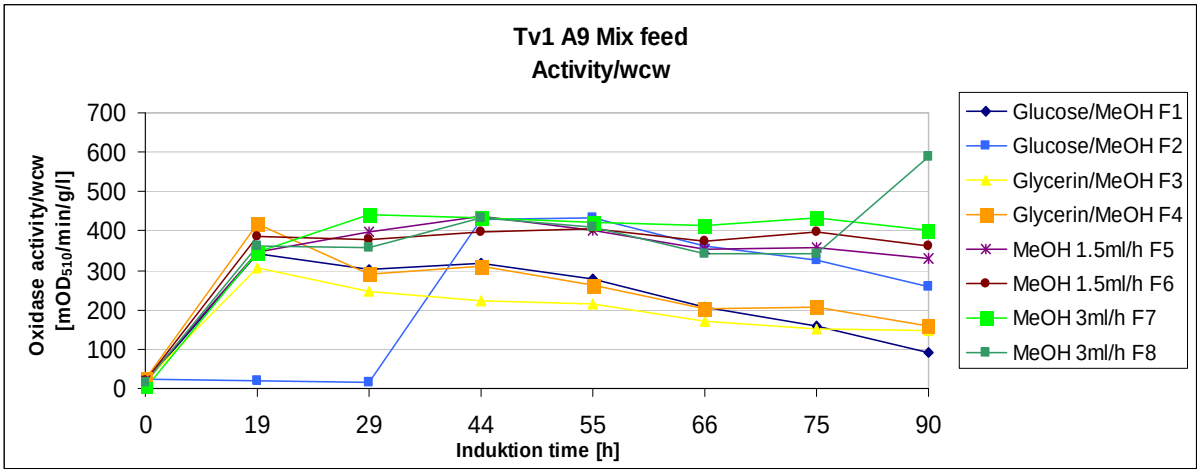

Figure 39:*P.pastoris* Tv1 A9 high cell density fermentation with 1.5 and 3mL/h MeOH, as well as mix-feed glucose/MeOH and glycerin/MeOH.
Photometrical activity determination of the fermentation course. **MixGlucose F1/F2** Tv1 A9 induced with glucose/MeOH feed mix with ratio 0.33/0.66 (fermenter 1+2). **MixGlycerin F3/F4** Tv1 A9 induced with glycerin/MeOH feed mix with ratio 0.33/0.66 (fermenter 3+4). **MeOH 3mL/h F5/F6** Tv1 A9 induced with 3mL/h MeOH feed (fermenter 5+6). **MeOH 1.5mL/h F7/F8** Tv1 A9 induced with 1.5mL/h MeOH feed (fermenter 7+8) **wcw** wet cell weight [g].

The promising results of the mix feed strategy from fermentation of Tv3 could not be confirmed with Tv1 mix feed cultivation.

Mix feed induction led to a 40% increase in biomass yield (~ 200 g wcw) without compromising the specific activity of the cells. The superiority of MeOH/glycerin and MeOH/glucose could not be reproduced for Tv1.

The best result of the intracellular activity was obtained with the reduced methanol rate (1.5 mL/h), which gave an 1.4-fold enhancement of specific activity as compared to the standard methanol feed of 3 mL/h. However the biomass yield was still low (~ 140 g/L). Figure 40 shows clearly, that the mix feeds improve the biomass yield, but not the specific activity and that the reduced methanol rate only improves activity without a marked growth advantage.

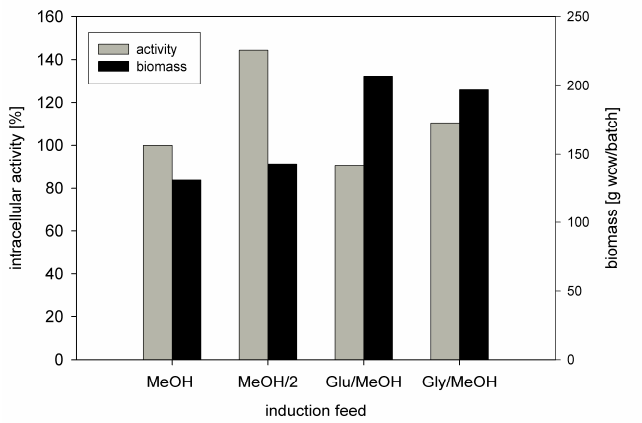

Figure 40: High-cell-density bioreactor cultivation of *P. pastoris* strain Tv1 A9 using induction with a mix-feed strategy.
Values are referenced to oxidase activity obtained using the standard methanol feed and set as 100%.
MeOH: 3 mL/h methanol. **MeOH/2:** 1.5 mL/h methanol, **Glu/MeOH:** 3mL/h glucose-methanol mixture, **Gly/MeOH:** 3 mL/h glycerol-methanol mixture

Even though the culture optimization did not improve the specific activities as seen for Tv3 H2 it has to be noted, that the activities reached with Tv1 measured with the oxygen consumption method exceed the values of other known *Tv*DAO expression strains Table 27.

Tv1 A9 is the most efficient host for production of *Tv*DAO that has been reported so far. Comparison with earlier reports shows that this strain reached an expression level up to one order of magnitude above the expression levels reported for *E. coli* and *P. pastoris* [59, 62]. Furthermore other yeasts such as *S. cerevisiae* and *K. lactis* showed significant lower *Tv*DAO production [60].

Table 27: Comparison of relevant host strains used for production of *Tv*DAO
Differences in the conditions used for assaying the enzyme activity are problematic for a direct and quantitative comparison of the published data. However, a clear trend is recognized. Results as already accepted for publication in [99].

Expression host	Activity	Reference
Trigonopsis variabilis [a]	4,620 U/L[e]	[48]
Pichia pastoris	7,596 U/L[f]	[64]
Pichia pastoris	23,000 U/L [e]	[100]
Pichia pastoris	12,532 U/L[e]	[59]
Pichia pastoris [b]	218,926 U/L [f]	Tv1 A9
Pichia pastoris [c]	338,616 U/L [f]	Tv3 H2
Saccharomyces cerevisiae	~110 U/g dcw [f]	[60]
Kluveromyces lactis	~150 U/g dcw [f]	[60]
Escherichia coli [d]	12,340 U/L [f]	[59]

[a] Induced with N-carbamoyl-D-alanine
[b] Induced with reduced methanol feed
[c] Induced with Glycerin/MeOH mix-feed
[d] Induced with D-methionine results in 4.5 fold expression improvement
[e] Activity measured with D-alanine as substrate
[f] Activity measured with D-methionine as substrate
[dcw] dryed cell weight

4.6.6 Fermentation IV (cloning constructs)

4.6.6.1 Strains and culture conditions

In this final fermentation the strains generated via cloning technique based on the T2-plasmid were cultivated. One aim of this fermentation was to evaluate the various engineering steps which led to the development of the optimized construct Tv1.

Table 28: *Pichia pastoris* *Tv*DAO expression strains chosen for high cell density fermentation.
Tv1A9 was fermented as a control. syn. codon. opt. codon optimized synthetic *Tv*DAO. -SKL amino acid sequence at the C-terminus. –PNL amino acid sequence at the C-terminus. **ZeoR** Zeocin resistance gene, promoter and terminator are characteristic for B1- or T2-plasmids. B1/T2 P.p. expression constructs for integration.

Fermenter	Strain	*Tv*DAO gene	PTS1	ZeoR (plasmid)	Biomass [g wcw/Batch]
F1	cTv1a G11	native	SKL	T2	275
F2					277
F3	Tv1 A9	syn- codon opt.	SKL	B1	114
F4	cTv1 B10	syn- codon opt.	SKL	T2	144
F5	cTv5 H3	syn- codon opt.	PNL	T2	259
F6					264
F7	cTv5a G4	native	PNL	T2	221
F8					212

To compare the performance of the cloning strains with the oePCR strains the culture conditions were kept the same as in fermentation I. The standard *P. pastoris* fermentation protocol with a standard MeOH feed rate 3 mL/h was followed. The best strain from the first fermentation Tv1 A9 was cultivated again as benchmark strain.

4.6.6.2 Analysis of oxidase activity

During fermentation samples were taken every 12 hours, starting prior to induction phase (90 hours). OD_{600}, wcw and activity via photometric assay was determined of each sample. Results can be seen in Figure 41.

After 90 hours cell material was harvested via centrifugation, frozen for oxygen consumption measurements which were performed with french press lysed cells.

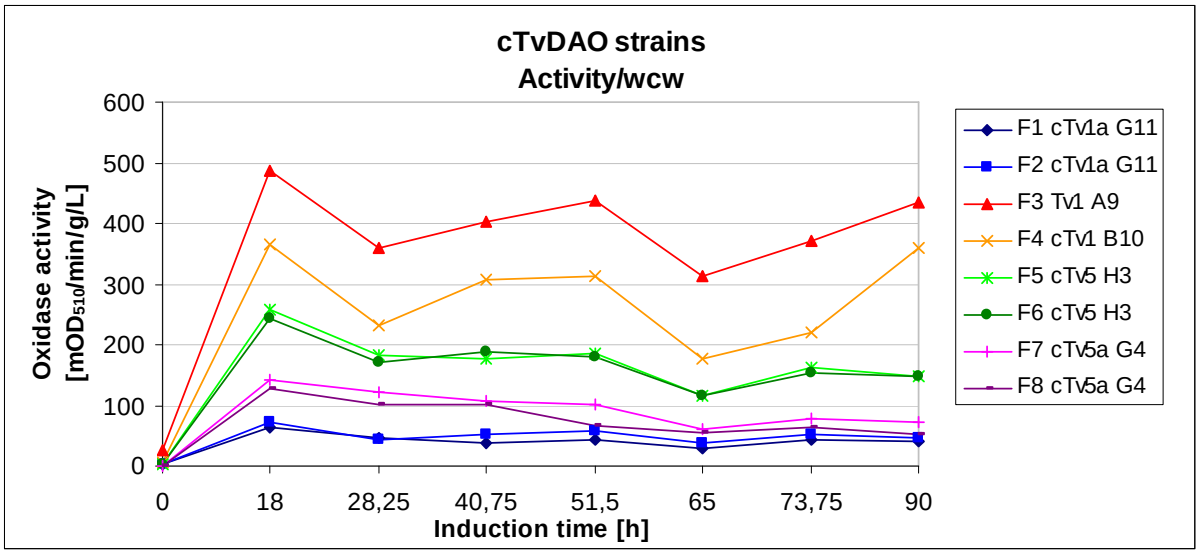

Figure 41: *P.pastoris* high cell density fermentation with standard 3mL/h MeOH induction feed. Photometrical activity determination of the fermentation course. **F1/F2 cTv1a G11** Best expressing strain harbouring native *Tv*DAO with engineered peroxisomal targeting sequence -SKL (fermenter 1 + 2). **F3 Tv1 A9** Best expressing strain harbouring codon optimized synthetic *Tv*DAO gene with engineered native peroxisomal targeting sequence -SKL, expression cassette constructed via oePCR (fermenter 3). **F4 cTv1 B10** Best expressing strain harbouring codon optimized synthetic *Tv*DAO gene with engineered peroxisomal targeting sequence -SKL (fermenter 4). **F5/F6 cTv5 H3** Best expressing strain harbouring codon optimized synthetic *Tv*DAO gene with native peroxisomal targeting sequence PNL (fermenter 5 + 6). **F7/F8 cTv5a G4** Best expressing strain harbouring native *Tv*DAO with native peroxisomal targeting sequence -PNL (fermenter 7 + 8).

Tv1/cTv1, harbouring the codon optimized gene and an exchanged peroxisomal targeting sequence -SKL, showed the highest activity. Noteworthy is that a difference in terms of activity could be observed between strains harbouring the same *Tv*DAO coding sequence but different Zeocin resistance marker.

The strain cTv5a, harbouring the unmodified *Tv*DAO showed relatively low activity. Surprisingly the first engineering step, the exchange of the peroxisomal targeting sequence (cTv1a) resulted in an unexpected activity decay of ~ 35%.

Starting from the unmodified *Tv*DAO strain cTv5a, a first improvement could be obtained by optimization of the codon usage based on high methanol expression in *P. pastoris* (cTv5) in

combination with the native peroxisomal targeting sequence –PNL. This synthetic gene showed a 2.5-fold improvement compared to the original sequence found in *Trigonopsis variabilis*. By combination of the codon-optimized gene with the engineered C-terminal targeting sequence –SKL, a dramatic expression enhancement could be observed in construct Tv1. This strain displayed 5.5-fold improvement compared to the original *Trigonopsis variabilis* sequence. Detailed results of the activity determination via oxygen consumption method can be found in Table 29.

Table 29: Intracellular activity comparison between *P.pastoris* strains harbouring native and engineered *Tv*DAO expression variants.
Cells were cultivated in a high cell density fermentation with standard 3mL/h MeOH induction feed. **F1/F2 cTv1a G11** Best expressing strain harbouring native *Tv*DAO with engineered peroxisomal targeting sequence -SKL (fermenter 1 + 2). **F3 Tv1 A9** Best expressing strain harbouring codon optimized synthetic *Tv*DAO gene with engineered native peroxisomal targeting sequence -SKL , expression cassette constructed via oePCR (fermenter 3). **F4 cTv1 B10** Best expressing strain harbouring codon optimized synthetic *Tv*DAO gene with engineered peroxisomal targeting sequence -SKL (fermenter 4). **F5/F6 cTv5 H3** Best expressing strain harbouring codon optimized synthetic *Tv*DAO gene with native peroxisomal targeting sequence PNL (fermenter 5 + 6). **F7/F8 cTv5a G4** Best expressing strain harbouring native *Tv*DAO with native peroxisomal targeting sequence -PNL (fermenter 7 + 8).

Fermenter	Strain	Intracellular activity [U/g fcw]	Biomass [g fcw/Batch]	Copy number
F1	cTv1a G11	90	274	1
F2		86	277	
F3	Tv1 A9	1118	114	18
F4	cTv1 B10	767	144	1
F5	cTv5 H3	348	259	1-2
F6		346	264	
F7	cTv5a G4	137	220	1
F8		133	212	

All strains except Tv1/cTv1 reached biomass yields above 200 g/L using the standard P. *pastoris* fermentation protocol. The lower biomass of Tv1 A9 and cTv1 B10 might be explained by the stress generated when large amount of recombinant DAO accumulates in the cell. The strain Tv1 grew normally in glycerol batch phase where *Tv*DAO expression was repressed, but not during the induction phase using methanol. Similar observations were made with other yeast host organisms, were reduced growth during induction due to apparent cell toxicity of recombinant DAO was reported [60, 64].

4.6.7 Overview of fermentation results

Summing up it can be said that the strains generated during this study exceed the activity of the *P. pastoris* WT1 benchmark strain (provided by Ingenza) by far.

Codon-optimization of the *Tv*DAO gene sequence led to improved activity (2.5-fold) as compared to the native *Trigonopsis variabilis* sequence. Drastic activity enhancement (5.5-fold) was observed at the combination of codon-optimization with exchange of the native C-terminal peroxisomal targeting sequence –PNL to an optimized –SKL sequence.

The *P. pastoris Tv*DAO expression strain Tv3 H2 is the most efficient host for production of *Tv*DAO that has been reported so far (see Table 27). Table 30 shows that in bioreactor cultivation the multi copy (mc) strain Tv1 A9 gave a twofold enhancement of specific oxidase activity as compared to the single copy (sc) strain cTv1 B10. This implies that the number of gene copies did not translate linearly into the titre of active *Tv*DAO.

Another interesting result is that the best expression strains only show relatively low biomass yields which can be explained by a possible toxicity effect of the recombinant *Tv*DAO or that the highly engineered expression strains suffer a metabolic load which leads to a slow growth behaviour.

Table 30: Enzyme and strain engineering for efficient expression of *Tv*DAO in *Pichia pastoris*
Enzyme activities are reported for *P. pastoris* cells obtained in 1.5-L bioreactor cultivation employing standard induction with 3mL/min methanol. Codon usage: native as in the original *Tv*DAO gene, or optimized for expression using the AOX1 promoter. *PTS1* – peroxisomal targeting using the native C-terminal tripeptide PNL or the engineered motif SKL. *CN*: Number of copies of the expression cassette integrated into the *P. pastoris* genome. Results as already accepted for publication in [99].

Strain	Sequence		CN	Intracellular activity	Biomass
	Codon usage	PTS1		[U/g wcw]	[g wcw/batch]
WT1	native	-	7-9	64	210
Tv1 A9	optimized	SKL	18-19	1283	117
Tv1a C4	native	SKL	5	550	240
Tv3 H2	optimized	SKL	16-17	1448	131
cTv1 B10	optimized	SKL	1	767	144
cTv1a G11	native	SKL	1	90	274
cTv5 H3	optimized	PNL	1-2	348	259
cTv5a G4	native	PNL	1	137	220

Concluding remark: The data of the bioreactor cultivation were obtained in collaboration with Sandra Abad and have already been accepted for publication in [99].

5 Conclusion

This study reports the successful development of an innovative *P. pastoris* DAO whole-cell biocatalysator that is suitable for application in industrial deracemization processes.

Sequence analysis of oxidases from different sources was used to obtain more detailed information about the target oxidases and chose suitable strategies for expression in *Pichia pastoris*. These constructs included the design of synthetic genes, which are codon-optimized for expression in *P. pastoris* under conditions of methanol induction and exchange of the C-terminal peroxisomal targeting sequence. Even though the sequences of several oxidases were analysed and constructs were designed, the main focus of this study lies on the expression of *Tv*DAO in *P. pastoris*.

Therefore two strategies to assembly the expression cassette for *P. pastoris* expression were chosen. Firstly assembly of a linear expression cassette by oePCR and secondly a classical cloning approach. These two strategies also used different plasmids (pPP-B1 and pPP-T2) as origin of the Zeocin cassette.

With these two strategies *P. pastoris* strains, harbouring the various constructs, were generated and cultivated in deep well plates. The clones were screened and rescreened for oxidase activity using an enzyme-coupled colorimetric assay. The best clones were selected for large scale cultivation in bioreactors to produce biomass under controlled parameters and characterize the generated strains. The data showed that all generated *P. pastoris* strains have a higher activity than the benchmark strain WT1 (provided by Ingenza). Furthermore it was shown that codon-optimization of the *Tv*DAO gene lead to 2.5-fold activity improvement. The additional exchange of the peroxisomal targeting sequence (PTS1) improved the activity further (5.5-fold as compared to the native *Trigonopsis variabilis* sequence in *P. pastoris*). To our knowledge this is the first report describing a combination of PTS1 exchange with codon optimization of a gene for improved expression. Additionally it was found out that multi copy strains show higher activities than single copy strains, but that the copy number does not correlate linearly to the activity.

Notwithstanding the generated *P. pastoris* strain Tv3 H2 is the most efficient host for production of *Tv*DAO that has been reported so far. Additional process development in terms of feeding strategy during bioreactor cultivation helped to overcome growth deficiencies in the cell and enhanced the biomass yield from 120g/L at standard conditions to 200 g/L.

The overall concept and additive as well as synergistic effects from different individual improvements lead to a highly active and robust biocatalyst which might be interesting for industrial applications.

6 References

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7 Appendix

7.1 Strains generated during diploma thesis

Table 31: Strains generated during this work, deposited in the culture collection

BT Number	Organism	Host Strain	Name	Comments	VNTI-file
5965	*E. coli*	TOP10F´	pJET-Tv1	*Tv*DAO: codon opt., -SKL, B1-ZeoR	pJET-Tv1
5966	*E. coli*	TOP10F´	pJET-Tv3	*Tv*DAO: codon opt., -SKL, N-term citrine fusion, B1-ZeoR	pJET-Tv3
3633	*E. coli*	TOP10F´	pJET-Tv1a	*Tv*DAO: native sequence., -SKL, B1-ZeoR	pJET-Tv1a
3623	*E. coli*	TOP10F´	T2- Tv1	*Tv*DAO: codon opt., -SKL, T2-ZeoR	T2- Tv1
3624	*E. coli*	TOP10F´	T2-Tv1a	*Tv*DAO: native, -SKL, T2-ZeoR	T2-Tv1a
3625	*E. coli*	TOP10F´	T2-Tv5	*Tv*DAO: codon opt., -PNL, T2-ZeoR	T2-Tv5
3626	*E. coli*	TOP10F´	T2-Tv5a	*Tv*DAO: native, -PNL, T2-ZeoR	T2-Tv5a
3577	*P. pastoris*	Muts7	Tv1 A9	*Tv*DAO: codon opt., -SKL, B1-ZeoR,	pJET-Tv1
3634	*P. pastoris*	Muts7	Tv1a C4	*Tv*DAO: native sequence., -SKL, B1-ZeoR	pJET-Tv1a
3576	*P. pastoris*	Muts7	Tv3 H2	*Tv*DAO: codon opt., -SKL, N-term citrine fusion, B1-ZeoR	pJET-Tv3
3575	*P. pastoris*	KM71H	WT1	*Tv*DAO: codon opt., 5 pointmutations, Invitrogen material!	WT1
3629	*P. pastoris*	Muts7	T2- Tv1 B10	*Tv*DAO: codon opt., -SKL, T2-ZeoR	T2- Tv1
3630	*P. pastoris*	Muts7	T2-TV1a G11	*Tv*DAO: native, -SKL, T2-ZeoR	T2-Tv1a
3631	*P. pastoris*	Muts7	T2-Tv5 H3	*Tv*DAO: codon opt., -PNL, T2-ZeoR	T2-Tv5
3632	*P. pastoris*	Muts7	T2-Tv5a G4	*Tv*DAO: native, -PNL, T2-ZeoR	T2-Tv5a

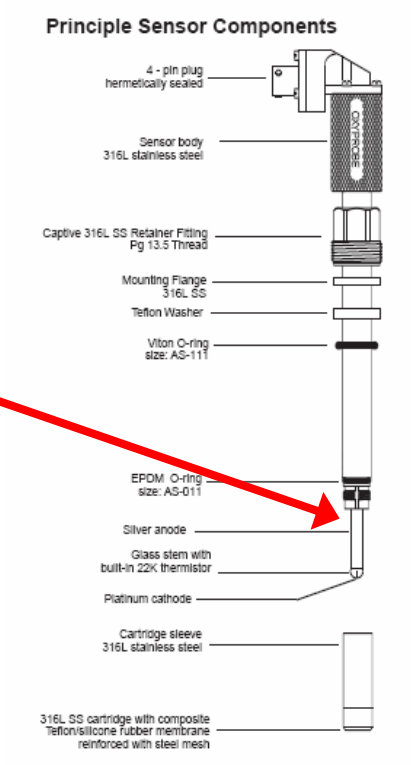
7.2 Protokoll DAS GIP Fermentation

DURCHFÜHRUNG

Tag 1 (Mi)

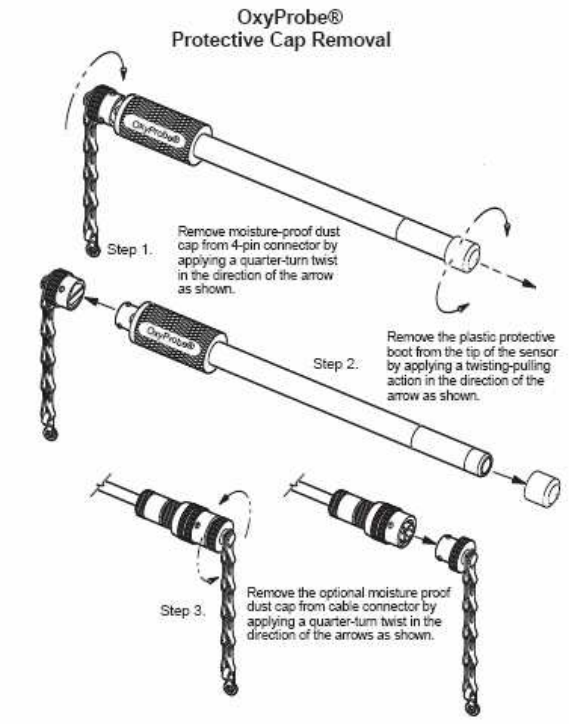
O2 Elektroden putzen

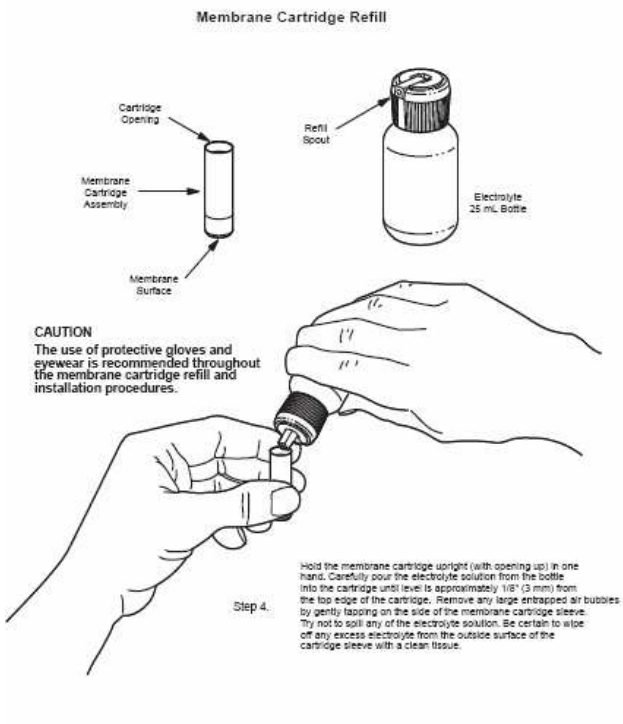
schwarze Schutzkappe abziehen
Elektrode aufschrauben
Elektrolytlösung ausleeren, mit Wasser spülen
Elektrode mit Zahnbürste und Zahnpasta putzen
(Vorsicht bei Glaskopf)
gründlich mit Wasser spülen
mit Zellstoff trocknen
neue Elektrolytlösung (Oxyprobe Elctrolyte, Broadley James Corp.) einfüllen
zusammenbauen, Schutzkappe aufsetzen

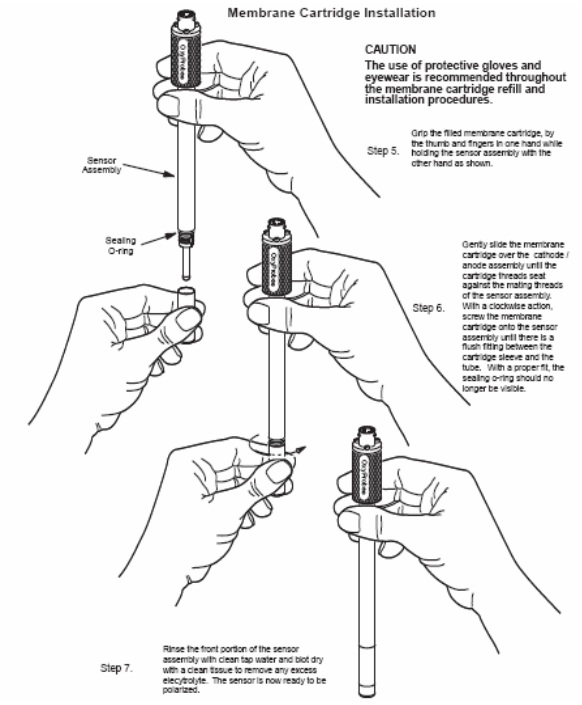


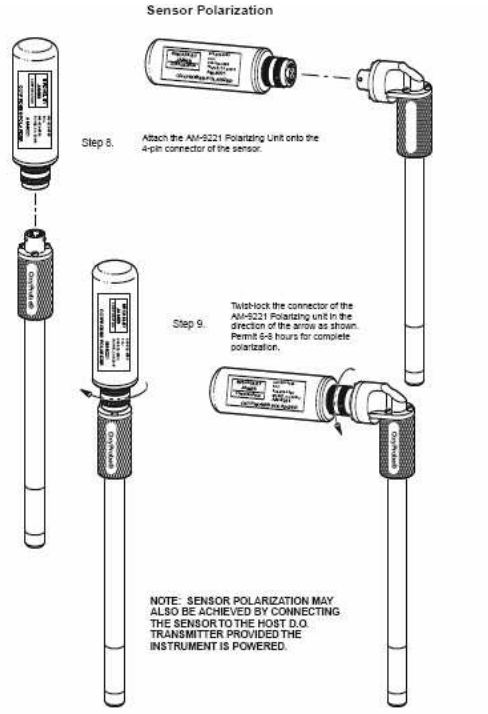
danach die Elektroden nicht mehr umlegen sondern aufrecht transportieren

zum **polarisieren** über Nacht an die Kabel hängen
mind. 6-8 Stunden









Fermenter putzen

mit H2O füllen und nochmals autoklavieren, nochmals reinigen, und über Nacht trocknen lassen

<u>Reinigung</u>

Schläuche, Filter runter
Feed lines durchspülen (Wasser, EtOH)
Glaskörper mit heißem Wasser und Seife schrubben, mit dest. Wasser nachspülen
Rührwerk mit Bürste abschrubben
Sparkler durchspülen, überprüfen ob richtig verschraubt
Leitfähigkeitselektrode festschrauben

VK1 ansetzen

Vorkultur in 50 mL BMGY (mit Glycerol oder Glucose) ansetzen

80 mg/L Zeocin verwenden um Kopiezahl Verlust zu verhindern
Achtung bei WT Stämmen kein Antibiotika verwenden!

Vorkultur Medium

VK Medium mit Glycerol

10 g	Yeast Extract	autoklavieren
20 g	Peptone	
100 mL	H2O	
+ 100 mL	YNB	
+ 100 mL	PPB	
+ 100 mL	10 X Glycerol (10% v/v)	

80-100 mg/ L Zeo

VK Medium mit Glucose

10 g	Yeast Extract	autoklavieren
20 g	Peptone	
140 mL	H2O	
+ 100 mL	YNB	
+ 100 mL	PPB	
+ 60 mL	10 X D	

80-100 mg/ L Zeo

Tag 2 (Do)

VK2 ansetzen

pro Fermenter 50 mL BMGY (mit Glycerol oder Glucose)
um 18 Uhr (Start der Fermentation, Inokulierung) soll die OD 10 sein (laut Andrea besser 15-20)

Autoklavieren

Spüllösungen und Lösungen zur pH Korrektur [9]
Feedlösungen
2x 1L Kolben zum Schläuche spülen
Messzylinder für MeOH
Bechergläser um VK zu poolen

Spüllösungen

80% EtOH (150 mLH2O + 600 mL EtOH)
2M NaOH (60g/ 750mL H2O)
H2O
Substrat

pH Korrektur

25% NH3
10% H3PO4 (360mL H2O + 40 mL Säure)

ACHTUNG!!!

EtOH, Säure, NH3, MeOH, … erst nach dem autoklavieren in der Cleanbench in die Feedflaschen füllen

[9] Spüllösungen können auch schon an Tag 1 hergestellt und autoklaviert werden

Fed Batch Feeds

Feed Gylcerol

			Menge	1,133333	kg	
		Molare Masse	ml/kg	g/kg	**Einwaage**	
autoklavieren	Glycerol 100% v/v	92,1		587,7	**666,06**	g
	VE Wasser			auf	**1,00**	L auffüllen
sterfiltrieren	PTM1 Trace Salts mit Biotin		12		**12,00**	mL

ODER

Feed Glucose

			Menge	0,63	kg	
		Molare Masse	ml/kg	g/kg	**Einwaage**	
autoklavieren	D(+) Glukose Monohydrat			632,3	**398,35**	g
	VE Wasser			auf	**0,50**	L auffüllen
sterfiltrieren	PTM1 Trace Salts mit Biotin		12		**12,00**	mL

Induktion

Feed Methanol

			Menge	1	kg	
		Molare Masse	ml/kg	g/kg	**Einwaage**	
	Methanol 100% v/v			1000	**1000,00**	g
sterfiltrieren	PTM1 Trace Salts mit Biotin		12		**15,17**	mL

1000g MeOH entspricht 1,27 L (ϱ=0,79 g/mL)

ODER

Mixfeed (Glucose – Methanol)

			Menge	1,00	kg	
		Molare Masse	ml/kg	g/kg	**Einwaage**	
	Methanol 100% v/v			452,24	**452,24**	g
autoklavieren	D(+) Glukose Monohydrat			346,34	**346,34**	g
	VE Wasser		auf	0,83	**171,08**	g
sterilifiltrieren	PTM1 Trace Salts mit Biotin		12		**15,17**	mL

454,24g MeOH entspricht 0,57 L (ϱ=0,79 g/mL)

ODER

Mixfeed (Glycerol – Methanol)

			Menge	1	kg	
		Molare Masse	ml/kg	g/kg	**Einwaage**	
	Methanol 100% v/v			452,24	**452,24**	g
autoklavieren	Glycerol 100% v/v			321,90	**321,90**	g
	Wasser		auf	0,80	**195,52**	g
sterilfiltrieren	PTM1 Trace Salts mit Biotin		12		**15,17**	mL

454,24g MeOH entspricht 0,57 L (ϱ=0,79 g/mL)

Batch Medien

600 mL Arbeitsvolumen

1.) Salze einwiegen
2.) C-Quelle einwiegen (Glycerol oder Glucose)
3.) mit Wasser auffüllen
4.) Phosphorsäure zugeben

Batch Medium mit Glycerol

		Summenformel	Menge für 600 mL	
autoklavieren	Phosphorsäure 85%	H_3PO_4	2.55	mL
	Calciumsulfat Dihydrat	$CaSO_4 \cdot 2H_2O$	0.10	g
	Kaliumsulfat	K_2SO_4	1.72	g
	Magnesiumsulfat Heptahydrat	$MgSO_4 \cdot 7H_2O$	1.39	g
	Kaliumhydroxid	KOH	0.38	g
	EDTA Disodiumsalz Dihydrat	$C_{10}H_{12}N_2Na_2O_8$ monohydrat	0.36	g
	Natriumchlorid	NaCl	0.13	g
	Glycerol 100% v/v	$C_3H_8O_3$	24	g
	Wasser + Inokulum (10%)	H_2O	auf 600	mL
	PTM1 Trace Salts mit Biotion		2.61	mL
	Antifoam (Acepol 10% v/v)		0,1	mL

ODER

Batch Medium mit Glucose

		Summenformel	Menge für 600 mL	
autoklavieren	Phosphorsäure 85%	H_3PO_4	2.55	mL
	Calciumsulfat Dihydrat	$CaSO_4 \cdot 2H_2O$	0.10	g
	Kaliumsulfat	K_2SO_4	1.72	g
	Magnesiumsulfat Heptahydrat	$MgSO_4 \cdot 7H_2O$	1.39	g
	Kaliumhydroxid	KOH	0.38	g
	EDTA Disodiumsalz Dihydrat	$C_{10}H_{12}N_2Na_2O_8$ monohydrat	0.36	g
	Natriumchlorid	NaCl	0.13	g
	Glucose Monohydrat	$C_6H_{12}O_6\ H_2O$	25.83	g
	VE Wasser + Inokulum (10%)	H_2O	auf 600	mL
	PTM1 Trace Salts mit Biotin		2.61	mL
	Antifoam (Acepol 10% v/v)		0,1	mL

pH Elektroden kalibrieren

Elektroden in richtiger Position anschließen
aus Elektrolytlösung in leeres PLASTIK Becherglas geben und mit dest. Wasser spülen
Prozedur pH Kalibration am PC starten
Elektroden in pH Kalibrationslösung (pH 7) halten
am PC „Start" drücken
Elektroden mit dest. Wasser spülen
Elektroden in pH Kalibrationslösung (pH 4) halten
am PC „Start" drücken
Elektroden mit dest. Wasser spülen
nicht mehr in Elektrolytlösung geben sondern direkt in Fermenter einbauen

O2 Elektroden kontrollieren

Prozedur „O2 Kalibration" am PC starten
schauen ob alle Elektroden reagieren

schwarze Kappe runter = 70 – 100 % O2
Elektrode mit N2 über Greiner-Schlauch-Konstruktion „spülen" (O2 Gehalt sollte sinken)
Kalibration wird aber nicht jetzt, sondern erst nach dem Autoklavieren, kurz vor dem Fermentations - Start durchgeführt

Fermenter zusammenbauen

kontrollieren ob Glasgefäß und Rührwerk sauber sind
Kühlungsröhren installieren
neue Schläuche und Filter für Zu-/Abluft installieren (auf INLET Richtung achten, IN soll in O2-Fluss Richtung sein), mit Kabelbinder fixieren
Batch Medium einfüllen und Rührwerk aufsetzen
Feed Anschlüsse mit Deckel verschließen
Probenahmeventil (lila) montieren und mit lila Deckel verschließen, weiße Klemme schließen
Filter mit Alu verschließen
Seitenausgänge des Fermenters verschließen: grüner Deckel, roter Deckel mit Membran
Elektroden montieren und Autoklavierkappen aufsetzen, auf Farbcode achten
O2 Elektrode gegenüber vom Sparkler montieren




autoklavieren (Seitendeckel leicht geöffnet, Elektroden und Filter verschlossen)

Spül- und Feedlösungen fertig machen

Ethanol, Säure, Methanol, PMT1, NH3 unter sterilen Bedingungen in der Cleanbench in die Feedflaschen geben

Schläuche spülen

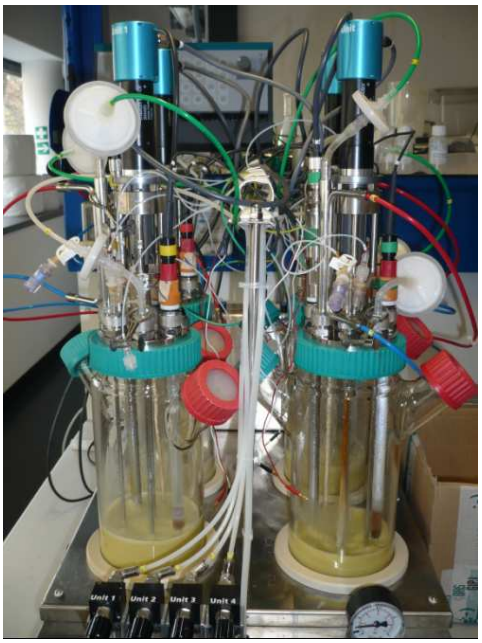
Feed Anschlüsse (je 4x gelb, rot, grün, blau) mit Nadeln versehen und in leere Kolben stechen (durch Alufolie)

alle Anschlüsse vor dem Öffnen mit EtOH desinfizieren
Waschen mit: 80% EtOH, 2M NaOH, H2O, Substratlösung

Anschluss der „Substratlösungen“:

Pumpe	Farbe	Lösung	
A	gelb	Säure	H_3PO_4
B	grün	Base	NH_3
C	rot	Feed (FedBatch Phase)	Glycerol oder Glukose
D	blau	Induktion	MeOH oder Mix Glycerol oder Mix Glukose

Fermenter anschließen



Achten auf Nummerierung bzw Farbcode:

1 gelb
2 grün
3 rot
4 blau
5 gelb – weiß
6 grün – weiß
7 rot – weiß
8 blau – weiß

Motor installieren (auf Nummerierung achten!!!)
Zuluftfilter an weiße Schläuche anschließen (grüne Schläuche führen zur Abgasanalytik, eventuell an Abluftfilter anschließen)
Kühlungsrohr: blauer Schlauch unten, roter oben (fest reinstecken durch rausziehen der Metallhülse fixieren), Wasser an der Rückseite aufdrehen
pH Elektrode anschließen (rote Anschlüsse, Nummerierung!!)
O2 Elektrode anschließen (silber Anschlüsse, Nummerierung!!)
Thermofühler in Hülse stecken
Erdung (grün/gelbes Kabel) an Deckel anschließen

2,6 mL PMT1 und 1 mL Antifoam (10% v/v) mit Sterilfilter-Spritze durch Membran in den Fermenter geben (lange Nadel verwenden)

O2 AN
H2O AN

pH einstellen auf pH 6

manuell Pumpen einschalten, Prozedur pH Kalibration zur pH Überwachung
NICHTS drücken sonst sind die Elektroden nicht mehr kalibriert!!!

pO2 Kalibrierung

Prozedur pO2 Kalibrierung am PC Starten

zuerst Sauerstoff einschalten, maximale Drehzahl bei der Rührung (=100% Sauerstoff)
dann Stickstoff anschließen(= 0% Sauerstoff)

anschließend O2 wieder einschalten!

neuen Arbeitsablauf starten

→ basierend auf …
→ bearbeiten
→ ausführen

Rührer AN
pH AN
Temperatur AN
Pumpen AN

Inokulieren

50 mL VK2 durch Membran in den Fermenter spritzen (lange Nadeln verwenden)

Start Fermentation

Fermentations Parameter

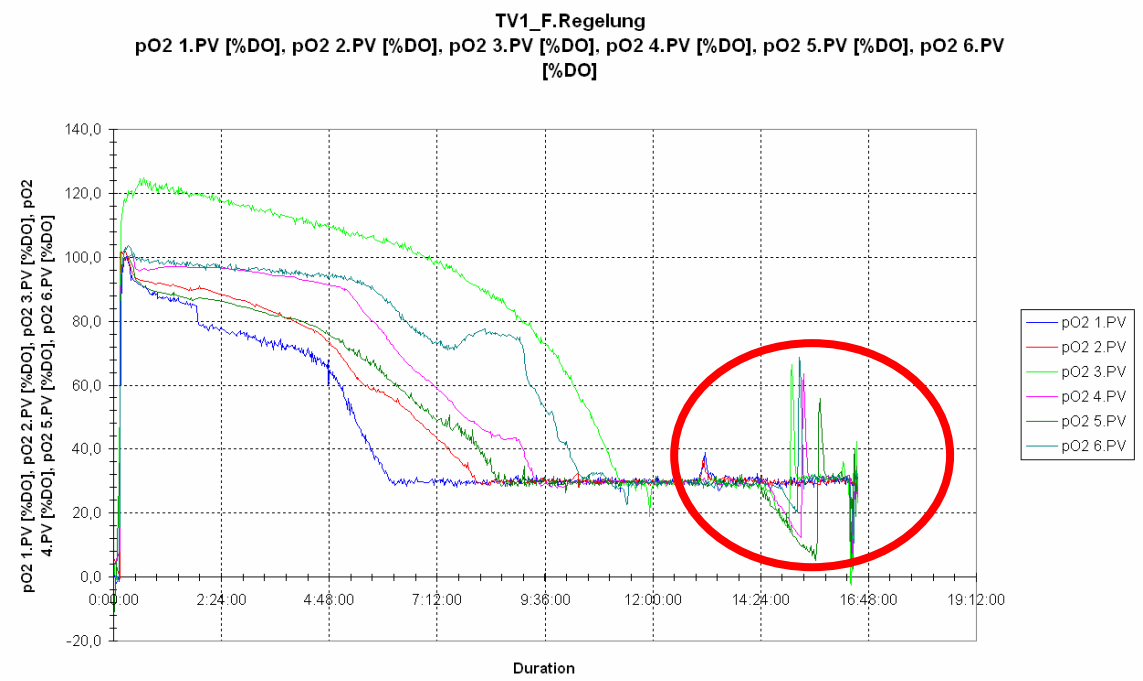
Temperatur	28°C
Rührerdrehzahl	500 – 1250 rpm
DO Kontrolle über Rührung	30%
pH Kontrolle mit 25% NH3	pH 6
Hungerphase	ca. 30 min
Feed	6 Stunden
Expressionsphase	90 Stunden (normal 3 mL MeOH / h)

OD VK2

OD der VK2 bestimmen

Tag 3 (Fr)

Zellen wachsen über Nacht und verbrauchen C-Quelle im Batch Reaktor, wodurch O2 verbraucht wird. Sobald die Zellen keine C-Quelle mehr haben stoppt der Metabolismus, dadurch wird kein/weniger O2 verbraucht und der DO Wert steigt an. Es bildet sich ein Peak(siehe roter Kreis) in der DO Kurve. Dies wird durch Verringerung der Rührerdrehzahl ausgeglichen wodurch der DO Wert wieder sinkt, ein Wechselspiel zwischen O2-Gehalt und Rührerdrehzahl entsteht.



Trigger auslösen

warten auf Hungerpeak (siehe Bild, ca. 12-16 Stunden nach Inokulation)
dann Pumpen C und D bei Hauptregelung einschalten → Trigger (offline value A) auslösen → 6 Stunden später fängt die Induktion mit MeOH (bzw. Mix feeds) an

Fermentation läuft 90 Stunden ab dem Zeitpunkt der Induktion

Programm Tv1_F (etc.) hat eine längere Induktionszeit (100 h) eingestellt, um das Ende der Induktion nicht zu versäumen.

Probe nehmen

Probe 0 (kurz vor Induktionsstart)

Probe aus Fermenter entnehmen
Verschluss desinfizieren mit EtOH
Spritze mit Drehverschluss and er Öffnung fixieren
Klemme öffnen
ca. 10 mL entnehmen
Klemme und Verschluss schließen
Entnommenes verwerfen
Verschluss erneut desinfizieren
Spritze mit Drehverschluss an der Öffnung fixieren
Klemme öffnen
Probe entnehmen (ca. 2 mL)
Deckel und Klemme schließen

ZNG bestimmen
Eppi abwiegen
500 µL Zellen in Eppi pippetieren
zentrifugieren (10 min, 4°C, 13.200 rpm)
Überstand verwerfen
zentrifugieren (2 min, 4°C, 13.200 rpm)
restlichen Überstand mit einer Pippetenspitze abnehmen
wiegen
ZNG= Eppi mit Zellen – Eppi
Probe einfrieren (-20°C) für spätere Aktivitätsmessung

OD600 bestimmen
Verdünnung mit 100 mM PPB pH 6
während Fermentation ca. 1:1000 verdünnen

Tag 4 (Sa), 5 (So), 6 (Mo)

Probe nehmen

jeweils in der Früh und am Abend (am besten ca. 12 Stunden auseinander)

Zentrifuge reservieren für Ernte!!!

Tag 7 (Di)

Ernte

benötigte Utensilien
Sackerl
Gummiringe
Pipettboy + Spitzen
Pipette + Spitzen
gr. Becherglas als Müll
kl Becherglas zum Austarieren
100 mM PPB pH 6
50 mL Greini

ERNTE
Zentrifuge auf 4°C vorkühlen

Fermenter einzeln beenden(damit Zellen nicht pelletieren)
am PC: bei jedem Fermenter (Setup1-8) BEENDEN klicken
warten bis Rührer ausgeschaltet ist
Fermenter abbauen
ACHTUNG Schutzbrille tragen (in den Feed Anschlüssen ist NH3, Säure und MeOH)

Fermenter in Zentrifugenraum tragen
Sackerl mit einer Ecke nach unten in den Zentrifugenbecher geben
Zellen reinleeren (2 Becher pro Fermenter)
zentrifugieren (Rotor JA 10, 4000 rpm, 10 min, 4°C)
Überstand verwerfen
Zellpellet mit 50 mL 100 mM PPB pH 6 waschen
Sacker mit einer Ecke nach unten in den Becher geben
zentrifugieren (Rotor JA 10, 4000 rpm, 10 min, 4°C)
Überstand verwerfen

Zellpellet abwiegen und einfrieren (an Jozef übergeben)

Feed Anschlüsse an Nadeln anschließen, in die Waschkolben stecken
Schläuche mit sterilem dest. Wasser waschen

Fermenter totautoklavieren

Darauf achten dass die Elektroden mit Schutzkappen verschlossen sind
Filter evt. abnehmen oder mit Alu verschließen

Putzen

Schläuche, Filter runter
Feed Anschlüsse durchspülen (Wasser, EtOH)
Glaskörper mit heißem Wasser und Seife schrubben, mit dest. Wasser nachspülen
Rührwerk mit Bürste abschrubben

Daten exportieren

fertiger Arbeitsablauf → rechte Maustaste → Daten exportieren → Daten werden in Ordner „DAS GIP Export" am Desktop exportiert
Regelungsdatei öffnen und als .exe Datei speichern

Tag 8 (Mi)

Aktivitätsbestimmung der Proben

500 µL der Zellsuspension wurden 10 min, 13000 rpm, 4 °C zentrifugiert
Überstand verworfen und weiter 2 min zentrifugiert. Überstand wieder verworfen.
ZNG wurde bestimmt.
Das Pellet wurde eingefroren.
Pellet wurde aufgetaut und mit 300 µL Yeastbuster mit der Pipette resuspendiert
Suspension 10 min, 13000 rpm,4°C zentrifugiert
ca.300 µL in einer Mikrotiterplatte transferiert
Aktivitätsmessung bei 30 °C, 510 nm

Zelltrümmer wurden wieder für 10 min zentrifugiert.
Restlicher Überstand entnommen, und mit 200 µL Yeastbuster resuspendiert
Suspension 10 min, 13000 rpm, 4 °C zentrifugiert
Aktivitätsmessung bei 30°C, 510 nm

Auswertung

jeweiliges Volumen bei den Probenahmen und Endvolumen aus exportierten Daten herausschreiben

berechnen von:
Aktivität
Aktivität / ZNG
Aktivität / OD
Aktivität / Volumen

MEDIEN

Vorkultur

VK Medium mit Glycerol

10 g	Yeast Extract	} autoklavieren
20 g	Peptone	
100 mL	H2O	
+ 100 mL	YNB	
+ 100 mL	PPB	
+ 100 mL	10 X Glycerol (10% v/v)	

80-100 mg/ L Zeo

VK Medium mit Glucose

10 g	Yeast Extract	} autoklavieren
20 g	Peptone	
140 mL	H2O	
+ 100 mL	YNB	
+ 100 mL	PPB	
+ 60 mL	10 X D	

80-100 mg/ L Zeo

Spurenelementlösung PTM1 (mit Biotin)

PTM1 (alt) – Fermentation 04/2008 – P. pastoris CBS7435 – T2-Stämme

	M [g/mol]	**(4.35 mL/L)**	
Biotin		150	mg
$CuSO_4 \cdot 5\ H_2O$	249.69	4.5	g
NaI	149.89	60	mg
$MnSO_4 \cdot H_2O$	169.02	2.25	g
$Na_2MoO_4 \cdot 2\ H_2O$	241.95	150	mg
H_3BO_3	61.833	15	mg
$CoCl_2 \cdot 6\ H_2O$	237.93	687	mg
$ZnCl_2$	136.28	15	g
$FeSO_4 \cdot 7\ H_2O$	278.0176	48.75	g
H_2SO_4		3.75	mL
Endvolumen		0.75	L

sterilfiltrieren und bei 4°C lagern

Batch Medien

600 mL Arbeitsvolumen

1.) Salze einwiegen
2.) C-Quelle einwiegen (Glycerol oder Glucose)
3.) mit Wasser auffüllen
4.) Phosphorsäure zugeben

Batch Medium mit Glycerol

		Summenformel	Menge für 600 mL	
autoklavieren	Phosphorsäure 85%	H_3PO_4	2.55	mL
	Calciumsulfat Dihydrat	$CaSO_4 \cdot 2H_2O$	0.10	g
	Kaliumsulfat	K_2SO_4	1.72	g
	Magnesiumsulfat Heptahydrat	$MgSO_4 \cdot 7H_2O$	1.39	g
	Kaliumhydroxid	KOH	0.38	g
	EDTA Disodiumsalz Dihydrat	$C_{10}H_{12}N_2Na_2O_8$ monohydrat	0.36	g
	Natriumchlorid	NaCl	0.13	g
	Glycerol 100% v/v	$C_3H_8O_3$	24	g
	Wasser + Inokulum (10%)	H_2O	auf 600	mL
	PTM1 Trace Salts mit Biotion		2.61	mL
	Antifoam (Acepol 10% v/v)		0,1	mL

ODER

Batch Medium mit Glucose

		Summenformel	Menge für 600 mL	
autoklavieren	Phosphorsäure 85%	H_3PO_4	2.55	mL
	Calciumsulfat Dihydrat	$CaSO_4 \cdot 2H_2O$	0.10	g
	Kaliumsulfat	K_2SO_4	1.72	g
	Magnesiumsulfat Heptahydrat	$MgSO_4 \cdot 7H_2O$	1.39	g
	Kaliumhydroxid	KOH	0.38	g
	EDTA Disodiumsalz Dihydrat	$C_{10}H_{12}N_2Na_2O_8$ monohydrat	0.36	g
	Natriumchlorid	NaCl	0.13	g
	Glucose Monohydrat	$C_6H_{12}O_6\ H_2O$	25.83	g
	VE Wasser + Inokulum (10%)	H_2O	auf 600	mL
	PTM1 Trace Salts mit Biotin		2.61	mL
	Antifoam (Acepol 10% v/v)		0,1	mL

Feed Berechnungen

	C	H	O	H_2O	M	C-Anteil	Dichte kg/L
	12,0107	1,00794	15,9994	18,01528	g/mol	gC/gZucker	(25°C)
Glycerin	3	8	3	0	92,094	0,391	1,25
Glucose	6	12	6	0	180,156	0,400	
Glucose Monohydrat	6	12	6	1	198,171	0,364	
Methanol	1	4	1	0	32,042	0,375	0,791

Feed	Dichte		conc glu/gly		conc MeOH		conc C	conc MeOH C
glucosemonohydrat Medium	1,27	$kg\ L^{-1}$	0,63	$g_{glucosel}\ g_{H2O}^{-1}$			0,23	
glycerol Medium	1,13	$kg\ L^{-1}$	0,59	$g_{glyceol}\ g_{H2O}^{-1}$			0,23	
methanol Medium	0,79	$kg\ L^{-1}$			1	%		0,37
glucose- methanol	0,97	$kg\ L^{-1}$	0,35	$g_{glucosel}\ g_{H2O}^{-1}$	0,45	$g_{MeOH}\ g_{H2O}^{-1}$	0,13	0,17
glycerol- methanol	0,92	$kg\ L^{-1}$	0,32	$g_{glyceol}\ g_{H2O}^{-1}$	0,45	$g_{MeOH}\ g_{H2O}^{-1}$	0,13	0,17

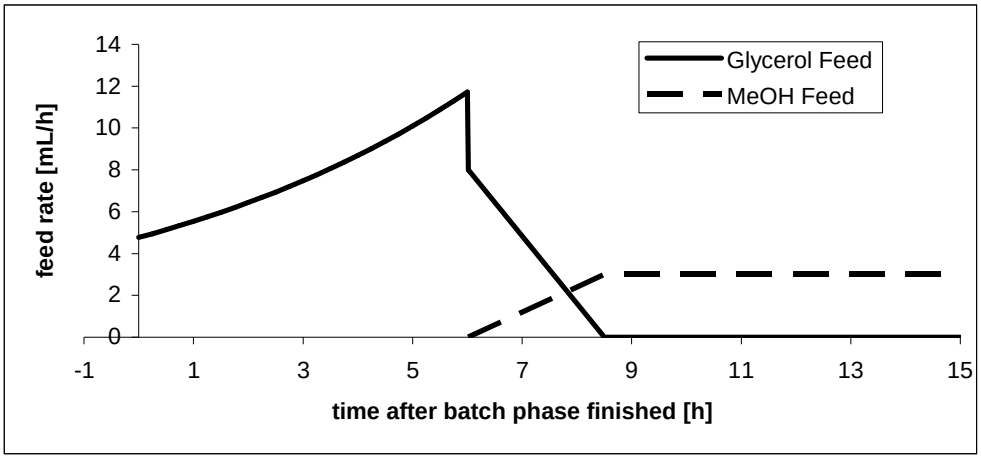
	feed (mL h-1)	feed (g h-1)	C-Quelle zucker		C-Quelle methanol
Methanol	3	2,37	0,89		
Glycerolfeed	3,41	3,86	0,89	x	
Glucosefeed	3,04	3,86	0,89	x	
Mixfeed (glu/meth)	3,10	3,01	0,38		0,51
Mixfeed (gly/meth)	3,25	3,01	0,38		0,51

Medium	Menge	Anzahl reaktoren	Dauer Fedbatchphase
glucosemonohydraht	772,75	2	100
glycerol	2318,24	6	100
methanol	1000,00	4	106
mix (glucose-methanol	1000,00	3	110,87
mix (glycerol-methanol)	1000,00	3	110,87

Feed Protokoll

Standard MeOH 3mL/h

t [h]	V [mL]	c_X [g L^{-1}]	M_X [g]	r_S [g h^{-1}]	r_{feed} [g h^{-1}]	v_{feed} [mL h^{-1}]			
0.00	650	24	15.6	3.90	5.57	4.77	μ	0.15	h^{-1}
0.25	651.2	24.9	16.2	4.05	5.78	4.95	Y_{SX}	0.6	g g^{-1}
0.50	652.4	25.8	16.8	4.20	6.01	5.14	C_{feed}	700	g kg^{-1}
0.75	653.7	26.7	17.5	4.36	6.23	5.33			
1.00	655.0	27.7	18.1	4.53	6.47	5.54	q_S	0.25	g $(g\ h)^{-1}$
1.25	656.4	28.7	18.8	4.70	6.72	5.75			
1.50	657.9	29.7	19.5	4.88	6.98	5.97	ρ_{Glyc}	1.26	g L^{-1}
1.75	659.4	30.8	20.3	5.07	7.24	6.20	ρ_{Feed}	1.17	g L^{-1}
2.00	660.9	31.9	21.1	5.26	7.52	6.43			
2.25	662.5	33.0	21.9	5.47	7.81	6.68			
2.50	664.2	34.2	22.7	5.67	8.11	6.94			
2.75	665.9	35.4	23.6	5.89	8.42	7.20		1,264	g L^{-1}
3.00	667.7	36.6	24.5	6.12	8.74	7.48			
3.25	669.6	37.9	25.4	6.35	9.07	7.76			
3.50	671.5	39.3	26.4	6.59	9.42	8.06			
3.75	673.5	40.6	27.4	6.84	9.78	8.37			
4.00	675.6	42.1	28.4	7.11	10.15	8.69			
4.25	677.8	43.5	29.5	7.38	10.54	9.02			
4.50	680.1	45.1	30.6	7.66	10.94	9.36			
4.75	682.4	46.6	31.8	7.95	11.36	9.72			
5.00	684.8	48.2	33.0	8.26	11.79	10.09			
5.25	687.4	49.9	34.3	8.57	12.25	10.48			
5.50	690.0	51.6	35.6	8.90	12.71	10.88			
5.75	692.7	53.4	37.0	9.24	13.20	11.29			
6.00	695.5	55.2	38.4	9.59	13.70	11.72			
6.02	695.5	55.2	38.4	6.55	9.35	8.00	0	0	
8.50	705.5	61.3	43.2	0.00	0.00	0.00	3	2.48	h
15.00						mL/h	3	5	mL



Mix Feed

t [h]	V [mL]	c_X [g L^{-1}]	M_X [g]	r_S [g h^{-1}]	r_{feed} [g h^{-1}]	$v_{feedglycerol}$ [mL h^{-1}]	$v_{feedglucose}$ [mL h^{-1}]
0,00	650	24	15,6	3,90	6,63	5,85	5,26
0,25	651,5	24,9	16,2	4,05	6,89	6,08	5,47
0,50	653,0	25,8	16,8	4,20	7,15	6,31	5,67
0,75	654,6	26,7	17,5	4,36	7,42	6,55	5,89
1,00	656,2	27,6	18,1	4,53	7,71	6,80	6,12
1,25	657,9	28,6	18,8	4,70	8,00	7,06	6,35
1,50	659,7	29,6	19,5	4,88	8,31	7,33	6,59
1,75	661,5	30,7	20,3	5,07	8,62	7,61	6,84
2,00	663,4	31,7	21,1	5,26	8,95	7,90	7,11
2,25	665,4	32,9	21,9	5,47	9,30	8,20	7,38
2,50	667,4	34,0	22,7	5,67	9,65	8,52	7,66
2,75	669,5	35,2	23,6	5,89	10,02	8,84	7,95
3,00	671,8	36,4	24,5	6,12	10,40	9,18	8,26
3,25	674,1	37,7	25,4	6,35	10,80	9,53	8,57
3,50	676,4	39,0	26,4	6,59	11,21	9,89	8,90
3,75	678,9	40,3	27,4	6,84	11,64	10,27	9,24
4,00	681,5	41,7	28,4	7,11	12,09	10,66	9,59
4,25	684,1	43,1	29,5	7,38	12,55	11,07	9,96
4,50	686,9	44,6	30,6	7,66	13,03	11,49	10,34
4,75	689,8	46,1	31,8	7,95	13,52	11,93	10,73
5,00	692,8	47,7	33,0	8,26	14,04	12,39	11,14
5,25	695,9	49,3	34,3	8,57	14,58	12,86	11,57
5,50	699,1	50,9	35,6	8,90	15,13	13,35	12,01
5,75	702,4	52,6	37,0	9,24	15,71	13,86	12,47
6,00	705,9	54,4	38,4	9,59	16,31	14,39	12,95
6,02	705,9	54,4	38,4	5,33	9,07	8,00	7,20
8,50	715,8	59,2	42,3	0,00	0,00	0,00	0,00
15,00						mL/h	

μ	0,15	h^{-1}
Y_{SX}	0,6	g g^{-1}
C_{feed}	588	g kg^{-1}
q_S	0,25	g (g $h)^{-1}$
$\rho_{Glufeed}$	1,26	g L^{-1}
$\rho_{glyfeed}$	1,13	g L^{-1}
	1.264	g L^{-1}

Fed Batch Feeds

Feed Gylcerol

				Menge	1,133333	kg	
		Molare Masse		ml/kg	g/kg	**Einwaage**	
autoklavieren	Glycerol 100% v/v	92,1			587,7	**666,06**	g
	VE Wasser				auf	**1,00**	L auffüllen
sterfiltrieren	PTM1 Trace Salts mit Biotin			12		**12,00**	mL

ODER

Feed Glucose

			Menge	0,63	kg	
		Molare Masse	ml/kg	g/kg	**Einwaage**	
autoklavieren	D(+) Glukose Monohydrat			632,3	**398,35**	g
	VE Wasser			auf	**0,50**	L auffüllen
sterfiltrieren	PTM1 Trace Salts mit Biotin		12		**12,00**	mL

Induktion

Feed Methanol

			Menge	1	kg	
		Molare Masse	ml/kg	g/kg	**Einwaage**	
	Methanol 100% v/v			1000	**1000,00**	g
sterfiltrieren	PTM1 Trace Salts mit Biotin		12		**15,17**	mL

1000g MeOH entspricht 1,27 L (ϱ=0,79 g/mL)

ODER

Mixfeed (Glucose – Methanol)

		Molare Masse	Menge ml/kg	1,00 g/kg	kg **Einwaage**	
	Methanol 100% v/v			452,24	**452,24**	g
autoklavieren	D(+) Glukose Monohydrat			346,34	**346,34**	g
	VE Wasser		auf	0,83	**171,08**	g
sterilifiltrieren	PTM1 Trace Salts mit Biotin		12		**15,17**	mL

454,24g MeOH entspricht 0,57 L (ϱ=0,79 g/mL)

ODER

Mixfeed (Glycerol – Methanol)

		Molare Masse	Menge ml/kg	1 g/kg	kg **Einwaage**	
	Methanol 100% v/v			452,24	**452,24**	g
autoklavieren	Glycerol 100% v/v			321,90	**321,90**	g
	Wasser		auf	0,80	**195,52**	g
sterilfiltrieren	PTM1 Trace Salts mit Biotin		12		**15,17**	mL

454,24g MeOH entspricht 0,57 L (ϱ=0,79 g/mL)

Zusätzliche Lösungen

ACHTUNG!!!
EtOH, Säure, NH3, MeOH, … erst nach dem autoklavieren in der Cleanbench in die Feedflaschen füllen

<u>Spüllösungen</u>
80% EtOH (150 mLH2O + 600 mL EtOH)
2M NaOH (60g/ 750mL H2O)
H2O
Substrat

<u>pH Korrektur</u>
25% NH3
10% H3PO4 (360mL H2O + 40 mL Säure)

BERECHNUNGEN

Formeln zur Berechnung des Feeds

Gleichung 1: Berechnung der C- Konzentration

conc c = conc glu / gly . C Anteil Zucker [g C / g Zucker]

Gleichung 2: Berechnung der C Konzentration im Methanol

conc MeOH C = conc MeOH . C Anteil Zucker [g C / g Zucker]

Gleichung 3: Berechnung der Feedgeschwindigkeit

feed [g/h] = feed [mL/h] . ϱ

Gleichung 4: Berechnung der C-Quelle Zucker (für MeOH)

C-Quelle Zucker (MeOH) = feed [g/h] . conc MeOH . C Anteil [g C / g Zucker]

Gleichung 5: Berechnung der C-Quelle Zucker

$$\text{C-Quelle Zucker} = \frac{\text{C-Quelle Zucker (MeOH)} \cdot \text{conc C}}{\text{conc C + conc MeOH C}}$$

Gleichung 6: Berechnung feed [mL/h]

feed [mL/h] = feed [g/h] / ϱ [g/mL]

Gleichung 7: Berechnung feed [g/h]

$$feed[g/h] = \frac{\frac{CQuelleZuc\,\text{ker}}{CAnteil[gC/gZuc\,\text{ker}]}}{conc\ glu/gly} + \frac{CQuelleMeOH}{CAnteilMeOH}$$

Gleichung 8: Berechnung der Menge an Feedlösung

Menge [g] = feed [g/h] . Dauer [h] . Anzahl Reaktoren

Formeln zur Berechnung des Feed Protokolls

Gleichung 9: Berechnung der spez. Wachstumsrate μ

$$\mu = \frac{r_x}{x}$$

μ	spez. Wachstumsrate [h^{-1}]
r_x	Wachstumsgeschwindikeit [g/h]
x	Biomassekonzentration [g]

Gleichung 10: Berechnung des Ausbeutekoeffizienten $Y_{X/S}$

$$Y_{X/S} = \frac{\Delta X}{\Delta S} = \frac{X - X_0}{S_0 - S}$$

$Y_{X/S}$	Ausbeutekoeffizient [g/g]
ΔX	Zuwachs an Zellen [g]
ΔS	Verbrauch von Substrat [g]

Gleichung 11: Berechnung der spez. Substratverbrauchsgeschwindigkeit q_s

$$q_s = \frac{\mu}{Y_{X/S}}$$

q_s	spez. Substratverbrauchsgeschwindigkeit [g (g h)$^{-1}$]
μ	spez. Wachstumsrate [h^{-1}]
$Y_{X/S}$	Ausbeutekoeffizient [g/g]

Gleichung 12: Berechnung der vol. Substratverbrauchsgeschwindigkeit r_s

$$r_S = q_S \cdot x$$

r_s	vol. Substratverbrauchsgeschwindigkeit [g/h]
q_s	spez. Substratverbrauchsgeschwindigkeit [g (g h)$^{-1}$]
x	Biomassekonzentration [g]

Gleichung 13: Berechnung von r_{feed}

$$r_{feed} = \frac{r_s}{c_{feed}}$$

r_{feed}	Feedgeschwindigkeit [g/h]
r_s	vol. Substratverbrauchsgeschwindigkeit [g/h]
C_{feed}	C-Quelle im Feed [g/kg]

Gleichung 14: Berechnung der Biomasse zur Zeit t $M_{X(t)}$

$$M_{X(t)} = M_{X(0)} \cdot e^{\mu \cdot (t - t_0)}$$

$M_{x(t)}$	Menge an Biomasse zur Zeit t [g]
$M_{x(0)}$	Menge an Biomasse zur Zeit 0 [g]
μ	spez. Wachstumsrate [h^{-1}]
t	Zeit t [h]
t_0	Zeit 0 [h]

Gleichung 15: Berechnung der Biomasse Konzentration c_x

$$c_x = \frac{M_x}{V}$$

c_x	Konzentration der Biomasse [g/L]
M_x	Menge an Biomasse [g]
V	Volumen [L]

Gleichung 16: Berechnung der Feedgeschwindigkeit v_{feed}

$$v_{feed} = \frac{r_{feed}}{\rho_{feed}}$$

v_{feed}	Feedgeschwindigkeit [mL/h]

r_{feed} vol. Substratverbrauchsgeschwindigkeit [g/h]
ϱ_{feed} Dichte des Feeds [g/mL]

Gleichung 17: Berechnung des Volumens zu einer bestimmten Zeit V_t $V_t = V_0 + v_{feed} \cdot (t - t_0)$

V_t Volumen zur Zeit t [mL]
V_0 Volumen zur Zeit 0 [mL]
v_{feed} Feedgeschwindigkeit [mL/h]
t Zeit t [h]
t_0 Zeit 0 [h]

CHECKLISTE

Zeitpunkt	Aufgabe	erledigt?
Tag 1 (Mi)	Fermenter putzen	□
	O2 Elektroden putzen + polarisieren	□
	VK1 ansetzen	□
Tag 2 (Do)	VK2 ansetzen	□
	Spüllösungen vorbereiten	□
	Feedlösungen vorbereiten	□
	autoklavieren	□
	Batch Medien einwiegen	□
	pH Elektroden kalibrieren	□
	O2 Elektroden kontrollieren	□
	Fermenter zusammenbauen	□
	Fermenter autoklavieren	□
	Spül- und Feedlösungen fertig machen	□
	Schläuche spülen	□
	Fermenter anschließen	□
	pH einstellen	□
	pO2 Elektroden kalibrieren	□
	neuen Arbeitsablauf anlegen	□
	Inokulieren = Start Fermentation	□
	OD von VK2 bestimmen	□
Tag 3 (Fr)	Trigger auslösen	□
	Probe nehmen	□
Tag 4 (Sa)	Probe nehmen	□
	Probe nehmen	□
Tag 5 (So)	Probe nehmen	□
	Probe nehmen	□
Tag 6 (Mo)	Probe nehmen	□
	Probe nehmen	□
Tag 7 (Di)	Probe nehmen	□
	Ernte	□
	Fermenter totautoklavieren	□
	Fermenter putzen	□
	Daten exportieren	□
Tag 8 (Mi)	Aktivitätsbestimmung	□
	Auswertung	□