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Improving recombinant protein secretion in Pichia pastoris

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Kurzfassung

Transkriptionsfaktoren (TF) sind Proteine, welche die Genexpression in Zellen regulieren. Dabei können TF durch verschiedene Stresssituationen (de)aktiviert werden und allfällige Probleme, zum Beispiel bei der Faltung und Sekretion von Proteinen, überwinden. Ziel dieser Arbeit war es auf Basis eines rekombinanten Enzymes, das unter der Kontrolle des konstitutiven GAP bzw. des mit Methanol induzierbaren *AOX1* Promotors exprimiert wurde, den Einfluss von drei TF auf die Enzym-Sekretion in *P. pastoris* zu untersuchen. Dabei wurden Transformanten, welche die verschiedenen TF überexprimierten, in Screening, Re-screening und Bioreaktor-Kultivierungen mittels Aktivitätsassay, ELISA und SDS-PAGE analysiert. Dabei wurde, zusätzlich zum rekombinanten Protein, eine interessante, ca. 130 kDa schwere Proteinbande beobachtet, die mittels Peptid-MS als Flokkulin identifiziert wurde. Um weitere Informationen über die Rolle der spezifischen TF zu erhalten, wurden FM4-64 Färbungen durchgeführt, sowie die Sensitivität der TF-Überexpressionsstämme gegenüber diversen Chemikalien getestet.

Abstract

Transcription factors (TFs) are proteins that regulate gene expression within cells. TFs get (de)activated by various signals and stresses and can help to overcome different bottlenecks in protein assembly such as for example in folding or secretion. Topic of this study was the secretion of a recombinant carboxylesterase under control of the constitutive GAP and methanol-inducible *AOX1* promoter, respectively, in *P. pastoris* and the influence of three TFs thereon was studied. Transformants overexpressing the different TFs were studied upon screening, re-screening and bioreactor cultivations by activity assay, ELISA and SDS-PAGE. Thus, over-expression of a membrane-associated TF yielded in an interesting protein band at about 130 kDa, which was identified by peptide-MS as flocculin protein. For this reason, additional experiments using FM4-64 staining and sensitivity tests against toxic compounds were performed to gain more information on the function of this specific TF in *P. pastoris*.

Abbreviations

4PL	Four parameter logistic curve
DMSO	Dimethylsulfoxid
DO	Dissolved oxygen
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
Evc	Empty vector control
FM4-64	N-(3-triethylammonium-propyl)-4-(p-diethylaminophenylhexa-trienyl) pyridinium dibromide
gDNA	Genomic DNA
Gen	Geneticin
GOI	Gene(s) of interest
Kan	Kanamycin
LB	Lysogeny broth
MeOH	Methanol
PCI	Phenol/Chloroform/Isoamylalkohol
PCR	Polymerase chain reaction
pNSi	p-Nitrophenyl-2-(trimethylsilyl) ethyl carbonat
POI	Protein(s) of interest
RT	Room temperature
RT-PCR	Real time-polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamid-gelelectrophoreses
TF	Transcription factor
Zeo	Zeocin

1 Introduction

1.1 *Pichia pastoris* expression system

Yeasts have been used as host organisms to produce many recombinant proteins for years. The first large scale industrial process using *P. pastoris* was the production of *Hevea brasiliensis* hydroxynitrile lyase at more than 20 g/L of recombinant protein [1]. Nowadays, human serum albumin, tumour necrosis factor α or α-amylase are some of the proteins which get produced at high titers [2]. From the yeast clade, the methylotrophic *P. pastoris* has many advantages as expression host. On the one hand, genetic manipulations can be easily performed. *P. pastoris* is also able to perform post-translational protein modifications such as glycosylation and disulphide-bond formation. On the other hand, this well-characterized yeast grows to very high cell densities combined with the ability to secrete high titers of active, post-translationally processed protein into the surrounding media [3] [4]. These characteristics make *P. pastoris,* currently reclassified as *Komagataella pastoris,* a unique and successful system for heterologous protein production.

Gene(s) of interest (GOI) can be integrated upon electroporation into the *P. pastoris* genome. Therefore, a shuttle vector is amplified in *Escherichia coli* containing important *E. coli* and *P. pastoris* components (e.g. resistance marker, origin of replication, promotor, gene of interest). *P. pastoris* can be transformed with the linearized vector using homologous recombination or random insertion for a stable integration of the expression cassette into the genome [2]. The choice of the vector system, cultivation conditions, promotor-terminator combination together with the proper selection marker and secretion signal has strong influence on the secreted protein levels [5]. Not every protein can be produced and secreted by *P. pastoris* at high titers. Particularly, successful expression and secretion depends on the origin and complexity of the protein. New methods for protein and cell engineering have been developed recently to increase protein production levels [6]. Especially, the development of new promotors, selection markers and cofactors facilitate the heterologous expression of complex proteins, e.g. membrane-attached or protease-sensitive protein(s) of interest (POI).

One strategy to obtain a high-level protein expression strain is to screen a substantial number of transformants. High-throughput methods may bed necessary to find highly

producing and/or secreting transformants with one or more copy numbers of the expression cassette. Therefore, transformants may be plated directly on selection plates containing enhanced amounts of selection agent. Frequently the protein titers from screening in small scale, e.g. deepwell plate cultivation, will not reflect the ultimate protein levels in bioreactor cultivations. Especially, media buffering for pH stabilisation, maximum aeration and addition of peptone or casamino acids to prevent proteolysis are essential for a promising screening method [4] [6].

1.1.1 Classic *P. pastoris* promotors

Many studies have been focused on the promoters of the methanol utilization pathway (MUT) genes alcohol oxidase 1 (P_{AOX1}) and on the promoter of the constitutive glycolysis gene glyceraldehyde 3-phosphate dehydrogenase (P_{GAP}).

P_{AOX1} is a strong and tightly regulated methanol-inducible promotor, which is repressed in the presence of glucose/glycerol and is strongly induced by methanol. The *AOX1* gene is more strongly transcribed than the *AOX2* gene in *P. pastoris*, resulting in up to 30 % of total intracellular protein being Aox1p [7]. The *P. pastoris* culture can be grown to high cell densities using glucose or glycerol as carbon source and, subsequently, protein production can be induced by methanol. However, using methanol - a potential fire hazard - might be inappropriate for industrial applications.

Alternatively the constitutive P_{GAP} can be used and often provides similar expression levels on glucose compared to P_{AOX1} on methanol [2] [7]. The strength of this constitutive promotor is depending on the carbon source. Waterham et al. (1997) discovered that β-Lactamase activity under P_{GAP} is highest when grown on glucose (=100%), about 75% of this activity is achieved on glycerol and about 30-35% of the maximum activity was detected on methanol as carbon source [8]. There is no shift of carbon source needed (in contrast to the P_{AOX1}) for induction of heterologous protein production, however such a shift may be beneficial for the heterologous expression of toxic genes and gene products to separate growth and production phase. Another important factor is the appropriate oxygen supply.

Yet, a positive effect of hypoxic cultivation conditions leading to an increased productivity of recombinant proteins was found out by Baumann et al. [9]. The one-stage cultivation using P_{GAP} decreases process time and handling efforts.

Despite the advantages of the *AOX1* and *GAP* promotors, their strong expression is not always the best choice to produce heterologous proteins. In some cases, a weaker

promotor would be an alternative for rate-limiting steps such as correct folding or secretion in *P. pastoris* [10]. For example, Ruth et al. (2010) demonstrated with P_{AOX1} variants that weaker promotor variants showed higher trypsinogen levels than the wild type version [11]. Additionally, the P_{FLD1} (promotor of formaldehyde dehydrogenase 1) with similar strength as P_{AOX1} is not only regulated by methanol but also by the nitrogen source. This promotor was used by Resina et al. (2005) and was induced by co-feeding methylamine and sorbitol. This methanol-free production process reached better results than compared to the P_{AOX2} [12].
The constitutively express PGK (phosphoglycerate kinase) promotor has been used in different yeasts such as *S. cerevisiae* and *Candida maltosa* [13] [14]. P_{PGK} was used in *P. pastoris* in 2005 and showed about 10 % of the activity of the P_{AOX1} [15]. To date, less knowledge about these promotors is published than for P_{AOX1} and P_{GAP}.

1.1.2 Protein secretion in *P. pastoris*

Heterologous proteins can be expressed either intracellularly or extracellularly. The advantages of protein secretion are that no cell lysis is required and native proteins are secreted at very low levels resulting in an easier recovery of the heterologous protein from the culture media. The intial step of protein secretion, i.e. entry of a protein into the endoplasmic reticulum (ER) lumen, requires the presence of a signal sequence. The most commonly used and effective secretion signals in *P. pastoris* are the ones derived from α-MF and *SUC2* of *S. cerevisiae* and *PHO1* of *P. pastoris* [6].
Secretory proteins are folded and modified in the ER with the help of ER-resident proteins. Folded proteins can then be exported to the Golgi where they get further modified and transported via secretory granules from the cell, to vacuoles or other organelles. Misfolded proteins are retained in the ER, which may cause cellular stress and the activation of unfolded protein response (UPR) and stimulation of the ER-associated degradation (ERAD) pathway (Figure 1.1). This system inhibits further transcription/translation events and translocation by membrane-enclosed transport vesicles. Additionally, chaperones get upregulated and this often becomes a rate-limiting step (bottleneck) in heterologous protein production [5].

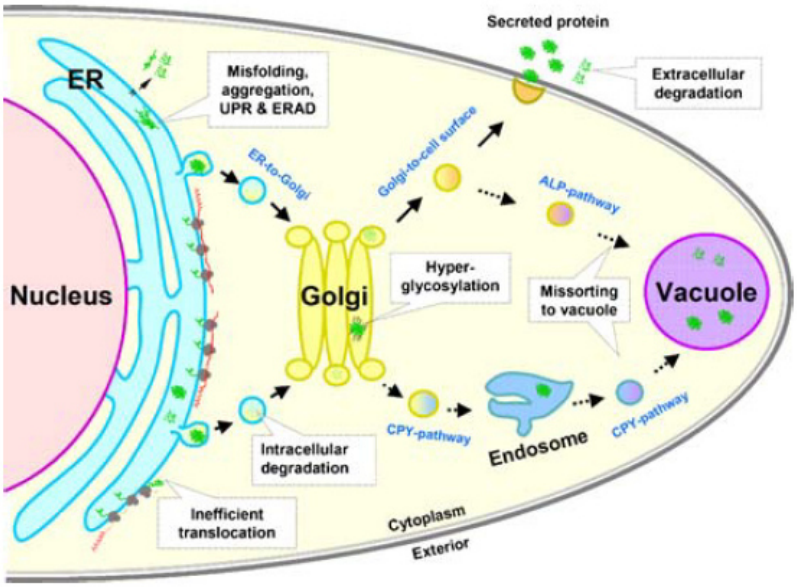


Figure 1.1 Schematic diagram of the secretory pathway of heterologous protein in *P. pastoris* with characteristic bottlenecks [5]**.** The main secretion and vacuolar pathways are illustrated. ERAD - ER-associated degradation. UPR - unfolded protein response. CPY – carboxypeptidase Y. ALP – alkaline phosphatase.

Modification effects of one step might be protein or host specific. Overexpression or knock-out of specific genes, e.g. chaperones, folding helpers, as well as transcription factors can positively effect secreted protein titers and may yield in consistent improvement for different applications [16].

1.2 Transcription factors

Environmental constraints, e.g. temperature, pH and stress, may have a high influence on productivity. TFs are stress related genes whose overexpression might increase the heterologous protein production in yeast [17]. Especially, stress response, protein processing and secretion are highly interrelated. TFs have outermost potential to overcome bottlenecks in the cellular protein production pathway. Already, it has been shown that the transcriptional activator Hac1p regulates the UPR pathway, gets induced by accumulation of unfolded/misfolded protein in the ER and activates expression of chaperones [16]. Overexpression of Hac1p could be used to increase the production and the correct processing of heterologously expressed genes [18]. Therefore, promotors hold specific DNA-binding sites for TFs for transcriptional regulation. These TFs can activate or repress DNA elements [19]. The possible function and effects of the three chosen TFs in this work had mainly been studied in *S. cerevisiae* before. There is not much known about their role in *P. pastoris*.

1.2.1 Upc2: regulator of ergosterol biosynthesis

In yeast, ergosterol influences membrane stability, fluidity and permeability. Upc2 is a regulator of ergosterol biosynthesis [20]. Additionally, *ERG* genes encoding enzymes for ergosterol biosynthesis are transcriptionally regulated by Upc2 in case of ergosterol depletion [21]. The production of ergosterol needs a lot of energy and is oxygen dependent [22]. Moreover, sterol metabolism modulates the relative proportion of the yeasts' cell wall constituents, such as glucans, mannoproteins and chitin, depending on growth conditions [23].

Under anaerobic conditions, sterols will not be produced and must be taken up from the growth media for survival of the cells [22]. Upc2 also regulates the hypoxical induction of *DAN/TIR* genes. These genes encode for mannoproteins, which are highly glycosylated, and responsible for anaerobic cell wall synthesis [20] [23]. Among others, Hickman et al. (2011) proposed that ergosterol depletion alters membrane fluidity in yeast [24]. This assumption was confirmed by Zara et al. (2009). They found that the loss of the ability to desaturate fatty acids and the incomplete ergosterol pathway led to a dramatic loss of viability [25]. Oxygen limitation affect strongly the lipid, membrane and cell wall composition [26]. Upc2 might be a major factor for the adaption to low oxygen levels in yeasts [22] [21]. Under certain conditions, *P. pastoris* may be more tolerant than *S. cerevisiae* upon oxygen deprivation. At least, the positive effect on the production of recombinant protein had been demonstrated by Baumann et al. [9]. The overexpression of Upc2 under aerobic conditions may, thus, positively affect protein secretion. To date, predictions are difficult to make, because not all genes regulated by Upc2 are characterized currently [22].

1.2.2 Mit1: putative regulator of pseudohyphal growth

The TF Mit1 of *S. cerevisiae* putatively corresponds to the TF of *P. pastoris* used in this study. This TF has sequence similarity to *S. pombe* Gti1 (gluconate transport inducer) and *C. albicans* Wor1 (white-opaque regulator) [27] [28]. Mit1 is involved in S*. cerevisiae* pseudohyphal growth pathway. The cells become elongated at a lack of nutrition in the media and attach to each other by forming chains of cells [27]. Rebnegger et al. (2014) found a negative correlation of the expression of *MIT1* to the specific growth rate. However, recombinant protein secretion is positively correlated to specific growth rates [29]. The regulation of filamentous growth is complex and controlled by the MAPK pathways, which is described in detail by Cullen et al. [30].

The effect of *MIT1* co-expression in *P. pastoris* on secretory protein titers has not been studied, yet. Additionally, transcription regulators are essential for expressing genes involved in MUT pathway. Wang et al. (2016) identified *MIT1* in *P. pastoris* as an essential regulator of P_{AOX1} with strict repression on glycerol and strong induction on methanol [31].

1.2.3 Rpn4: regulator of the ubiquitin-proteasome system

The stress-response transcription factor Rpn4 in *S. cerevisiae* is a transcriptional activator of proteasomal genes. This short-lived - about 2 min - protein interacts with a subunit of the 26S proteasome and gets depleted by the active, assembled proteasome. The activation of genes by Rpn4p plays an essential role in a wide variety of cellular processes such as differentiation, stress response and protein production [32] [33]. Proteasomes serve in cytosolic degradation of misfolded proteins that are mainly tagged by ubiquitin [34]. The Rpn4-proteasome negative feedback circuit is involved in a broad range of tasks including protein processing, recovery and proteolysis. It is a major system in cellular quality control and defence. The higher proteasome expression level induced by Rpn4p supports additionally the ER-associated degradation of misfolded proteins [35]. Chen et al. (1994) demonstrated that ubiquitin overexpression yielded in up to 10-fold increased heterologous secretion of human leucocyte elastase inhibitor [36]. The over-expression of Rpn4 might increase the secretory pathway of heterologously produced proteins by preventing accumulation of incorrectly folded protein in the ER and, thus, enhance cell viability under stress conditions, too.

It needs to be considered that Rpn4 target gene induction may be toxic if the TF cannot be processed rapidly by the proteasome to shut off the activation. Rpn4p degradation in cellular stress is ensured by two pathways, ubiquitin-dependent and –independent routes. These dual Rpn4p degradation pathways underscore the importance of fine-tuning Rpn4p abundance [33] [37].

1.3 Scale-up for bioreactor cultivation

Besides the choice of suitable expression vectors and host strains, screening for highly expressing transformants is important. Due to the likely integration of different gene copy numbers into the genome, generally numerous *P. pastoris* transformants are screened on small scale. A scalable screening method is essential for a successful

large-scale application using high-throughput screening for strain development. Especially, isolating variants on a solid transformation plate and cell sorting using flow cytometry are often used techniques for screening [38]. However, transformants may behave differently due to variable cultivation conditions in small scale and bioreactor cultivation. The absence of pH and aeration control, and no feeding at growth-limiting rates, are potential reasons for a lack of transferability [4] [6]. Cultivation of *P. pastoris* in a defined media containing carbon source, salt, trace elements and biotin is typically a three-stage process independently of the promoter. The batch phase using glycerol is the first stage to accumulate biomass. Afterwards, glycerol is fed at growth-limiting rates (fed-batch phase) to increase the biomass concentration and prepare the cells for induction. Finally, the heterologous protein production phase is induced by methanol after depletion of glycerol. Then, the GOI is fully expressed with little further cell proliferation [4].

An additional feeding strategy is the mixed-feed applying glycerol and methanol as a carbon source at the same time. Advantages of mixed feeding strategies are higher cell mass, shorter induction phases, improved viability of the cells and often higher recombinant protein production rates [3]. *P. pastoris* is not able to tolerate high methanol concentrations in the media. Zhang et al. (2003) found that methanol levels above 3.65 g/L inhibit growth [39]. In addition, the accumulation of formaldehyde and hydrogen peroxide, from methanol utilization pathway, in the cells is toxic.

Yeasts regulate gene expression as a reaction to environmental stressful situations till adapted to the new conditions. This process is not yet fully understood and different stress situations can lead to changes in the expression of genes [40]. The optimum growth temperature for *P. pastoris* is usually about 28°C. A reduced temperature during the production phase has been shown to improve recombinant protein production. The lower production temperature might reduce death rate and the release of host cell proteases into the culture medium [41]. Li et al. (2001) described a positive effect at 23°C on secreted protein yield and cell vitality [42]. Additionally, a lower pH of about 5 combined with a lower temperature has been shown to reduce cell lysis and proteolysis. Jahic et al. (2003) correlated a lower concentration of proteases in the culture medium to increased product yield due to reduced cell lysis [41].

Temperature and pH may not be the only stress factors. Changes in media components such as carbon source, oxygen supply and osmolality can also cause cell

stress. Certain stress responses might increase heterologous protein production but need to be characterized for industrial processes [40].

2 Objectives

Overexpression of TFs may be a promising tool to increase the production of heterologous proteins and industrial efficiency. The aim of this work is to link heterologous protein secretion of a model protein with overexpression of certain TFs to increase the secreted protein titers in *P. pastoris*. Three different TFs (Upc2, putative Mit1, Rpn4) will be characterised as putative expression helpers under the constitutive *GAP* promotor. Secreted carboxylesterase will be used as a model protein. The project aim is to compare secreted protein levels of different transformants compared to the *P. pastoris* parent strains (P_{AOX1} Parent and P_{GAP} Parent) by activity assay, ELISA and SDS-PAGE. The parent strains and TFs were studied and identified already at an earlier stage of this project. Transformants will be screened in small scale (24-well) to identify high, medium and low secreting transformants. The best transformants will be studied in bioreactor cultivations. Different feeding profiles for glycerol and methanol induction will be applied.

3 Materials and Methods

3.1 Instruments and Devices

Table 3.1 Instruments and Devices.

Task	Instrument/Device	Manufacturer
24 deep well plate cultivation	Multitron	Infors AG, Bottmingen, Switzerland
	Gas permeable adhesive seals	Fisher Scientific - Austria GmbH, Vienna, Austria
	Whatman® UNIPLATE microplates 24 well (10 mL)	Sigma-Aldrich, Schnelldorf, Germany
Absorbance measurement	BioPhotometer Plus	Eppendorf, Germany
	Spectrophotometer	
	Semi-Micro-Cuvettes, PS, 10 x 10 x 45 mm	Greiner bio-one AG, Germany
Agarose gel electrophoresis	PowerPac™ Basic + Sub-Cell GT	Bio-Rad, USA
Bioreactor cultivation	DasGip® Parallel Bioreactor Systems	Eppendorf, Germany
	DasGip® pH Sensors	Eppendorf, Germany
	DasGip® DO Sensors	Eppendorf, Germany
	DasGip® control	Eppendorf, Germany
Cell harvest/Centrifugation	Tabletop centrifuge 5810R	Eppendorf, Germany
Determination of DNA concentration	NanoDrop 2000 UV-Vis Spectrophotometer	Thermo Fisher Scientific, USA
Electrotransformation	MicroPulser™	Bio-Rad, USA
	Electroporation Cuvettes (2 mm gap)	Life Technologies, USA
Immunoblotting (Western blotting)	PowerEase500 XCell SureLock™	Life Technologies, USA
	Electrophoresis SureLock® cell and blot module	Thermo Fisher Scientific, USA
	Nitrocellulose Blotting Membrane, 0.45 µm	GE Healthcare Life Sciences, USA
	Bioimager GBox HR16	SynGene, UK
Incubator (28°C and 37°C)	BINDER Kühlbrutschränke	Binder GmbH, Germany

Task	Instrument/Device	Manufacturer
Microplate assay	Microplate, 96 well, PS, U-bottom, MICROLON	Greiner bio-one AG, Germany
	Synergy Mx monochromator-based multi-mode microplate reader	BioTek Instruments, Winooski, USA
	Titramax 1000	Heidolph Instruments GmbH, Schwabach, Germany
Microscopy	DGC350FX	Leica Microsystems, Austria
	Cover slip, 24x50 mm	Menzel-Gläser, Germany
	Object plate, 76x26 mm	Carl Roth GmbH, Karlsruhe, Germany
Mixing (small volumes)	Vortex – Genie 2	Scientific Industries Inc., USA
PCR reaction	2720 Thermal cycler	Applied Biosystems, USA
pH measurement	inoLab WTW 720 pH meter	WTW GmbH, Germany
Protein electrophoresis	NuPAGE® SDS-PAGE Gel System	Life Technologies, USA
	NuPAGE® 4-12.5% SDS-Gels, 15 wells	Life Technologies, USA
RT-PCR	AbiPrism 7300	Applied Biosystems, USA
	Multitron Rotilabo®-sealing	Infors AG, Bottmingen, Switzerland
Shaker (small volumes)	Thermo Fishermixer comfort	Eppendorf, Germany
Sterile work	Clean Air biohazard	UNIQUIP, USA
Weighing	Lab scale: SI-202	Denver Instrument, USA
	Precision scale: Kern Scale ABS 220-4	Kern &Sohn GmbH, Germany

3.2 Chemicals

Table 3.2 Chemicals.

Reagent	Supplier
1,4-Dithiothreitol (DTT)	Carl Roth GmbH, Karlsruhe, Germany
6x DNA Loading Dye	Thermo Fisher Scientific, Vienna, Austria
Acetic acid	Carl Roth GmbH, Karlsruhe, Germany
Agarose LE	Biozyme, Germany
Ammonium acetate, 25 %	Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany

Reagent	Supplier
Ammonia solution	Carl Roth GmbH, Karlsruhe, Germany
Aqua bidest. (ddH_2O)	Fresenius Kabi Austria GmbH, Graz, Austria
BactoTM agar	Sigma-Aldrich Handels GmbH, Vienna, Austria
BactoTM phytone-peptone	Becton, Dickinson and Company, Sparks, USA
BactoTM yeast extract	Becton, Dickinson and Company, Sparks, USA
Bicine	Carl Roth GmbH, Karlsruhe, Germany
Biotin	Carl Roth GmbH, Karlsruhe, Germany
Biozym LE agarose	Biozym BioTech Trading GmbH, Vienna, Austria
Boric acid	Carl Roth GmbH, Karlsruhe, Germany
Calciumchlorid	Carl Roth GmbH, Karlsruhe, Germany
Calcofluor white	Sigma-Aldrich Handels GmbH, Vienna, Austria
Citric acid monohydrate	Carl Roth GmbH, Karlsruhe, Germany
Cobalt (II) chloride	Carl Roth GmbH, Karlsruhe, Germany
Copper (II) sulfate	Carl Roth GmbH, Karlsruhe, Germany
Congo Red	Sigma-Aldrich Handels GmbH, Vienna, Austria
D-Glucose-monohydrate	Carl Roth GmbH, Karlsruhe, Germany
Di-ammonium hydrogenphosphate	Carl Roth GmbH, Karlsruhe, Germany
Dichloromethyl	Sigma-Aldrich Handels GmbH, Vienna, Austria
DifcoTM Yeast Nitrogen Base w/o amino acid	Sigma-Aldrich Handels GmbH, Vienna, Austria
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Handels GmbH, Vienna, Austria
Di-Potassium hydrogenphosphate	Carl Roth GmbH, Karlsruhe, Germany
Di-Sodium hydrogenphosphate	Carl Roth GmbH, Karlsruhe, Germany
D-Sorbitol	Carl Roth GmbH, Karlsruhe, Germany
Ethidium bromide	Carl Roth GmbH, Karlsruhe, Germany
Ethylene glycol	Carl Roth GmbH, Karlsruhe, Germany
Ethylenediamine tetraacetic acid (EDTA)	Carl Roth GmbH, Karlsruhe, Germany
FeedBeads® Glucose (Ø 12mm)	Adolf Kühner AG, Basel, Switzerland
FM4-64	Thermo Fisher Scientific, USA

Reagent	Supplier
Carboxylesterase	BIOMIN Holding GmbH, Tulln, Austria
GeneRuler™ 1 kb DNA Ladder Mix	Thermo Fisher Scientific, USA
Glanapon 2000	Bussetti, Vienna, Austria
Glycerol	Carl Roth GmbH, Karlsruhe, Germany
Glycin	Carl Roth GmbH, Karlsruhe, Germany
Iron (II) sulfate	Carl Roth GmbH, Karlsruhe, Germany
J. T. Baker ® abs. Ethanol	VWR International GmbH, Vienna, Austria
Magensiumsulphate heptahydrate	Carl Roth GmbH, Karlsruhe, Germany
Magnesium sulphate	Carl Roth GmbH, Karlsruhe, Germany
Manganese (II) sulfate monohydrate	Carl Roth GmbH, Karlsruhe, Germany
Methanol (MeOH)	Carl Roth GmbH, Karlsruhe, Germany
NuPage® LDS Sample Buffer (4x)	Life Technologies, USA
NuPage® MES SDS buffer	Life Technologies, USA
NuPage® Sample Reducing Agent (10x)	Life Technologies, USA
PageRuler Plus Prestained Protein Ladder	Thermo Fisher Scientific, USA
pNPP (4-Nitrophenyl phosphate) liquide substrate system	Sigma-Aldrich Handels GmbH, Vienna, Austria
Phosphoric acid, 85 %	Carl Roth GmbH, Karlsruhe, Germany
p-Nitrophenyl-2-(trimethylsilyl) ethyl carbonat (pNSi)	Sigma-Aldrich Handels GmbH, Vienna, Austria
Potassium chloride	Carl Roth GmbH, Karlsruhe, Germany
Potassium dihydrogenphosphate	Carl Roth GmbH, Karlsruhe, Germany
Potassium hydroxide	Carl Roth GmbH, Karlsruhe, Germany
Potassium sulfate	Carl Roth GmbH, Karlsruhe, Germany
Power SYBR Green Master Mix	Applied Biosystems, USA
Roti®-Phenol/Chloroform/Isoamylalkohol (25:24:1)	Carl Roth GmbH, Karlsruhe, Germany
SimplyBlue™ SafeStain	Thermo Fisher Scientific, USA
Sodium acide	Carl Roth GmbH, Karlsruhe, Germany
Sodium chloride	Carl Roth GmbH, Karlsruhe, Germany
Sodium dodecyl sulphate (SDS)	Carl Roth GmbH, Karlsruhe, Germany

Reagent	Supplier
Sodium fluoride	Sigma-Aldrich Handels GmbH, Vienna, Austria
Sodium hydroxide	Carl Roth GmbH, Karlsruhe, Germany
Sodium iodide	Carl Roth GmbH, Karlsruhe, Germany
Sodium molybdate	Carl Roth GmbH, Karlsruhe, Germany
Sodium Orthovanadate	Sigma-Aldrich Handels GmbH, Vienna, Austria
Sulforic acid	Carl Roth GmbH, Karlsruhe, Germany
Tris	Carl Roth GmbH, Karlsruhe, Germany
Triton X-100	Carl Roth GmbH, Karlsruhe, Germany
Tween® 20	Carl Roth GmbH, Karlsruhe, Germany
Zinc chloride	Carl Roth GmbH, Karlsruhe, Germany

3.3 Media, Buffer and stock solutions

Table 3.3 Media, Buffer and Stock Solutions.

Medium/Buffer	Composition
1 M Dithiothreitol	152.2 g/L DTT
1 M Phosphate buffer	30.13 g/L K_2HPO_4, 118.13 g/L KH_2PO_4, pH 6
1 mM pNSi reaction solution	0.1 mL 100 mM pNSi stock solution are mixed with 9.9 mL 1x FCE buffer
1 M Tris(hydroxymethyl)-aminomethan	121.14 g/L TRIS
15 mM Sodium azide	0.63 g/L NaN_3 disolved in ddH_2O
15 mM Sodium fluoride	0.975 g/L NaF disolved in ddH_2O
100 mM pNSi stock solution	141.68 mg pNSi disolved in 5 mL abs. ethanol
16 mM FM4-64	1 mg FM4-64 solved in 102.9 µL DMSO, -20°C
10x D	220 g/L D-glucose-monohydrate
10x FCE buffer	200 mM Tris-Cl, 1 mg/mL BSA, pH 8
10x TBS	30.3 g/L Tris, 87.6 g/L NaCl, pH 7.5
10x TF buffer	30.3 g/L Tris, 144 g/L glycin
10x YNB	134 g/L Difco™ Yeast Nitrogen Base w/o amino acid
1x TF buffer	50 mL/L 10x TF buffer, 200 mL/L methanol, 750 mL/L H_2O

Medium/Buffer	Composition
4 M Ammonium acetate	308.32 g/L ammonium acetate
500x Biotin	200 mg/L biotin
50x Tris-Acetat-EDTA buffer (TAE)	242 g/L Tris, 18.61 g/L EDTA, 57.1 mL/L acetic acid
Base	12.5 % NH_3
BEDS solution	10 mM bicine-NaOH (pH 8.3), 3 % (v/v) ethylene glycol, 5 % (v/v) DMSO, 1 M sorbitol
Blocking buffer	100 mL washing buffer with 1 g BSA
BM Medium	100 mL/L 1 M phosphate buffer, 2 mL/L 500x biotin, 100 mL/L 10x YNB, specific carbon source if needed
BSM medium	13.5 mL/L H3PO4 (85%), 0.5 g/L CaCl * 2 H2O, 7.5 g/L MgSO4 * 7 H2O, 9 g/L K2SO4, 2 g/L KOH, 40.0 g/L glycerol, 0.25 g/L NaCl, 4.35 mL/L PTM1 (after autoclaving), 4.35 mL/L 500x biotin (after autoclaving)
Coating buffer	1.8 g/L Na_2HPO_4 * 2 H_2O, 0.24 g/L KH_2PO_4, 0.2 g/L KCl, 8.0 g/L NaCl, pH 7.4
Dilution buffer	1.8 g/L Na_2HPO_4 * 2 H_2O, 0.24 g/L KH_2PO_4, 0.2 g/L KCl, 8.0 g/L NaCl, 0.1 g/L BSA, 1 mL Tween®20, pH 7.4
Glycerol feed	60 % (w/w) glycerol, 12 mL/L PTM1, 12 mL/L 500x Biotin
LB agar	10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl, 20 g/L agar
LB medium	10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl
M2 Medium	3.15 g/L $(NH_4)_2HPO_4$, 0.49 g/L $MgSO_4$, 0.8 g/L KCl, 0,0268 g/L $CaCl_2$ * 2 H_2O, 22 g/L citric acid, 1.47 mL/L PTM1, 2 mL/L 500x biotin, pH 5
M2D Medium	3.15 g/L $(NH_4)_2HPO_4$, 0.49 g/L $MgSO_4$, 0.8 g/L KCl, 0,0268 g/L $CaCl_2$ * 2 H_2O, 22 g/L citric acid, 1.47 mL/L PTM1, 2 mL/L 500x biotin, 22 g/L D-glucose, pH 5
Methanol feed	pure methanol
PTM1	5 mL/L H_2SO_4, 65 g/L $FeSO_4$ * 7 H_2O, 25 g/L $ZnCl_2$, 7.5 g/L $CuSO_4$ * 5 H_2O, 4.2 g/L $MnSO_4$, 1.025 g/L $CoCl_2$ * 6 H_2O, 0,2 g/L Na_2MoO_4 * 2 H_2O, 0.1 g/L NaI, 0.025 g/L H_3BO_3
TBST	100 mL/L 10x TBS, 500 µL/L Tween®20
TBST-milk	100 mL TBST and 5 g skim milk powder
TE buffer	1.21 g/L Tris-HCl, pH 7.5, 0.29 g/L EDTA, pH 8.0
Washing buffer	1 L coating buffer with 1 mL Tween®20

Medium/Buffer	Composition
Yeast lysis buffer	20 mL/L Triton X-100, 100 mL/L 10 % SDS, 20 mL/L 5 M NaCl, 2 mL/L 0.5 M EDTA, 10 mL/L 1 M Tris, pH 8.0
YPD agar	10 g/L yeast extract, 20 g/L phytone-peptone, 100 mL/L 10x D-glucose, 20 g/L agar
YPD medium	10 g/L yeast extract, 20 g/L phytone-peptone, 100 mL/L 10x D-glucose
YPhyG	10 g/L yeast extract, 20 g/L phytone-peptone, 20 g/L glycerol

Table 3.4 Antibiotic stock solutions.

Name	Concentration in solution	Used for	Source
Geneticin disulphate (G418 Sulphate)	450 ng/µL	*P. pastoris*	Carl Roth GmbH, Karlsruhe, Germany
Kanamycin sulphate	100 ng/µL	*E. coli*	Carl Roth GmbH, Karlsruhe, Germany
Zeocin™ powder	50 ng/µL	*P. pastoris*	InvivoGen, San Diego, USA

3.4 Plasmids and Strains

Table 3.5 Plasmids. The different untagged plasmids were amplified in *E. coli* TOP10 F´. * c.c - culture collection number of the Institute of Molecular Biotechnology.

Plasmid	Feature	c.c *
pPUZZLE_KanR_pGAP_evc	Backbone, Empty vector	5333
pPUZZLE_KanR_pGAP_PP7435_chr2_1193	Upc2	6894
pPUZZLE_KanR_pGAP_PAS_chr3_0251	putative Mit1	7548
pPUZZLE_KanR_pGAP_PP7435_chr3_0511	Rpn4	7549

Table 3.6 *E. coli* and *P. pastoris* strains. The strains in bold were produced during this work. * c.c - culture collection number of the Institute of Molecular Biotechnology.

Strains	Description	c.c *
PDI 1 copy ref strain	RT-PCR calibration strain	[43]
E. coli Top10F'	F'{lacI^q Tn10 (Tet^R)} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80 lacZΔM 15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG	1482
P. pastoris AFR WCB P_{GAP} Parent	P_{GAP} carboxylesterase multicopy model strain	6601

Strains	Description	c.c *
P. pastoris AFR WCB P_{GAP} – Upc2	Carboxylesterase secretion Upc2 overexpression	Pichler strain collection
P. pastoris AFR WCB P_{GAP} – Mit1	Carboxylesterase secretion Mit1 overexpression	-
P. pastoris AFR WCB P_{GAP} – Rpn4	Carboxylesterase secretion Rpn4 overexpression	-
P. pastoris BT3445 P_{AOX1} Parent	P_{AOX} Carboxylesterase model strain	Pichler strain collection
P. pastoris BT3445 P_{AOX1} – Upc2	Carboxylesterase secretion Upc2 overexpression	-
P. pastoris BT3445 P_{AOX1} – Mit1	Carboxylesterase secretion Mit1 overexpression	-
P. pastoris BT3445 P_{AOX1} – Rpn4	Carboxylesterase secretion Rpn4 overexpression	-

3.5 Enzymes, Kits and Antibodies

Table 3.7 Enzymes and Kits.

Enzymes/Kits	Supplier
DreamTaq Green PCR Master Mix (2X)	Thermo Fisher Scientific, USA
FastDigest™ restriction enzymes	Thermo Fisher Scientific, USA
Gene Jet™ Plasmid Miniprep Kit	Thermo Fisher Scientific, USA
SuperSignal® WestPico Chemiluminescent Substrate	Thermo Fisher Scientific, USA
Wizard® SV Gel and PCR Clean Up System	Promega Corporation, USA

Table 3.8 Antibodies.

Antibody	No.	Source	Application
Rabbit anti-carboxylesterase IgG	6287	BIOMIN Holding GmbH, Austria	capture antibody
Goat anti-rabbit IgG-Peroxidase conjugate	A9169	SIGMA-ALDRICH Handels GmbH, Vienna, Austria	detection antibody (Western blot)
Goat anti-Rabbit IgG-alkaline phosphatase antibody produced in goat	A3687	SIGMA-ALDRICH Handels GmbH, Vienna, Austria	detection antibody (Elisa)

3.6 Methods

3.6.1 Plasmid Isolation

The pPuzzle_KanR_pGAP-TF plasmid containing *E. coli* strains were provided by Dr. Claudia Ruth. The plasmids were amplified in *E. coli* on LB-Kan agar over night at 37°C. Plasmid isolation was done with the GeneJET Plasmid Miniprep Kit. For linearization, 1 µg of the isolated plasmid was incubated for 1 h at 37°C with 1 U of *AscI* FastDigest™ with 10x FastDigest ™ buffer in a final volume of 20 µL. The restriction enzyme cuts between the AOX-TT sequences to allow integration by homologous recombination into the genome with both flanking ends (Figure 3.1). Before transformation, the linearized plasmid was purified by gel electrophoresis (1 % agarose gel, 1 h at 100 V) with the Wizard® SV Gel and PCR Clean-Up system. The plasmid concentration was determined with a UV-VIS spectrophotometer (Nanodrop, Thermo Fisher Scientific, USA). The sequencing was done at Microsynth AG and the analyses was done with the CLC workbench.

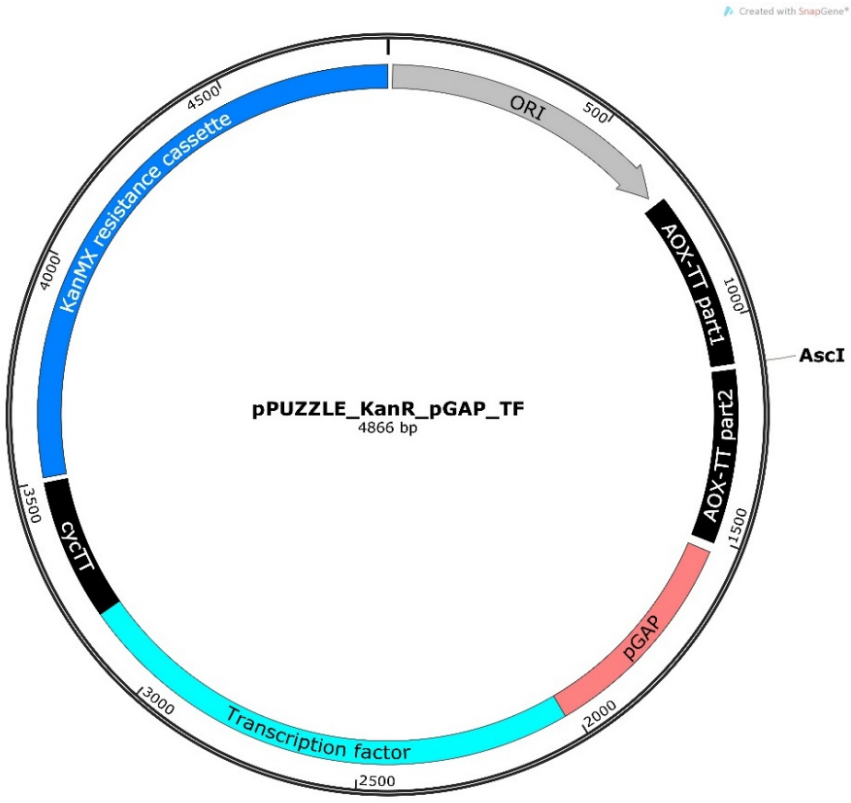


Figure 3.1 Plasmid map illustrating the *AscI* restriction site for linearization between AOX-TT sequences. ORI – origin of replication for *E. coli*, KanMX – kanamycin resistance, P_{GAP} and CYC-TT – promotor and terminator for TF expression in *P. pastoris*. The map was created with SnapGene.

3.6.2 PCR amplification

For PCR reactions, DreamTaq Green PCR Master Mix was used. About 50 ng of DNA were mixed with 0.5 µM of each primer to a final volume of 25 µl. PCR cycling conditions and pipetting instructions were performed according to the Thermo Scientific guidelines and are shown in Table 3.9. For analysis, 10 µL of each sample was loaded onto a 1 % agarose gel for 45 min at 100 V. Used primer pairs are listed in the Appendix (Table 7.2).

Table 3.9 PCR conditions for TF amplification using the DreamTaq Green PCR master mix.

Phase	Temperature	time
Initial Denaturation	95 °C	3 min
for 30 cycles		
Denaturation	95 °C	30 s
Annealing	58 °C	30 s
Elongation	72 °C	60 s
Final Elongation	72 °C	10 min
Hold	4 °C	∞

3.6.3 Transformation of *P. pastoris*

The condensed protocol of Lin-Cereghino was used for transformation of *P. pastoris* [44]. About 1 – 2 µg of linearized plasmid DNA were used for electroporation (2 kV, 200 Ω, 25 µF). Five hundred µL of 1 M sorbitol and 500 µL of YPD medium were added to the cells prior regeneration. After 2 h of regeneration at 28 °C, the cells were plated on YPD-Zeo/Gen agar plates and incubated for 3 days at 28 °C.

3.6.4 Cultivation of *P. pastoris* strains and culture supernatant analyses

24-deep well plates were used according to the project standard protocol: screening of P_{AOX1} production strains (AG Mattanovich, BOKU Vienna) and screening of P_{GAP} production strains (AG Mattanovich, BOKU Vienna, Version 3). One 24-deep well plate included at least two wells inoculated with the parent strain and two wells with a sterile control. The cultivation was performed at 25 °C, 250 rpm and 80 % humidity in a shaking incubator.

Briefly, P_{AOX1} carboxylesterase strains were inoculated in 2 mL M2D media containing one glucose feed bead per well with a single colony in screening and a single colony streaked out transformant in re-screening and were incubated for approximately 22 h. After washing and resuspending the culture in M2 (BMM) media, the main culture was inoculated to an OD_{600} of 4 and the expression was initiated by the addition of 0.5 % MeOH. MeOH was added to 1 % into each well after 6 h, 24 h and 30 h (Figure 3.2). For the P_{GAP} carboxylesterase strains, 2 mL YPD media were inoculated with a single colony in screening and a single colony streaked out transformant in re-screening and were incubated for approximately 22 h. After washing and resuspending the culture in M2 (BMM) media, the main culture was inoculated to an OD_{600} of 1 and the expression was initiated by the addition of one glucose feed bead per well. No additional feed was necessary (Figure 3.2). Harvest and cell mass determination (OD_{600}) were done after selection and 48 h after starting induction. Supernatants were analyzed immediately or stored at -20 °C.

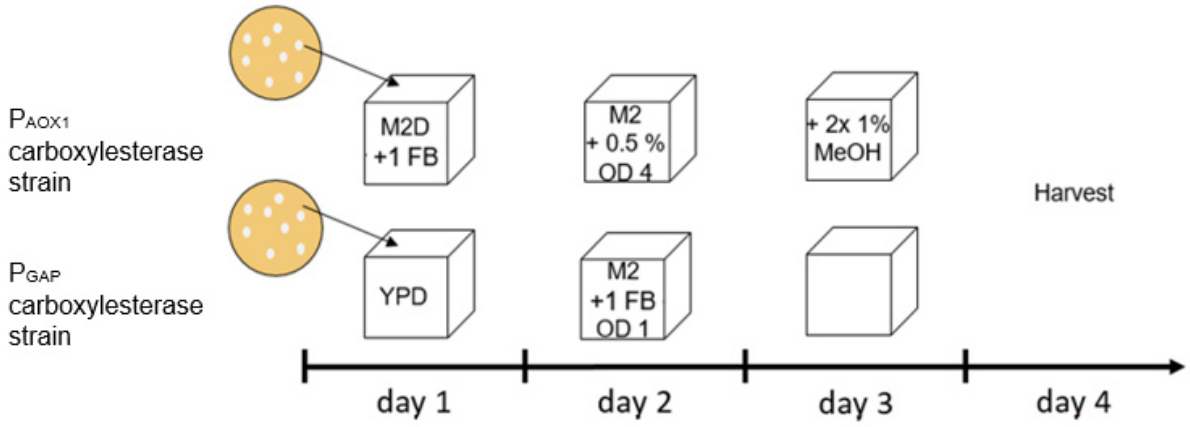


Figure 3.2 Screening cultivation process for *P. pastoris* P_{AOX1}/ P_{GAP} carboxylesterase secreting strains. One cube represents one well of a 24-deep well plate. The transformation plate illustrates that one colony was used per well. M2, M2D and YPD are the used media at each cultivation step. FB - glucose feed bead. OD - start OD main culture.

An ELISA assay was used for protein assessment and comparison of the different carboxylesterase-secreting transformants. All carboxylesterase standard dilutions were done with coating buffer to concentrations between 7.8 and 1000 ng/mL. High-binding microtiter plates were washed with 200 µL washing buffer for three times between all single ELISA steps (5 min, 500 rpm, RT). For coating, 100 µL of the carboxylesterase standard dilutions and undiluted culture supernatants were applied and incubated on a shaker at 4 °C over night (Figure 3.3). To block all protein binding positions on the plate, the wells were incubated at 37 °C for 1 h with 200 µL blocking

buffer. Afterwards, 100 µL rabbit anti- carboxylesterase IgG (1:10,000 diluted in dilution buffer) were filled into the wells for carboxylesterase binding and incubated for 1 h at 37 °C. For detection, 100 µL goat anti-Rabbit IgG-alkaline phosphatase (1:10,000 diluted with dilution buffer) were filled into the wells and again incubated for 1 h at 37 °C. Finally, 100 µL substrate solution were added and after approximately 15 min of incubation, the yellow colour change was measured at 405 nm and 620 nm (reference wavelength) on the plate reader. An absorption value of roughly 2 was used for the highest carboxylesterase standard concentration. The quantitative analyses of the enzyme concentration according to the standard curve was done with a four parameter logistic curve (www.myassays.com). The determination underlies following formula:

Formula 3.1 Calculation of the carboxylesterase concentration with a 4PL regression. a = the minimum value that can be obtained. d = the maximum value that can be obtained. c = point of inflection. b = Hill´s slope of the curve. x = concentration. y = measured absorption value.

$$y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^b}$$

In re-screening, three high, two medium and one low enzyme secreting transformants were studied.

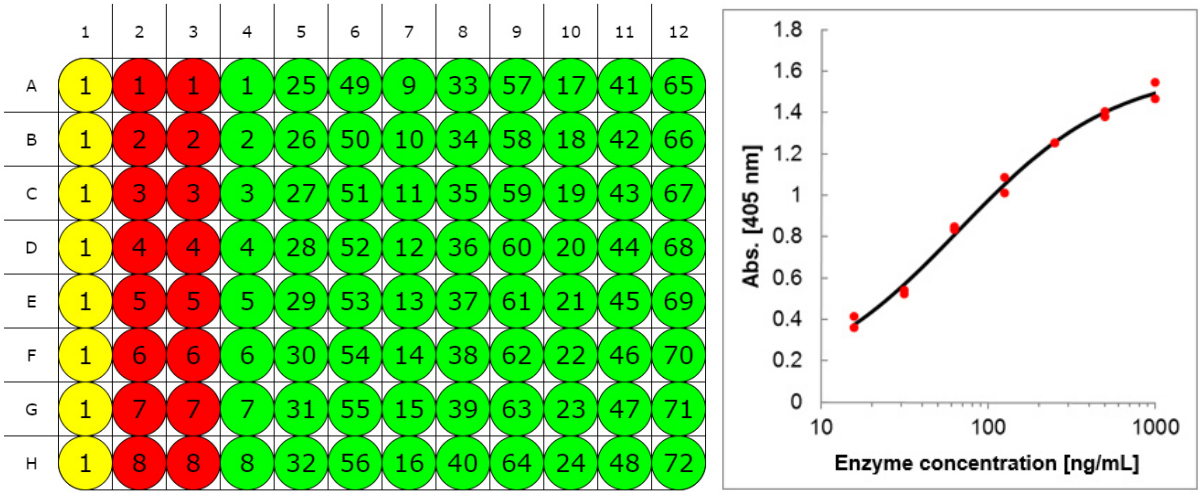


Figure 3.3 Pipetting scheme and 4PL standard curve for the ELISA screening assay. Left: 96 deep well ELISA plate. Yellow- Blank. Red- carboxylesterase Standard, starting with the highest concentration. Green- triplicate measurement of the 24 samples from the 24-deep well plate cultivation. Right: carboxylesterase standard curve after performing the ELISA assay. The red dots show the different dilutions. The course follows a 4PL regression by formula 3.1. The layout and curve were created with the tool from www.myassays.com.

Because of low enzyme concentrations in the culture supernatants, the cultivation media was changed to BM media instead of M2 media for selected transformants. Thereupon, the secreted enzyme amount increased significantly, and culture supernatants had to be diluted at least 1:200 with coating buffer before application.

3.6.5 Bioreactor cultivation of *P. pastoris* and culture supernatant analyses

The DasGip® bioreactor system (Eppendorf) with six simultaneously operating benchtop bioreactors was used for all *P. pastoris* cultivations. This system allows control of pH (by base feed), DO ≥ 30 % (by agitation between 500 – 1250 rpm), carbon feed and temperature in each single vessel. One set of experiments included one parent strain, one evc and up to four chosen strains from re-screening to gain comparable results related to the controls. The experiment was done according to a protocol provided by VTU.

Briefly, a pre-culture with 50 mL YPheG media was inoculated with one freshly streaked out colony and incubated at 28 °C and 120 rpm for approximately 20 h. The main culture was inoculated to an OD_{600} of 2 and was cultivated under the same conditions for approximately 6 h. Each bioreactor vessel contained 500 mL freshly prepared and autoclaved BSM media. The inoculation OD_{600} was 1.5 using the appropriate amount of the main culture (~ 40 mL). Each bioreactor cultivation was subdivided into batch (~ 18 h), fed-batch (~ 7 h), mixed feed (~ 18 h, only for P_{AOX1} carboxylesterase strains) and production phase (~ 70 h for P_{AOX1} carboxylesterase strains, ~ 85 h for P_{GAP} carboxylesterase strains). Mainly, the feed rate rose linear for the first 10 h of production phase (starting from 1 to 4 mL/h for P_{AOX1} and starting from 1.5 to 5 mL/h for P_{GAP}). After that, the feed rate was constant for further cultivation. The experimental set up of the parameters in the different phases is given in Table 3.10.

Table 3.10 Set up of important parameters and feeds in the different phases during bioreactor cultivation. The glycerol and MeOH amount is the total value at the end of the specific phase. The first feed number is the starting value that increased linearly to the final value during the phase.

	Phase	Duration [h]	Glycerol [mL]	MeOH [mL]	Feed [mL/h]	pH	Temp. [°C]
P_{AOX1} strains	Batch	~ 18	/	/	/	5.5	28
	Fed-batch	~ 7	~ 16	/	1 to 3	5.5	28
	Mixed Feed	~ 18	~ 36	~ 18	Glycerol 1 to 3 MeOH 0.5 to 1.5	5	24
	Prod. phase	~ 70	/	~ 190	1 to 4	5	24

	Phase	Duration [h]	Glycerol [mL]	MeOH [mL]	Feed [mL/h]	pH	Temp. [°C]
P_{GAP} strains	Batch	~ 18	/	/	/	5.5	28
	Fed-batch	~ 7	~ 25	/	1 to 3.5	5.5	28
	Prod. phase	~ 85	~ 400	/	1.5 to 5	5.5	24

During cultivation, samples were taken regularly (~ every 10 to 12 h) starting at the end of the batch phase until harvest. The first 4 mL of the sampled cultivation broth were always discarded. One mL of the freshly taken samples was transferred into a reaction vessel and spun down for 2 min at 13,200 rpm. The supernatant was transferred into a new vessel and immediately stored at -20 °C. The cell pellet was dried at 100 °C for at least 24 h before determination of the dry cell weight (DCW). All samples (starting from the production phase) were diluted 1:2, 1:5, 1:10 and 1:20 with 1x FCE buffer. Twenty µL of the diluted samples (parent, evc and TF overexpressing strains) and a negative control (1x FCE buffer) were filled into a microtiter plate. The activity assay was started by the addition of 180 µL of pNSI reaction solution. The reaction took place at 37 °C and the colour development was measured every 90 s for 1.5 h on a spectrophotometer. The enzyme activity was calculated using following formula:

Formula 3.2 Calculation of the enzyme activity with the alkaline phosphatase assay. F = dilution factor. mAU/min = measured absorption at 405 nm. Vmax = total volume in mL. ε = 13.9 mL/cm*µmol. V = sample volume in mL. d = layer thickness (0.45 cm).

$$\text{Activity}\left(\frac{\text{U}}{\text{L}}\right) = \frac{{}^{\text{mAU}}/_{\text{min}} * \text{f} * \text{Vmax}}{\boldsymbol{\varepsilon} * \text{V} * \text{d}}$$

Only values in the linear range of the absorption over time change were used for calculation and chosen manually for each sample and dilution.

To show the development of the recombinant protein in the supernatant during cultivation, an SDS-PAGE was performed with samples from different time points. Each sample was diluted 1:2 with water (finally 10 µL). Four µL NuPage® LDS sample Buffer (4x) and 1.5 µL NuPage sample reducing agent (10x) were added and finally heated for 7 min at 80 °C. Samples were applied to a 4 -12 % Bis-Tris Gel and run in 1x NuPage® MES Running buffer at 200 V for 35 min. The gels were stained for 1 h at RT with SimplyBlue Staining solution on a shaker. Additionally, Western blotting was performed for some selected transformants. Therefore, an SDS-PA gel was run and

blotted onto a nitrocellulose membrane. Between all antibody binding steps, the membrane was washed 3 times with 1x TBST for 10 min at RT. Each membrane was incubated for 1 h on a shaker with the antibodies: primary antibody was rabbit anti-carboxylesterase IgG and secondary antibody was goat anti-rabbit IgG-Peroxidase conjugate (1:10,000 diluted with TBST-milk). Protein bands were visualized by covering the membrane with 1.5 mL peroxide and 1.5 mL enhancer solution (SuperSignal® WestPico Chemiluminescent Substrate for HRP). The recording of images was done with the GeneSnap program (G-Box of SynGene, UK). For quantification, 50, 100 and 200 ng of enzyme were plotted on the same SDS gel.

3.6.6 Genomic DNA Isolation

The genomic DNA isolation was adapted from Hoffman and Winston [45]. A 50 mL YPD culture was incubated for approximately 20 h at 28 °C to an OD_{600} between 4 and 6. The equivalent volume of 100 OD_{600} units was transferred into a 50 mL tube and centrifuged for 5 min at 1500 rpm. The cell pellet was resuspended in 500 µL sterile water, transferred into a 1.5 mL centrifugation vessel and spun down shortly at 13,200 rpm. Afterwards the supernatant was discarded and 200 µL yeast lysis buffer, 200 µL PCI and 0.3 g of acid-washed beads were added. The vessel was shaken for 3 min and afterwards 200 µL of TE buffer were added and were centrifuged for 5 min at 13,200 rpm. The aqueous phase was transferred into a new vessel, 1 mL of 100 % ethanol was added, the vessel was gently inverted and spun down for 1 min at 13,200 rpm. The supernatant was removed and the pellet was resuspended in 400 µL of TE buffer and 5 µL RNAse A (10 mg/mL). After 2 h of incubation at 37 °C, 10 µL of 4 M ammonium acetate and 1 mL of 100 % ethanol were added. The vessel was centrifuged for 1 min at 13,200 rpm and the supernatant was discarded. Finally, the pellet was washed with 1 mL of 70 % ethanol and was dried before resuspension in 50 µL ddH_2O. The DNA concentration was measured with a UV-Vis spectrophotometer. Purity values were measured ($A_{260}/A_{280} \geq 1.8$, $A_{260}/A_{230} \geq 2.0$) before RT-PCR.

3.6.7 Copy number detection using RT-PCR

The copy number of the expression cassette of the best transformants of each construct was determined by quantitative RT-PCR following a protocol described by Abad et al. (2010) using following primer pairs: ARG4_RT_fw/ARG4_RT_rv and

Kan_RT_fw/Kan_RT_rv (Table 7.2). All steps were done on ice and under clean and fast working conditions using the ABI PRISM 7300 Real Time PCR System [43]. For the calibration curve, the gDNA of a single copy *P. pastoris* reference strain (PDI-1-copy) was used at the following concentrations: 11, 3.6, 1.2, 0.4, 0.1 and 0.033 ng/µl. All other strains with unknown copy number and the reference strain were diluted to a concentration of 0.66 ng/µL with ddH_2O.

3.6.8 Specific membrane associated tests for Upc2 strains

3.6.8.1 Plate spotting tests

Prior to spotting, a pre-culture (chapter 3.6.5) was cultivated for 48 h in YPD media, assuming that the cells are in a stationary phase. A total of five 1:10 dilutions starting at an OD_{600} of 1 were made for each strain and 3 µL were spotted onto the respective plates (Figure 3.4). The YPD plates contained the following chemicals: 5 mg/L calcofluor white, 1.5 mM sodium orthovanadate and 4 mg/L Congo red. For growth control, a YPD plate was spotted as well. The spotted plates were incubated at 28 °C for 3 days and photographed afterwards.

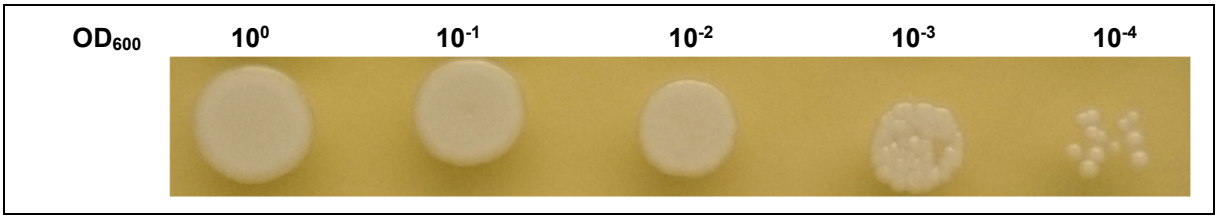


Figure 3.4 Culture spotting scheme for the plate test specifying the dilutions. The picture shows one strain growing on YPD agar after 3 days of incubation at 28 °C. 3 µL of each dilution were spotted.

3.6.8.2 FM4-64 staining

For an even FM4-64 uptake, the cells needed to be in exponential growth phase. Therefore, a *P. pastoris* pre-culture in YPD was prepared (chapter 3.6.5) and used to inoculate a YPD main culture to an OD_{600} of 0.1. The main culture was grown for 4 h at 28 °C and 120 rpm. Afterwards, 2 mL of the culture were centrifuged for 1 min at 5000 rpm and the pellet was resuspended in 90 µL of YPD media. The cells were incubated with 10 µL of 400 µM FM4-64 (1:40 dilution from the 16 mM stock solution) on ice for 30 min. To get rid of the remaining FM4-64 that was not taken up, the cells were washed twice with 500 µL ice-cold YPD medium and spun down at 5000 rpm for 1 min. The pellet was resuspended in 200 µL of YPD medium and incubated at 30 °C

and 300 rpm in a Thermo Fisher thermomixer covered with aluminium foil. Fifty µL aliquots were taken after 15 min, 30 min, 45 min and 180 min of incubation and pipetted into 500 µL of ice-cold media containing 15 mM each of NaN_3 and NaF to stop the uptake. The cells were centrifuged at 5000 rpm for 1 min and washed twice with 200 µL medium containing NaN_3 and NaF. Before microscopy, the cell pellet was resuspended in 10 µL medium with NaN_3 and NaF. Fluorescence microscopy was done with an excitation maximum of 558 nm, an emission maximum of 734 nm and 90 s exposure time. All phase contrast images were obtained with 40x magnification and immersion oil. The fluorescence images were analyzed using ImageJ for area, mean grey value and integrated density values. For each labeled strain, two images were chosen and 30 cells were selected randomly and circled with the button freehand selection. For each cell a background correction of the same size and next to the cell was considered and subtracted. The average total grey value was calculated based on the area and integrated density and was finally divided by the number of cells (= 30). The chosen two images should ideally show the same average value.

4 Results and Discussion

To validate whether overexpression of each of the transcription factors (Upc2, putatively Mit1 and Rpn4) had an effect on carboxylesterase secretion, i.e. secreted yields and titers, in *P. pastoris*, the TF overexpressing transformants were studied in screening, re-screening and bioreactor cultivations. In addition, spotting test, MS analysis and FM4-64 staining were performed for Upc2 overexpressing transformants. For a clear overview, the fold-change yield values are illustrated. The titer values were variable because of growth problems using the M2 media. A detailed listing (with highlighted transformants for re-screening) of all results is given in the Appendix (Table 7.3 – 7.24).

4.1 Screening and re-screening of TF over-expressing transformants

The parent strains (P_{GAP}-Parent and P_{AOX1}-Parent) were transformed with the three TFs and the empty vector pPuzzle_P_{GAP}_evc plasmid. The average fold-change yield of P_{GAP}-Parent-evc was 1.03 (fold-change titer = 1.08). In contrast, for P_{AOX1}-Parent-evc the average fold-change yield was 0.59 (fold-change titer = 0.59), which may be due to overexpression of the resistance marker or a copy number effect.
ELISA screening data are depicted as fold-change values in M2 media normalised to the average of the parent strain (average of 3-4 replicates). The fold-change yield values are given in Figure 4.1 to Figure 4.5. In re-screening, three high, two medium and one low secreting transformant were studied in triplicates to evaluate promising candidates for bioreactor cultivation.

4.1.1 Screening of transformants upon Upc2 overexpression

In *S. cerevisiae*, the transcription factor Upc2 is a sterol regulatory element binding protein involved in ergosterol biosynthesis [20]. In this work, the influence of its *P. pastoris* homolog on recombinant protein secretion was studied. At least eleven Upc2 overexpressing transformants were screened per parent strain and were evaluated for secreted enzyme levels. An average fold-change yield of 0.94 was reached for the P_{AOX1}-Parent-Upc2 and 0.80 for the P_{GAP}-Parent-Upc2 transformants (Figure 4.1), indicating rather a negative effect. Yield values of P_{AOX1}-Parent-Upc2 were mainly located between 0.4 and 1.1. Notably, the best transformants reached yield fold-change values of 1.82 and 2.09. These two transformants could be “outliers”

because of their remarkably higher fold-change yields compared to the 12 other transformants. Nevertheless, three P_{AOX1}-Parent-Upc2 transformants secreting low (0.37 – Upc2#10), medium (0.84 – Upc2#1) and high (1.82 – Upc2#5) levels were chosen for bioreactor cultivation to confirm these assumptions.

The values of P_{GAP}- Parent-Upc2 were clustered between 0.3 and 1.4 (Figure 4.1) and did not indicate a distinct tendency. One explanation could be that a different copy number of the plasmid caused the variety in enyzme yields of the different transformants. Since it was primarily not planned to use this TF in bioreactor cultivation, no re-screening was performed. Anyway, to validate the screening, three P_{GAP}- Parent-Upc2 transformants with medium (1.15 – Upc2#7) and high (1.22 – Upc2#1, 1.36 – Upc2#5) fold-change yields were chosen for bioreactor cultivation.

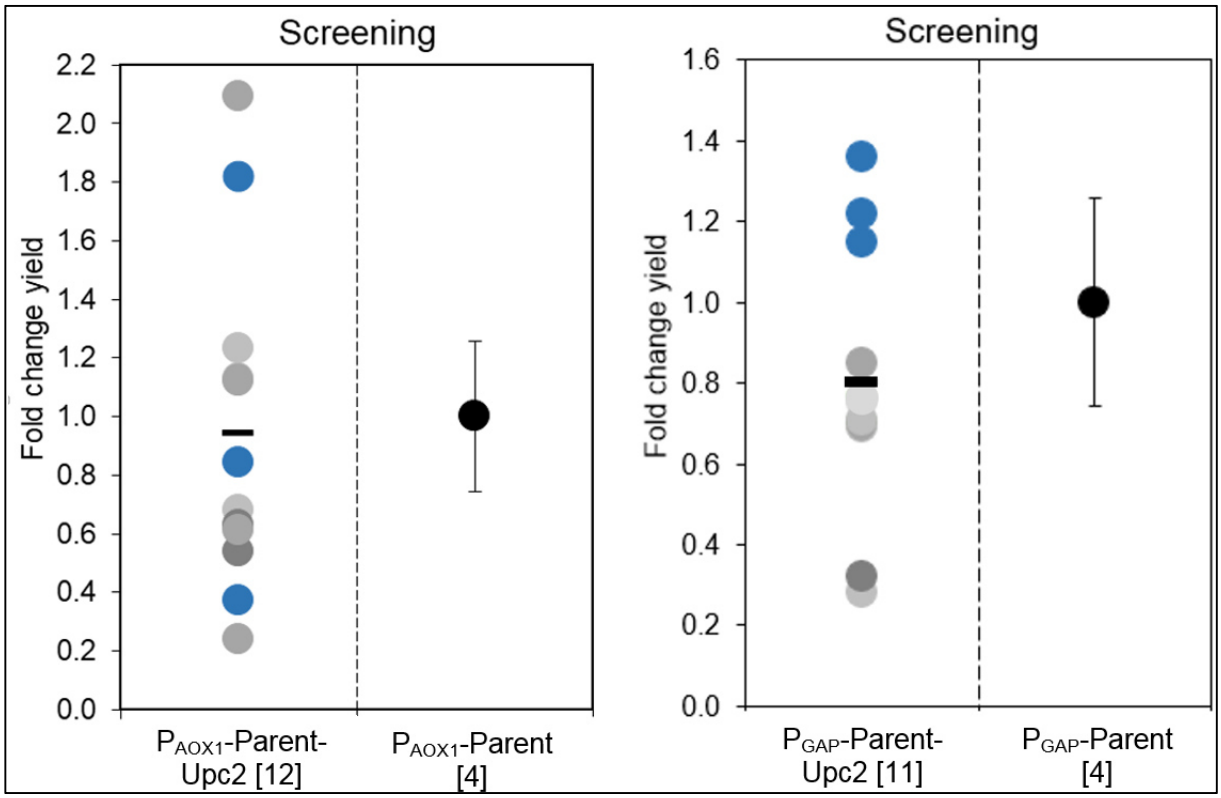


Figure 4.1 Screening results of P_{AOX1}-Parent-Upc2 and P_{GAP}-Parent-Upc2 transformants. Enzyme levels were determined by ELISA and are illustrated as fold-change levels of yield. The fold-change yields are related to P_{AOX1}-Parent at 12.9 ± 3.69 ng/mL/OD_{600} and P_{GAP}-Parent at 8.16 ± 1.20 ng/mL/OD_{600}. Transformants used in bioreactor cultivation are depicted in blue. The black bars the average levels. The brackets indicate number of screened transformants and biological replicates of parent strains.

4.1.2 Screening and rescreening of transformants upon Mit1 overexpression

The putative Mit1 might be a transcriptional regulator of pseudohyphal growth in *P. pastoris*, similar to *S. cerevisiae* [27]. For comparison of Mit1 overexpressing transformants to their parent strain, 24 transformants were screened and evaluated for secreted enzyme levels. In summary, an average screening fold-change yield of 1.66 was reached for the P_{AOX1}-Parent-Mit1 and 0.94 for the P_{GAP}-Parent-Mit1 transformants (Figure 4.2 and Figure 4.3), indicating a positive effect of Mit1 overexpression on enzyme secretion using P_{AOX} and a slightly negative effect using P_{GAP}.

About 80 % of the transformants of the strain P_{AOX1}-Parent-Mit1 were significantly improved with fold-change levels between 1.10 and 2.73. It seems possible that transformants with a certain copy number of the additionally gene do exhibit a positive effect on enzyme secretion. To evaluate their potential, three high improved transformants were studied in re-screening. Additionally, one lower and two medium secreting transformants were studied to verify the screening results.

Re-screening confirmed the fold-change yields for three of the six chosen transformants. The two-medium secreting transformants showed comparable values (up to 74 %) compared to the expected yields from screening (Figure 4.2 reddish and yellowish coloured sports). In contrast, the transformant with the highest fold-change yield in screening showed reduced protein levels and lost about 50 % compared to its screening value of 2.73. For further analysis, following transformants were selected for bioreactor cultivation: ● Mit1#16, ● Mit1#9, ● Mit1#21 and ● Mit1#20.

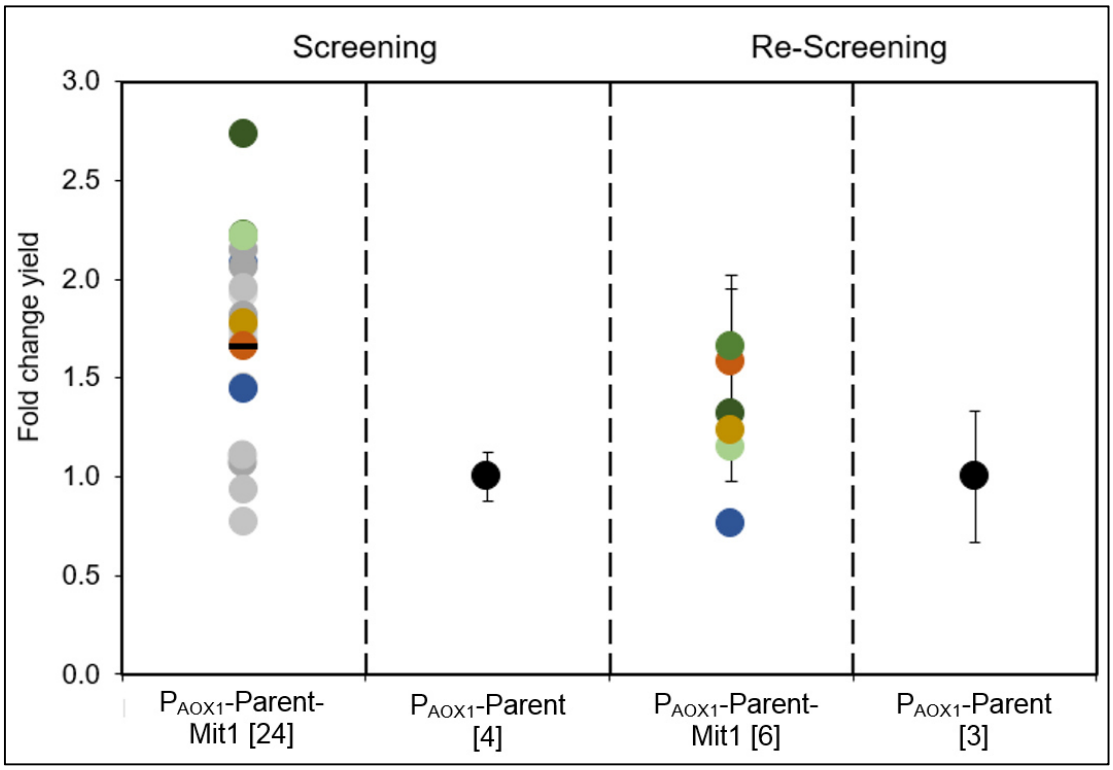


Figure 4.2 Screening and re-screening results of P_{AOX1}-Parent-Mit1 transformants. Enzyme levels were determined by ELISA and are illustrated as fold-change levels of yield. The fold-change yields are related to P_{AOX1}-Parent with 4.10 ± 0.51 ng/mL/OD_{600} and 1.00 ± 0.33 ng/mL/OD_{600}, respectively. Transformants used in Re-screening are depicted in color. The black bar illustrates the average level of all Screening values. The brackets indicate number of screened transformants and biological replicates of P_{AOX1}-Parent.

The fold-change yield values of P_{GAP}-Parent-Mit1 transformants were clustered between 0.5 – 0.9 and 1.1 - 1.7 (Figure 4.3). Around 30 % of the transformants showed higher enzyme secretion levels compared to the parent strain (up to 1.7-fold). In contrast, 16 out of 24 transformants showed a lower enzyme secretion compared to the parent strain. Six P_{GAP}-Parent-Mit1 transformants from low (0.71), medium (1.03, 1.08) and high (1.38, 1.54, 1.70) fold-change yields were selected for re-screening.

The re-screening confirmed the clustering of the Mit1 overexpressing transformants, reaching on the one hand improved and on the other hand decreased enzyme yields. For further analysis, the following transformants were selected for bioreactor cultivation: Mit1#18, Mit1#23, Mit1#19, Mit1#13 and Mit1#3.

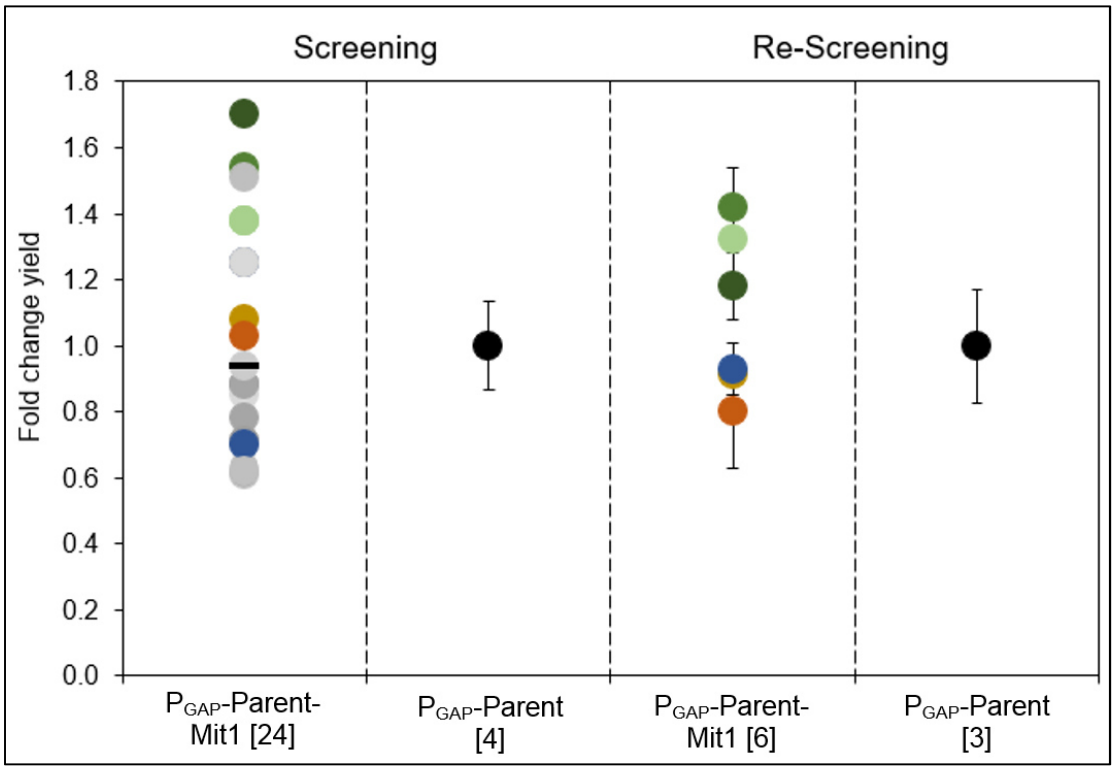


Figure 4.3 Screening and re-screening results of P_{GAP}-Parent-Mit1 transformants. Enzyme levels were determined by ELISA and are illustrated as fold-change levels of yield. The fold-change yields are related to P_{GAP}-Parent at 0.83 ± 0.11 ng/mL/OD_{600} and 0.76 ± 0.13 ng/mL/OD_{600}, respectively. Transformants used in re-screening are depicted in color. The black bar illustrates the average level of all screening values. The brackets indicate number of screened transformants and biological replicates of P_{GAP}-Parent.

4.1.3 Screening and rescreening of transformants upon Rpn4 overexpression

Rpn4 is a transcriptionally regulated short-lived protein that putatively stimulates the expression of proteasome-related genes to degrade incorrectly folded proteins in the ER in *P. pastoris* [37]. For comparison of Rpn4 overexpressing transformants to the parent strain, 27 P_{AOX1}-Parent-Rpn4 and 24 P_{GAP}-Parent-Rpn4 transformants were screened and evaluated for secreted enzyme levels compared to the parent strain. In total, overexpression reached an average fold-change yield of 1.16 (fold-change titer = 0.81) for the P_{AOX1}-Parent-Rpn4 and 1.18 (fold-change titer = 0.64) for the P_{GAP}-Parent-Rpn4 transformants in screening (Figure 4.4 and Figure 4.5).

The fold-change yield values for P_{AOX1}-Parent-Rpn4 ranged between 0.3 and 2.4 (Figure 4.4). Notably, more than 60% of the transformants showed higher enzyme secretion compared to the parent strain, indicating a putative positive effect. The best transformants reached fold-change values of 2.19 (fold-change titer = 1.60) and 2.35 (fold-change titer = 1.66), respectively. In contrast, two transformants with very low enzym secretion (fold-change yield 0.26 and 0.30) compared to the parent strain were

found. Both transformants showed significantly increased (70 %) OD_{600} values compared to the average OD_{600} levels and might for this reason not be representative. Six P_{AOX1}-Parent-Rpn4 transformants with low (0.63), medium (1.33, 1.57) and high (2.06, 2.19, 2.35) fold-change yields were selected for re-screening.

Re-screening confirmed the observed effects, showing higher enzyme yields for the best screening transformants compared to the parent strain. Notably, the values were generally lower compared to the screening. However, only low deviations between results from triplicates of the low, middle and high enzyme secreting transformants were observed, indicating that the experiment was valid. In this case, as observed for other model proteins, a possible change in TF copy number between screening and re-screening might also be the reason for the reduced improvements in re-screening compared to parent. To prove this, screening to re-screening copy number would have to be determined. For further analysis, following transformants were selected for bioreactor cultivation: ● Rpn4#5, ● Rpn4#22, ● Rpn4#6 and ● Rpn4#1.

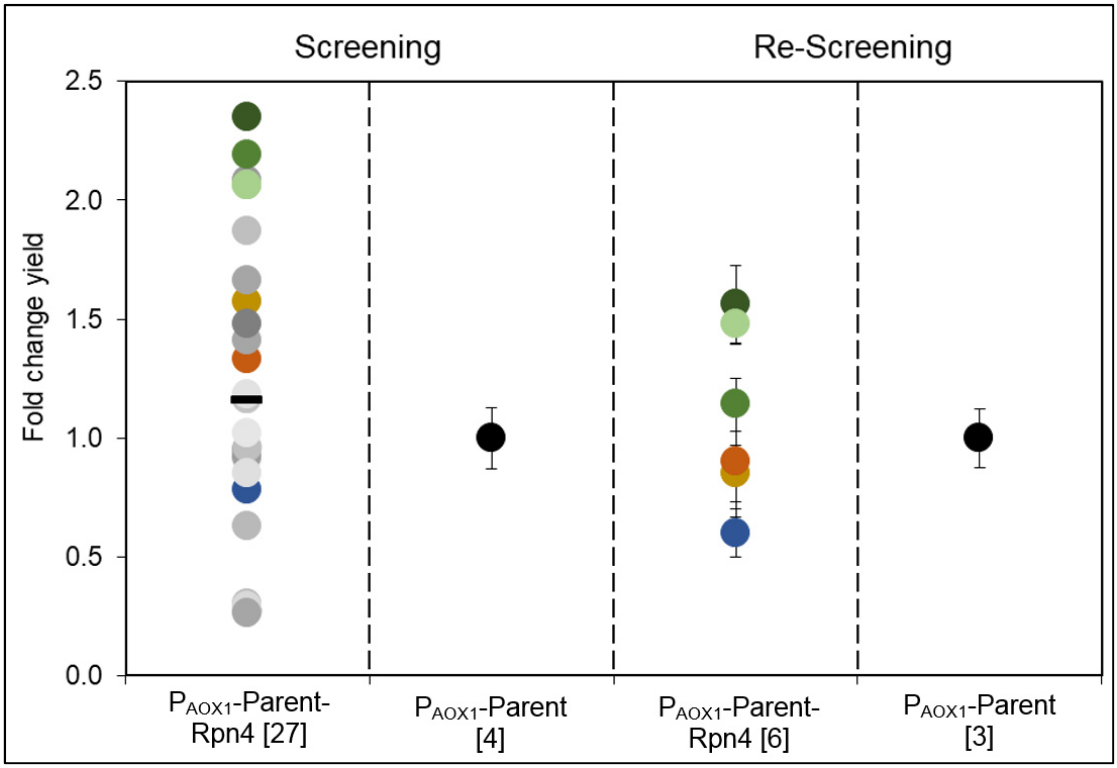


Figure 4.4 Screening and re-screening results of P_{AOX1}-Parent-Rpn4 transformants. Enzyme levels were determined by ELISA and are illustrated as fold-change levels of yield. The fold-change yields are related to P_{AOX1}-Parent with 12.9 ± 1.65 ng/mL/OD_{600} and 21.80 ± 1.81 ng/mL/OD_{600}, respectively. Transformants used in re-screening are depicted in color. The black bar illustrates the average level of all screening values. The brackets indicate number of screened transformants and biological replicates of P_{AOX1}-Parent.

The fold-change yield values for P_{GAP}-Parent-Rpn4 were found between 0.4 and 2.6 (Figure 4.5). Similar to P_{AOX1}-Parent-Rpn4, around 58 % of the transformants had higher enzyme secretion compared to the parent strain. The best five transformants reached fold-change values between 1.9 and 2.6. In contrast, 10 transformants showed a lower enzyme secretion (< 1.0-fold-change yield). Six P_{GAP}-Parent-Rpn4 transformants from low (0.74), medium (1.05, 1.51) and high (1.99, 2.07, 2.59) fold-change yields were selected for re-screening.

Re-screening confirmed the positive effect of Rpn4 overexpression on enzyme secretion, reaching an improved enzyme yield of 2.14 for the best transformant. Similar to P_{AOX1}-Parent, the observed fold-change yields were lower compared to the screening values and two transformants showed a high deviation in between the different biological replicates. Due to their constantly high values, the best three P_{GAP}-Parent-Rpn4 transformants were selected for further analysis in bioreactor: Rpn4#12, Rpn4#7 and Rpn4#2.

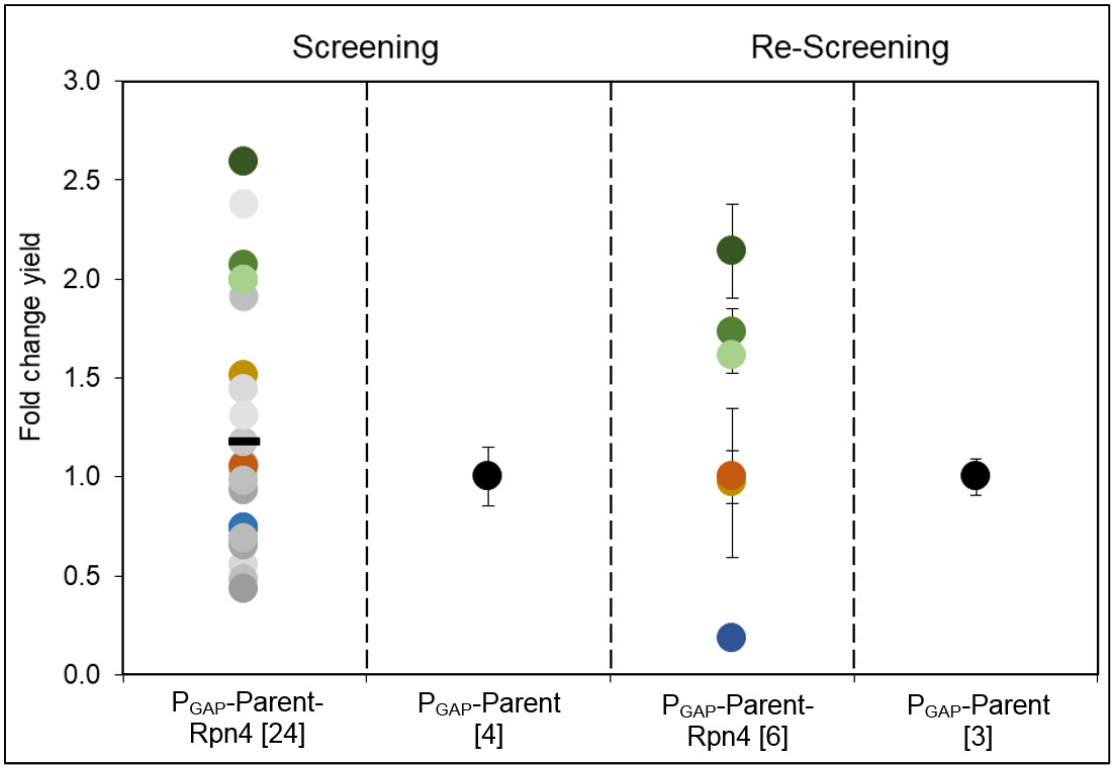


Figure 4.5 Screening and re-screening results of P_{GAP}-Parent-Rpn4 transformants. Enzyme levels were determined by ELISA and are illustrated as fold-change levels of yield. The fold-change yields are related to P_{GAP}-Parent with 8.16 ± 1.20 ng/mL/OD_{600} and 7.27 ± 0.66 ng/mL/OD_{600}, respectively. Transformants used in re-screening are depicted in color. The black bar illustrates the average level of all screening values. The brackets indicate number of screened transformants and biological replicates of P_{GAP}-Parent.

To sum up, it cannot be excluded that the observed differences between screening and re-screening are rather due to detection problems in the ELISA assay than effect of TF overexpression as the secreted enzyme levels were in the range of the lowermost area of the standard curve. Slightly different growth effects on the 24-deep well plate during cultivation (resulting in remarkable error bars) might in addition have influenced the results. Because of the low levels of secreted enzyme observed in ELISA (<1200 ng/mL), the cultivation media was changed to BM media instead of M2 media and selected transformants were again compared to verify the results from above screenings. The enzyme amount increased significantly to 100 – 400 µg/mL by using BM media and improved the reproducibility of ELISA measurements significantly. The full BM media is probably the better and more reliable media for screening.
Furthermore, the integration locus of the linearized plasmid in the *P. pastoris* genome or the copy number of the plasmid could have had an effect on the enzyme secretion yields and might be responsible for the grouped values at some screenings. For detection of the gene copy number in the *P. pastoris* genome, all transformants selected for bioreactor cultivation were tested with RT-PCR and specific primers as described in Materials and Methods (chapter 3.6). A copy number of one was determined for all transformants. However, whether the copy number changed between screening and re-screening remains still unclear.

4.2 Bioreactor cultivation of selected transformants

To assess whether the used screening protocol is a reliable method for scale up or not, selected transformants of the 24-deep well plate screening were studied in bioreactor cultivations. Using the DasGip system, up to six 1 L bioreactor vessels were used simultaneously. Unfortunately, the calibration of the oxygen electrodes was sometimes difficult, thus the quantification of the dissolved oxygen levels was frequently unreliable. The secretion of recombinant carboxylesterase in *P. pastoris* is very sensitive to changes in dissolved oxygen levels and may be increased under hypoxic conditions [46]. Each cultivation batch contained one parent strain for comparison. Culture samples were analysed for enzyme activity and dry cell weight over the period of cultivation (~ every 12-20 h). Additionally, final samples were applied to an SDS-PAGE gel. Results of TF overexpressing and enzyme secreting (P_{GAP}- and P_{AOX1}-) strains are described in the following. The bioreactor cultivations are classified in batches that differ in feeding amounts during the first hours of the production phase.

4.2.1 *P. pastoris* P_{AOX1}-Parent-TF bioreactor cultivations

Enzyme expression under the control of P_{AOX1} was divided into a glycerol-based growth phase (batch and fed-batch), a mixed glycerol and methanol phase and a methanol induction phase. While the transcription of carboxylesterase was repressed by glycerol access it was strongly induced by methanol. In contrast, the TF was expressed constitutively. Because of the low enzyme levels at the beginning of each cultivation, the activity was measured after 40 h of cultivation time until harvest.

Enzyme activity levels and biomass values of cultivation batch No. 1 containing P_{AOX1}-Parent-Upc2 and -Mit1 transformants is represented in Figure 4.6. During batch and fed-batch, nearly even biomass development was observed between the different transformants. However, upon methanol induction some growth differences were seen. Especially, the Mit1 overexpressing strains reached a higher cell density of 121 ± 7.8 g/L compared to the parent (116 g/L) and P_{AOX1}-Parent-Upc2#1 (111 g/L) strain. The enzyme activity of the parent strain (187 U/L) was almost similar to transformant P_{AOX1}-Parent-Mit1#21 (128 U/L). However, compared to the other strains lower enzyme levels were produced which is also seen at the activity level. Transformant P_{AOX1}-Parent-Upc2#1 reached significantly lower biomass compared to the other strains but highest enzyme activity at the harvest time point, i.e. 327 U/L, and thus a 2-fold improved activity compared to parent (187 U/L). Interestingly, for this transformant the enzyme activity significantly increased after 75 h which related to an observed decline in DCW between 50 h and 75 h of cultivation. One reason could be that the oxygen supply, i.e. levels of dissolved oxygen, was reduced at this time point because of cell clumping. The transformants P_{AOX1}-Parent-Mit1#16 and -Mit1#20 showed a four-times higher enzyme activity compared to the parent strain after 118 h of cultivation and the activity rose almost linearly until harvest to 674 U/L and 815 U/L, respectively. It seems that these transformants did not reach their maximum protein titers yet, because of a linear increase of enzyme activity until harvest. The lower activity of strain P_{AOX1}-Parent-Mit1#21, i.e. 187 U/L, might be explained by a stronger protein degradation observed by SDS-PAGE (Figure 4.6, line 5). The corresponding SDS-PAGE confirmed high levels of secreted enzyme (compared to parent) and showed only low protein degradation for P_{AOX1}-Parent-Mit1#16 and P_{AOX1}-Parent-Mit1#20 (Figure 4.6, line 4 and line 5). Interestingly, a protein band of high molecular weight was observed at about 130 kDa for transformant P_{AOX1}-Parent-Upc2#1 (Figure

4.6, line 3). In later experiments, it was found that this band is specific for Upc2 overexpression in *P. pastoris* and it was observed in all supernatant samples of Upc2 overexpressing strains.

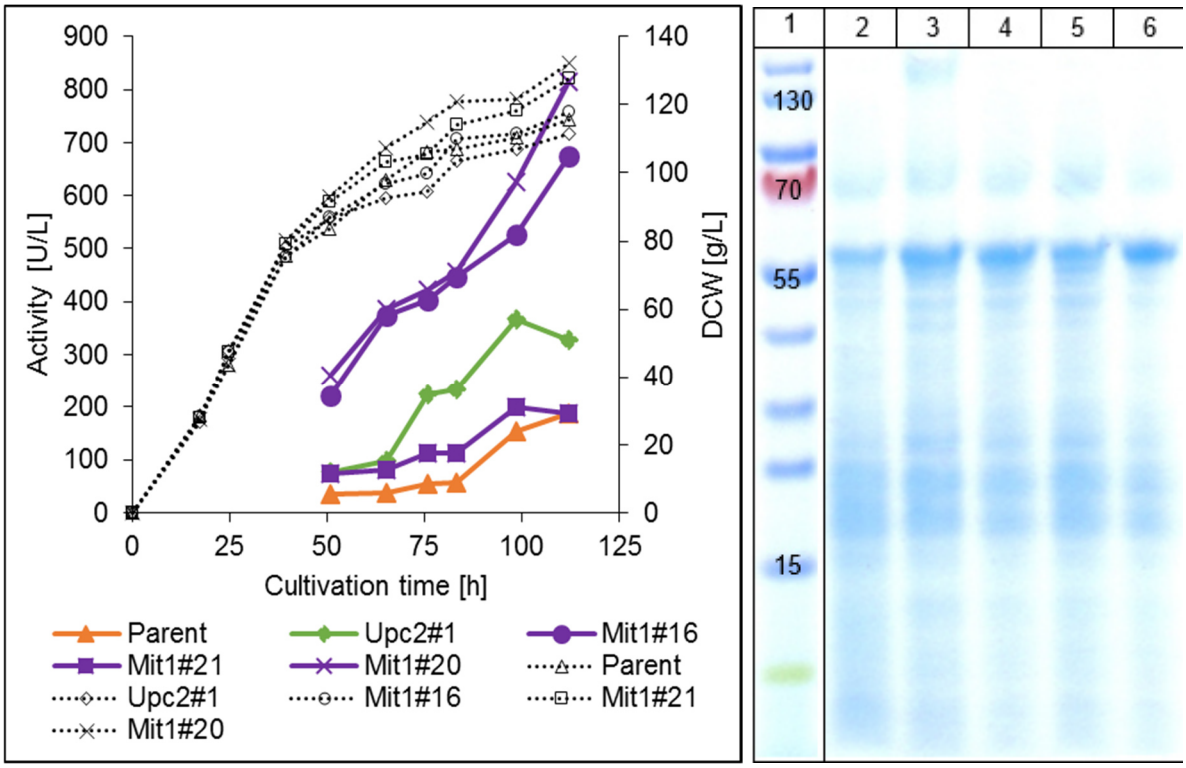


Figure 4.6 Comparable bioreactor cultivation results for P_{AOX1} strains (No. 1). Left: Enzyme activity using pNSi assay [U/L] – colored and DCW [g/L] – black symbols. Batch phase: 0 – 19 h. Fed-batch phase: 19 – 25 h. Mixed feed: 25 – 42 h. Methanol induction: 42 – 118 h. DCW – dry cell weight. Right: SDS-PAGE of samples after 118 h of cultivation. 5 µl of supernatant applied. The size of carboxylesterase is about 57 kDa. 1 – PageRuler prestained protein ladder (relevant marker bands are indicated, kDa). 2 – Parent strain. 3 – Upc2#1. 4 – Mit1#16. 5 – Mit1#21. 6 – Mit1#20.

The second batch of cultivation of P_{AOX1}-Parent-TF strains was performed to confirm the results of bioreactor cultivation No. 1. Therefore, the promising transformants, P_{AOX1}-Parent-Mit1#16 and P_{AOX1}-Parent-Mit1#20, were cultivated compared to the parent strain and, additionally, transformant P_{AOX1}-Parent-Upc2#5 was studied (Figure 4.7). All strains reached a similar biomass level of 121 ± 2.3 g/L. Only the parent strain biomass was about 10 % lower compared to all other transformants. Analyzing activity levels at harvest, transformant P_{AOX1}-Parent-Mit1#16 (367 U/L) had a three-times and P_{AOX1}-Parent-Mit1#20 (181 U/L) a 1.5-times higher enzyme activity compared to the parent strain. Also, the enzmye activity of transformant P_{AOX1}-Parent-Upc2#5, i.e. 236 U/L, was twice as high as the parent strain (133 U/L) and confirmed the positive effect of Upc2 overexpression on protein secretion under control of P_{AOX1}. However,

the enzyme activities of the two Mit1 overexpressing strains in this batch were only half as high as in batch No. 1. A variability in dissolved oxygen levels between the two batches seems likely. SDS-PAGE showed more or less the same protein band for the Mit1 overexpressing strains and the parent (Figure 4.7, line 2,3 and 4). The amount of carboxylesterase did not correlate with the gained enzyme activities of these strains. Again, the characteristic protein band at about 130 kDa was observed for transformant P_{AOX1}-Parent-Upc2#5 and a stronger protein band compared to the parent and confirmed the two-fold increased activity level (Figure 4.7, line 5).

Surprisingly, when genetically controlling the insertion of the expression cassettes by PCR using cassette specific primers, the Mit1 cassette could not be amplified. The reasons for this remained unclear. A determination of transcript levels might give a more detailed picture whether Mit1 is still overexpressed or not and might give indication upon genetic re-arrangements upon overexpression of Mit1 under P_{GAP}.

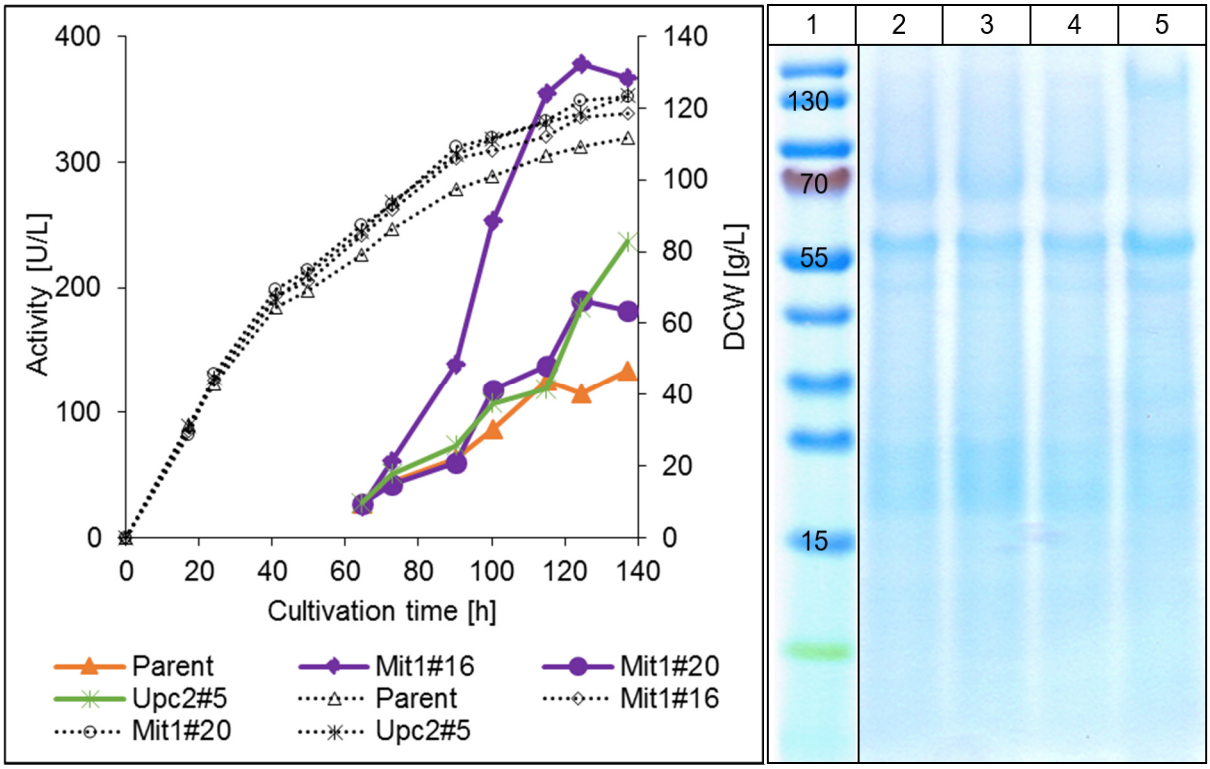


Figure 4.7 Comparable bioreactor cultivation results for P_{AOX1} strains (No. 2). Left: Enzyme activity using pNSi assay [U/L] – colored and DCW [g/L] – black symbols. Batch phase: 0 – 18 h. Fed-batch phase: 18 – 24 h. Mixed feed: 24 – 42 h. Methanol induction: 42 – 138 h. DCW – dry cell weight. Right: SDS-PAGE of samples after 138 h of cultivation. 5 µl of supernatant applied. The size of carboxylesterase is about 57 kDa. 1 – PageRuler prestained protein ladder (relevant marker bands are indicated, kDa). 2 – Parent strain. 3 – Mit1#16. 4 – Mit1#20. 5 – Upc2#5.

Enzyme activities of Rpn4 and Upc2 overexpressing transformants of cultivation batch No. 3 are represented in Figure 4.8. During batch and fed-batch, nearly even biomass formation was observed between the different transformants. Upon methanol induction, huge growth differences were observed. Especially, the Rpn4 overexpressing strains reached a two-fold higher cell density compared to their parent strain with 72.1 g/L after 117 h of cultivation. The enzyme activity levels of all strains increased steadily up to nearly 70 h of cultivation. Subsequently, a dramatic decrease in enzyme activity was observed until harvest while the DCW values were still increasing (Figure 4.8). Notably, a strong carboxylesterase protein band (57 kDa) was observed for the parent strain and transformant P_{AOX1}-Parent-Upc2#1 with hardly any protein degradation (Figure 4.8, line 2 and 3). Again, the characteristic protein band at about 130 kDa was observed for transformant P_{AOX1}-Parent-Upc2#1. Interestingly, for the Rpn4 overexpressing transformants only a half-intense carboxylesterase band with much more degradation was observed compared to parent (Figure 4.8, line4,5 and 6). The reasons for the observed loss in activity and protein degradation are unclear. However, based on previous experiments performed at BIOMIN Holding GmbH and BOKU, a media- and/or culture condition-dependency seems likely. In comparison to batch No. 1, the P_{AOX1}-Parent-Upc2#1 transformant and also the parent strain behaved atypically.

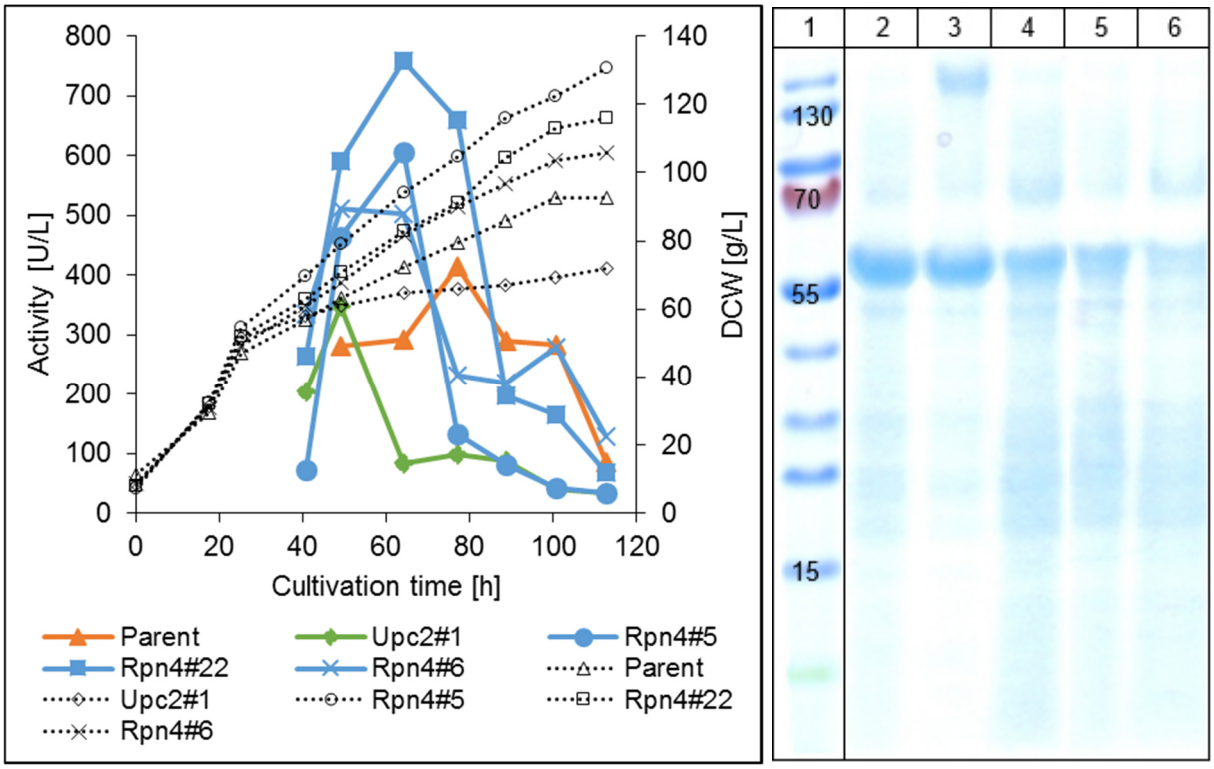


Figure 4.8 Comparable bioreactor cultivation results for P_{AOX1} strains (No. 3). Left: Enzyme activity using pNSi assay [U/L] – colored and DCW [g/L] – black symbols. Batch phase: 0 – 19 h. Fed-batch phase: 19 – 25 h. Mixed feed: 25 – 43 h. Methanol induction: 43 – 117 h. DCW – dry cell weight. Right: SDS-PAGE of samples after 117 h of cultivation. 5 µl of supernatant applied. The size of carboxylesterase is about 57 kDa. 1 – PageRuler prestained protein ladder (relevant marker bands are indicated, kDa). 2 – Parent strain. 3 – Upc2#1. 4 – Rpn4#5. 5 – Rpn4#22. 6 – Rpn4#6.

To complete the bioreactor cultivations of the methanol-inducible strains one weak P_{AOX1}-Parent-Upc2 and -Rpn4 transformant were chosen for batch No. 4 (Figure 4.9). In addition, one higher secreting P_{AOX1}-Parent-Mit1 from re-screening was studied. Additionally, parent strain and P_{AOX1}-Parent-evc#1 were cultivated to find out whether the empty plasmid has an effect on protein titers and activity. Biomass production was almost identical between all strains during cultivation. Only transformant P_{AOX1}-Parent-Mit1#9 showed about 20 % lower DCW level with 83.5 g/L prior harvest. This is actually untypically for Mit1 overexpressing strains which generally grew to higher cell densities compared to the parent strain in previous experiments (Figure 4.6, 4.7). Nevertheless, this strain showed a two-fold higher enzyme activity, i.e. 655 U/L, compared to the parent (282 U/L) and the most protein degradation on the SDS-PAGE gel (Figure 4.9, line 5). Furthermore, P_{AOX1}-Parent-Rpn4#1 showed at 1337 U/L a 4-fold higher enzyme activity compared to the parent and a huge carboxylesterase protein band at 57 kDa in SDS-PAGE (Figure 4.9, line 4). Surprisingly, the weakly secreting transformant from screening, strain Upc2#10, reached an eight-times higher enzyme activity, i.e.

2337 U/L, compared to the parent strain at the end of the cultivation with a nearly linear increase in production until harvest. Furthermore, SDS-PAGE showed low protein degradation (Figure 4.9, line 6) and improved protein production compared to parent. The characteristic high weight protein band at about 130 kDa was observed as for all other Upc2 overexpressing transformants (Figure 4.9, line 6). Enzyme activity of the parent strain increased similarly to the activity of the P_{AOX1}-Parent-evc#1 transformant. This was also confirmed by SDS-PAGe (Figure 4.9, line 2 and 3).

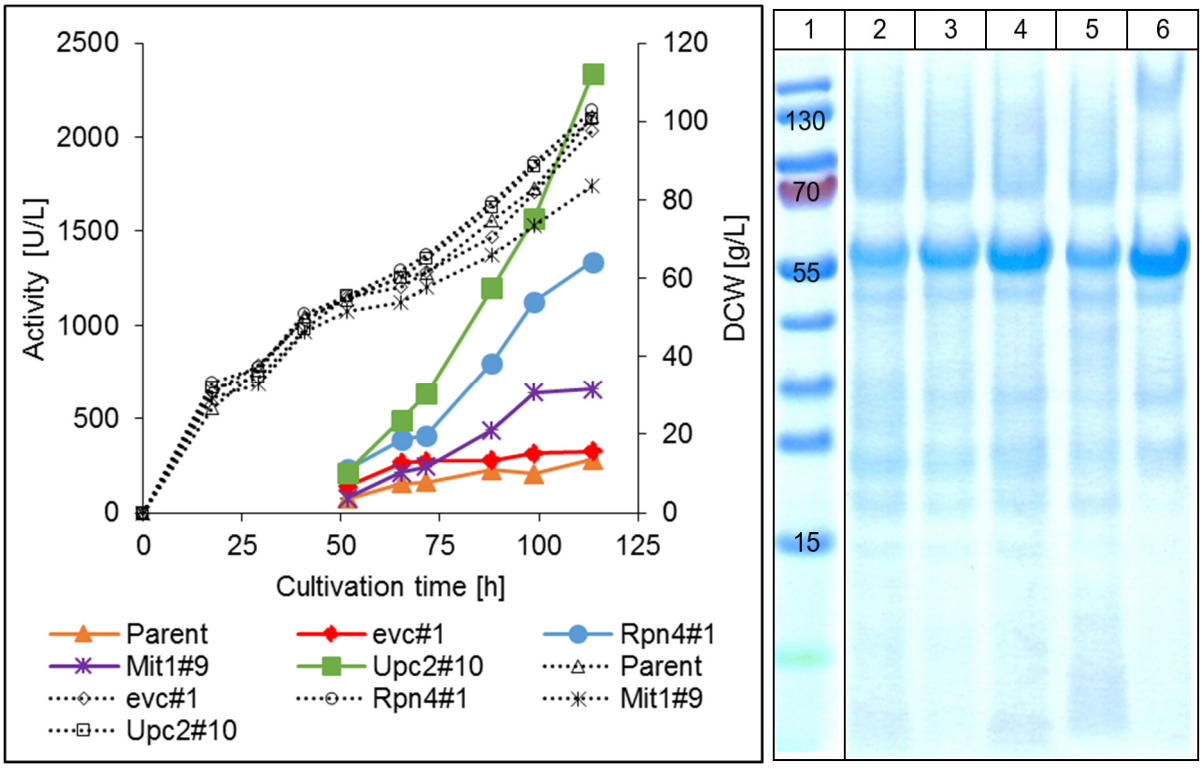


Figure 4.9 Comparable bioreactor cultivation results for P_{AOX1} strains (No. 4). Left: Enzyme activity using pNSi assay [U/L] – colored and DCW [g/L] – black symbols. Batch phase: 0 – 20 h. Fed-batch phase: 20 – 28 h. Mixed feed: 28 – 46 h. Methanol induction: 46 – 138 h. DCW – dry cell weight. Right: SDS-PAGE of samples after 138 h of cultivation. 5 µl of supernatant applied. The size of carboxylesterase is about 57 kDa. 1 – PageRuler prestained protein ladder (relevant marker bands are indicated, kDa). 2 – Parent strain. 3 – evc#1. 4 – Rpn4#1. 5 – mit1#9. 6 – Upc2#10.

The methanol-induced bioreactor cultivations did work well for each of the P_{AOX1}-Parent -Upc2 (#10, #5 and #1), -Mit1 (#20, #16 and #9) and –Rpn4 (#1) transformants. Overexpression of all three TFs yielded improved transformants compared to their parents, with the best transformants reaching eight-, three-, four-fold improvement, while the empty vector strain behaved as the parent, respectively. Especially, the results of the deep well plate screening of the P_{AOX1}-Parent-Mit1 transformants could be repeatedly confirmed. However, batch to batch variations in total activity levels were

observed for some of the Mit1 transformants. Genetic alterations as well as cultivation parameters could be reasons for the observed effects. The scale-up using the transcription factors Upc2 and Rpn4 was less successfully. Some transformants weak in screening reached the highest improvements in bioreactor compared to their parent strain.

4.2.2 *P. pastoris* P_{GAP}-Parent-TF bioreactor cultivations

Expression under control of P_{GAP} can be divided into a glycerol-based growth phase (batch and fed-batch - to reach higher cell densities) and a glycerol based induction phase under rate limiting conditions. Transcription of carboxylesterase and the overexpressed transcription factor was constitutively driven by glycerol during growth and induction phase.

Enzyme activity levels of cultivation batch No. 5 with the P_{GAP}-Parent-Rpn4 transformants #12, #2, #7 and –Upc2 transformant #1 is represented in Figure 4.10. All strains reached a final biomass level of 135.4 ± 7.3 g/L. Only transformant P_{GAP}-Parent-Rpn4#7 reached about 10 % higher biomass after 113 h of cultivation. The enzyme activities of all strains increased steadily up to a cultivation time of about 90 h. Subsequently, the activity of transformants P_{GAP}-Parent-Rpn4#2, P_{GAP}-Parent-Rpn4#7 and P_{GAP}-Parent decreased rapidly until harvest while the dry cell weight was still increasing. Compared to small scale, transformant –Rpn4#2 showed very low activity compared to its re-screening value and almost no secretory recombinant protein on SDS gel (Figure 4.10, line 5). The characteristic high molecular weight protein of about 130 kDa was observed for transformant -Upc2#1, while hardly any secreted carboxylesterase protein was observed (Figure 4.10, line 3). Transformant -Rpn4#12 showed a two-fold higher enzyme activity compared to the parent strain after 113 h of cultivation. However, the parent strain showed a significantly decreased value at this time point making a comparison difficult. Transformant -Rpn4#12 also showed strong protein degradation on SDS-PAGE. Similar activity losses were also observed for some of the P_{AOX1}-based, methanol driven cultivations and seemingly depended on bioprocess management. With small changes in bioprocess setup, e.g. feeding strategy, stirring rate, activity reductions could be avoided in subsequent cultivations.

Suspiciously and unexpectedly, the parent strain showed 3-fold higher activity compared to other batches, possibly due to changes in dissolved oxygen levels. Thus, results can only be compared with caution.

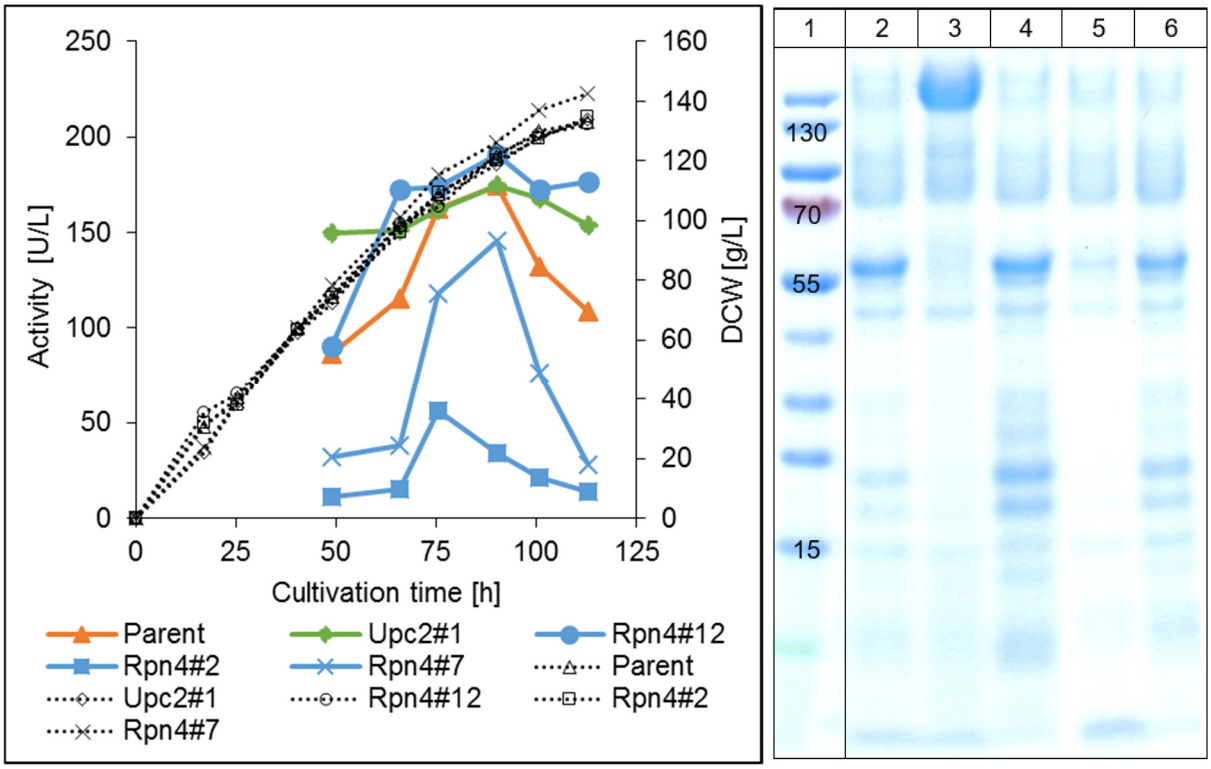


Figure 4.10 Comparable bioreactor cultivation results for P_{GAP} strains (No. 5). Left: Enzyme activity using pNSi assay [U/L] – colored and DCW [g/L] – black symbols. Batch phase: 0 – 19 h. Fed-batch phase: 19 – 26 h. Glycerol induction: 26 – 113 h. DCW – dry cell weight. Right: SDS-PAGE of samples after 113 h of cultivation. 5 µl of supernatant applied. The size of carboxylesterase is about 57 kDa. 1 – PageRuler prestained protein ladder (relevant marker bands are indicated, kDa). 2 – Parent strain. 3 – Upc2#1. 4 – Rpn4#12. 5 – Rpn4#2. 6 – Rpn4#7.

Enzyme activity levels of cultivation batch No. 6 of the P_{GAP}-Parent-Mit1 transformants #23, #18 and #13 is represented in Figure 4.11. Transformant P_{GAP}-Parent-Mit1#13 showed about 15 % higher biomass after 113 h of cultivation compared to the three other strains. However, this strain showed a 50% reduced final activity of 31.8 U/L compared to the parent, while the carboxylesterase protein bands on the SDS-PAGE were similarly strong (Figure 4.11, line 5). As observed before, a higher biomass yield rather correlated with lower protein expression levels. In contrast, transformant P_{GAP}-Parent-Mit1#23 showed a 1.5-fold higher activity compared to the parent strain and also improved secretion according to SDS-PAGE (Figure 4.11, line 4). The good performance of Mit1 transformants was further confirmed by transformant P_{GAP}-Parent-Mit1#18 which showed four-times higher activity compared to the parent strain (Figure 4.11, line 3 and 4), and also improvement on protein level. For confirmation, it was decided to study transformant P_{GAP}-Parent-Mit1#18 in bioreactor cultivation again (Figure 4.13).

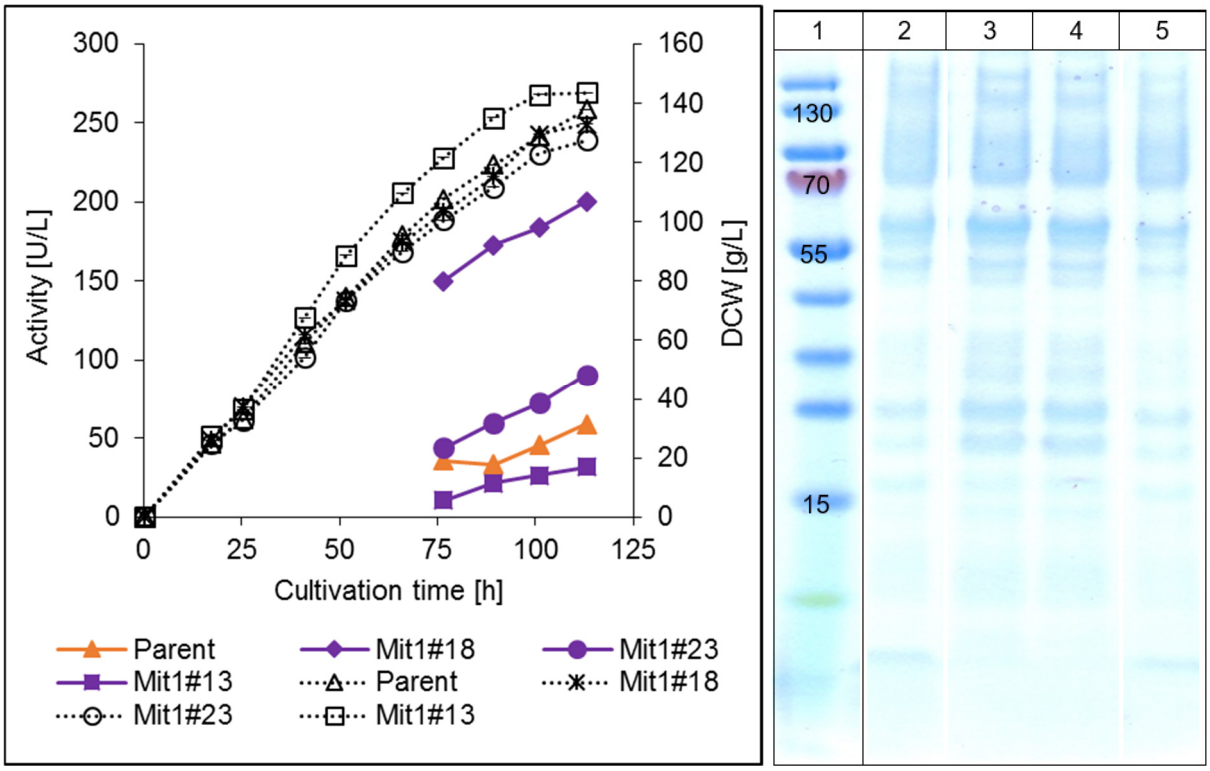


Figure 4.11 Comparable bioreactor cultivation results for P_{GAP} strains (No. 6). Left: Enzyme activity using pNSi assay [U/L] – colored and DCW [g/L] – black symbols. Batch phase: 0 – 21 h. Fed-batch phase: 21 – 27 h. Glycerol induction: 27 – 113 h. DCW – dry cell weight. Right: SDS page of samples after 113 h of cultivation. 5 µl of supernatant applied. The size of carboxylesterase is about 57 kDa. 1 – PageRuler prestained protein ladder (relevant marker bands are indicated, kDa). 2 – Parent strain. 3 – Mit1#18. 4 – Mit1#23. 5 – Mit1#13.

The third cultivation batch of P_{GAP}-Parent transformants was performed to review screening data using medium level secreting transformants in comparison to the parent strain (Figure 4.12), i.e. transformants P_{GAP}-Parent-Mit1#19 and -Mit1#3 and P_{GAP}-Parent-Upc2#7. Comparing biomass levels, the parent strain reached a 15 % higher final biomass compared to previous cultivations, but two-thirds less enzyme activity. This batch was run with a higher glycerol feed rate at the first few hours of production phase which might cause a higher biomass compared to other cultivations. During all other cultivations, the parent strain showed constant activity levels and biomass of 63.9 ± 29.4 U/L and 141 ± 8.3 g/L, respectively. Comparing activity levels, both P_{GAP}-Parent-Mit1 transformants showed slightly increased activity levels of 117 and 52.6 U/L, which was also confirmed by SDS-PAGE. In agreement with previous data, the higher-activity transformant P_{GAP}-Parent-Mit1#19 showed 20% lower biomass level and a more intense protein band at 57 kDa compared to transformant –Mit1#3 by SDS-PAGE (Figure 4.12, line 3 and 4). Notably, this effect of higher cell density and lower activity was also seen during other cultivations of transcription factor Upc2 and Mit1

overexpressing transformants. The bioreactor cultivations of Mit1 transformants confirmed data from re-screening, the enzyme activity of transformant P_{GAP}-Parent-Upc2#7 was found almost eight-times improved compared to the (usual) parent strain level, which is in contrast to its screening value. The good results of this transformant were confirmed by SDS-PAGE, which also showed only low protein degradation compared to the parent strain (Figure 4.12). As before, the characteristic high molecular weight protein band was found again at about 130 kDa for transformant P_{GAP}-Parent-Upc2#7 (Figure 4.12, line 5).

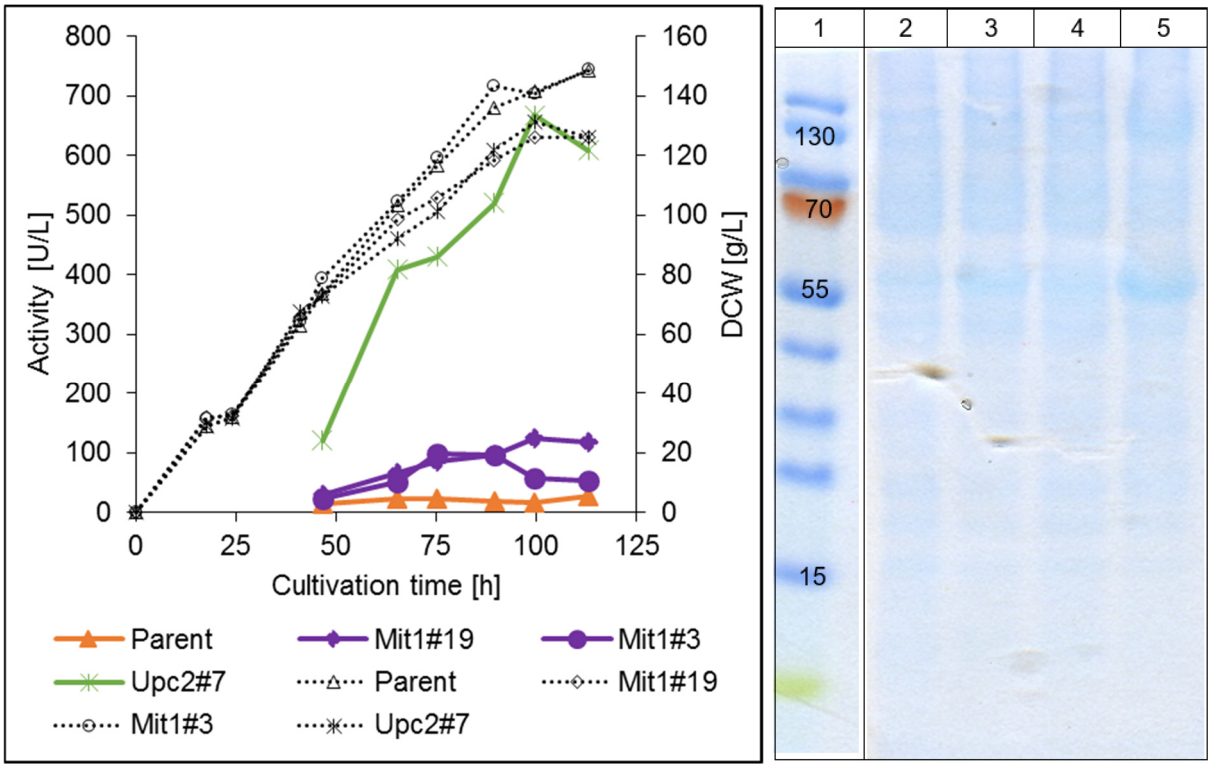


Figure 4.12 Comparable bioreactor cultivation results for P_{GAP} strains (No. 7). Left: Enzyme activity using pNSi assay [U/L] – colored and DCW [g/L] – black symbols. Batch phase: 0 – 18 h. Fed-batch phase: 18 – 24 h. Glycerol induction: 24 – 113 h. DCW – dry cell weight. Right: SDS-PAGE of samples after 113 h of cultivation. 5 µl of supernatant applied. The size of carboxylesterase is about 57 kDa. 1 – PageRuler prestained protein ladder (relevant marker bands are indicated, kDa). 2 – Parent strain. 3 – Mit1#19. 4 – Mit1#3. 5 – Upc2#7.

To complete the bioreactor cultivations of P_{GAP}-Parent TF overexpressing transformants, the transformant P_{GAP}-Parent-Mit1#18 (127 g/L, 258 U/L) and two new Upc2 transformants, i.e. -Upc2#1 and -Upc2#5, were studied in batch No. 8 (Figure 4.13). Additionally, the parent and empty vector strain P_{GAP}-Parent-evc#3 were cultivated to find out whether the empty plasmid influenced protein secretion and activity, or not.

The parent strain and empty vector control reached similar activity levels of 62.2 U/L and 64.4 U/L after 112 h of cultivation, respectively. This was also confirmed by SDS-PAGE, showing similarly intensive bands and degradation (Figure 4.13, line 2 and 3). In contrast to previous cultivations, transformant P_{GAP}-Parent-Upc2#1 did not show improved activity, while transformant -Upc2#5 showed 3-fold improvement compared to parent, which was also confirmed by SDS-PAGE. Both transformants showed the characteristic high molecular weight band (Figure 4.14, line 5 and 6). In contrast, previous good results were confirmed for transformant P_{GAP}-Parent-Mit1#18 showing 4-fold improved activity corresponding to a final activity of 258 U/L. The good performance of this transformant was also confirmed by SDS-PAGE showing significantly more protein compared to the parent.

Biomass levels were almost identical between all strains until about 50 h of cultivation time. Subsequently, both well-secreting transformants, -Mit1#18 and –Upc2#5, showed about 10 % lower biomass compared to the other strains.

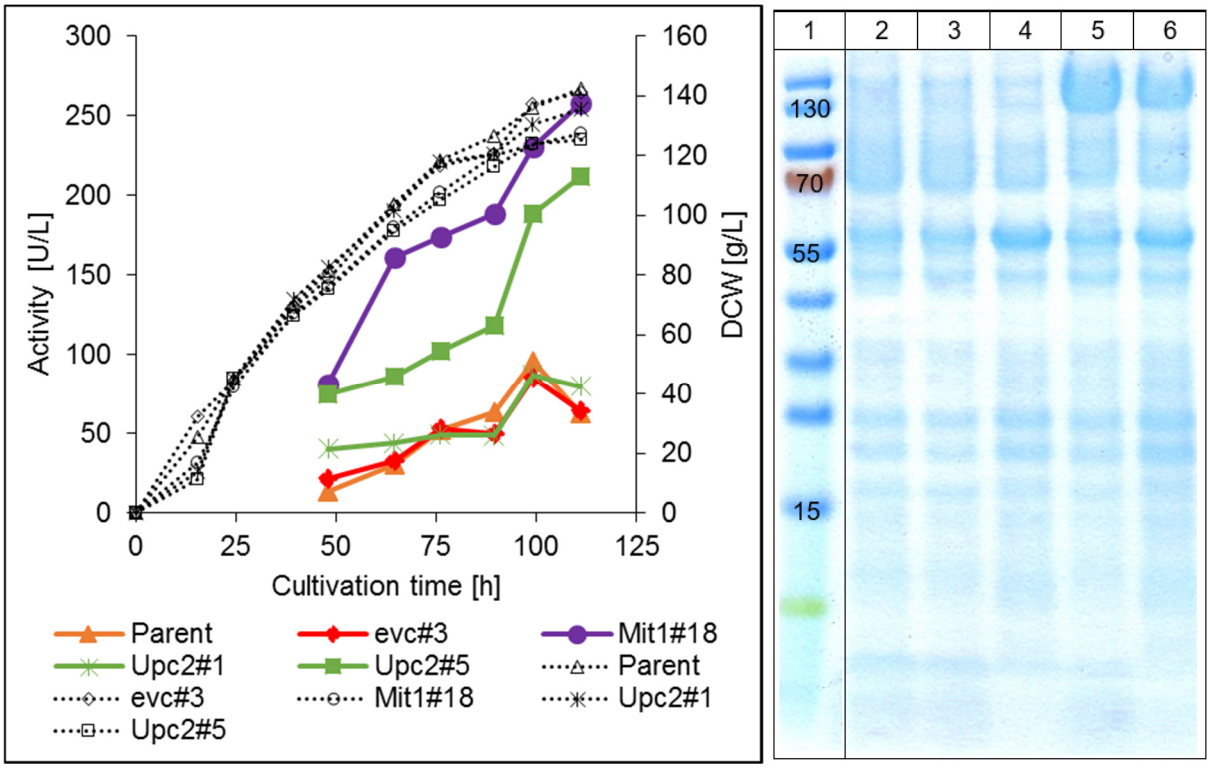


Figure 4.13 Comparable bioreactor cultivation results for P_{GAP} strains (No. 8). Left: Enzyme activity using pNSi assay [U/L] – colored and DCW [g/L] – black symbols. Batch phase: 0 – 17 h. Fed-batch phase: 17 – 24 h. Glycerol induction: 24 – 112 h. DCW – dry cell weight. Right: SDS-PAGE of samples after 112 h of cultivation. 5 µl of supernatant applied. The size of carboxylesterase is about 57 kDa. 1 – PageRuler prestained protein ladder (relevant marker bands are indicated, kDa). 2 – Parent strain. 3 – evc#1. 4 – Mit1#18. 5 – Upc2#1. 6 – Upc2#5.

Glycerol-driven bioreactor cultivations did work well for two P_{GAP}-Parent-Upc2 and one –Mit1 transformants (P_{GAP}-Parent-Upc2#5, -Upc2#7; P_{GAP}-Parent-Mit1#18). In addition, results of the deep well plate screening could be confirmed. Unfortunately, the assumption that transformant P_{GAP}-Parent-Upc2#1 produced actually three-fold more enzyme compared to the parent as observed in batch number 5 was not confirmed in batch No. 8. The reason for the observed difference remained unclear, however might arise from different dissolved oxygen levels. It also remained unclear whether overexpression of Rpn4 had an effect or not, due to the surprisingly high parent strain values observed in batch No. 5. However, the performance of the empty vector transformant -evc#3 (batch No. 8) confirmed the parent level of 63.9 ± 29.4 U/L.

4.3 Additional, Upc2-related experiments

Because of the interesting SDS-PAGE pattern (a strong carboxylesterase band at about 57 kDa and a second band at about 130 kDa) and high enzyme activity compared to the parent strain of Upc2 overexpressing transformants, additional experiments were performed to gain more information about the function of this TF in *P. pastoris*.

4.3.1 Report of the mass spectrometry

Immunoblotting was performed with cultivation supernatants of the batches No. 1, No. 3, No. 5 and No. 6. Interestingly, the high molecular weight protein(s) could not be detected by Western blotting using a carboxylesterase specific antibody. For further analysis of the unknown protein (or proteins), the 130 kDa band was cut from a SDS gel and sent for MS to the Omics Center Graz (BioTechMed-Graz).

The highest number of peptide spectrum matches was found for a lectin-like protein with similarity to the protein Flo1 (Table 4.1). The protein Flo1 is associated with flocculation by binding to mannose chains of other cells [47]. Upc2 is a putative regulator of *ERG* (ergosterol biosynthesis) genes, but also might be involved in the induction of *DAN/TIR* genes that encode mannoprotein genes under anaerobic conditions [48]. These glycosylated mannoproteins are located at the outer cell wall and are responsible for cell wall permeability [46]. Based thereon, it seems possible that the induction of *DAN/TIR* genes influenced the cell wall mannoproteins. The detachment of Flo1 might lead to increased cell wall porosity combined with increased enzyme secretion.

Table 4.1 Results from mass spectrometry of the high molecular weight protein(s) (~130 kDa) found in all samples of Upc2 overexpressing strains. PSM – peptide spectrum matches (total number of identified peptide sequences). Coverage – amino acids in all found peptides divided by the total number of the amino acids of the protein. pI – calculated isoelectric point of the protein.

Accession	Description	% Coverage	PSM	MW [kDa]	pI	Gene ID
254567221	Lectin-like protein with similarity to Flo1p [Komagataella pastoris GS115]	25.26	288	51.2	4.51	PAS_chr1-4_0584
328352741	putative secreted protein [Komagataella pastoris CBS 7435]	15.59	28	71.6	4.34	PP7435_Chr3-0167
254569190	hypothetical protein [Komagataella pastoris GS115]	27.83	27	30.5	4.36	PAS_chr2-1_0887

4.3.2 Spotting tests

Genetically modified strains often show growth discrepancies when streaked out on agar containing different toxins. To evaluate a possible membrane-associated effect of Upc2 overexpression in *P. pastoris*, a spotting test with three toxic substances was performed with the strains P_{AOX1}-Parent-Upc2#5 and P_{GAP}-Parent-Upc2#7 which showed increased enzyme activity in bioreactor cultivation. The wild type strain (CBS 7435) was used as a control. Different dilutions of an overnight culture were plated on YPD agar (Table 4.2). Methanol was not added for induction of protein secretion in P_{AOX1}-Parent strains.

A control plate with YPD media was plated in addition and showed that all strains were growing equivalently. Overexpression of carboxylesterase and/or Upc2 seemingly did no effect growth under standard conditions.

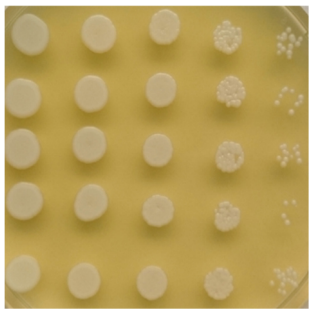
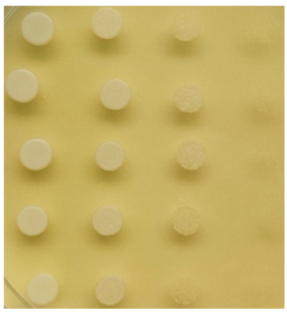
Calcofluor white is a fluorochromic dye which interacts with polysaccharides (e.g. chitin) and effects growth and causes incomplete separation of daughter and mother cell in yeast [49]. Both protein secretion and Upc2 overexpression resulted in a slightly reduced growth phenotype compared to the wild type. This effect was amplified upon combined protein secretion and Upc2 overexpression as illustrated by transformant

P_{GAP}-Parent-Upc2#7 which showed reduced growth in comparisons to the single overexpressing strains.

Sodium orthovanadate (NaOV) is a toxin that can be used to show defects in glycosylation which inhibits ATPases, i.e. loss of cell energy [50]. Growth of all strains was affected by addition of NaOV. Upon comparison, only transformant P_{GAP}-Parent-Upc2#7 (secreting carboxylesterase and overexpressing Upc2) showed slightly reduced growth.

Congo red generates complexes with parts of the cell wall network and causes a loss of cell wall rigidity. This dye affects growth and regeneration of protoplasts [51]. The effect of Congo red on the studied *P. pastoris* strains was enormous and only the undiluted spots grew under these conditions. Surprisingly, the two Upc2 overexpressing transformants seemed to be most affected (P_{AOX1}-Parent-Upc2#5 and P_{GAP}-Parent-Upc2#7) compared to the wild type and only enzyme secreting strains showed weaker growth indicated by a smaller spot size.

Table 4.2 Spotting test with different strains and dilutions using YPD media (containing glucose) with additional substances. The starting OD_{600} was 1. The incubation was for 48 h at 28 °C. CBS – *P. pastoris* control strain. YPD – yeast nitrogen base media. CFW – calcofluor white, 5 mg/L. NaOV – sodium orthovanadate, 1.5 mM. CR – Congo red, 4 mg/L. 1 – 10^0 dilution. 2 – 10^{-1} dilution. 3 – 10^{-2} dilution. 4 – 10^{-3} dilution. 5 – 10^{-4} dilution.

Strain	OD_{600} Dilutions														
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	1
CBS															
P_{AOX}-Parent															
P_{AOX}-Parent-Upc2#5															
P_{GAP}-Parent															
P_{GAP}-Parent-Upc2#7															
	YPD					CFW					NaOV				CR

The spotting test corresponded to a putative involvement of TFs in cell wall interactions, showing the most effect for NaOV and Congo red, both substances that influence cell wall interactions. Overexpression of this TF might decrease the resistance of the cells to these components because of a more porous cell wall compared to the parent strains. In contrast, sodium orthovanadate does not directly affect the cell wall of *P. pastoris*. So, almost no effect was observed.

4.3.3 FM4-64 staining

FM4-64 is a lipophilic dye that incorporates into cell membranes. During endocytosis, punctate endosomes get stained, migrate and fuse to vacuoles [52]. The fluorescence can be observed under the microscope and be determined in a given region. Again, the two P_{AOX1}-Parent-Upc2#5 and P_{GAP}-Parent-Upc2#7 transformants with increased enzyme activity in bioreactor cultivation were studied compared to their respective parent strains. As for the staining tests no methanol was added for carboxylesterase production of the strains P_{AOX1}-Parent and P_{AOX1}-Parent-Upc2#5. The uptake of FM4-64 into the cells at different incubation times is shown in Figure 4. During all experiments, fluorescence significantly increased with the duration of incubation (max. 180 min). At the beginning, small fluorescent dots were observed at the cell wall (15 min). These dots were later moving into the cytosol (30 min – 45 min). After 180 min, nearly all vacuoles were stained with the dye. For a better comparison between the strains, fluorescence per cell was quantified using ImageJ. An equal uptake of FM4-64 was observed after 15 min. After 30 min of incubation, the uptake of P_{AOX1}-Parent-Upc2#5 cells was about 10 % higher compared to the parent strain and 14 % higher after 45 min of incubation, indicating only a small effect upon single overexpression of Upc2. After 180 min cells were saturated showing all similar values.

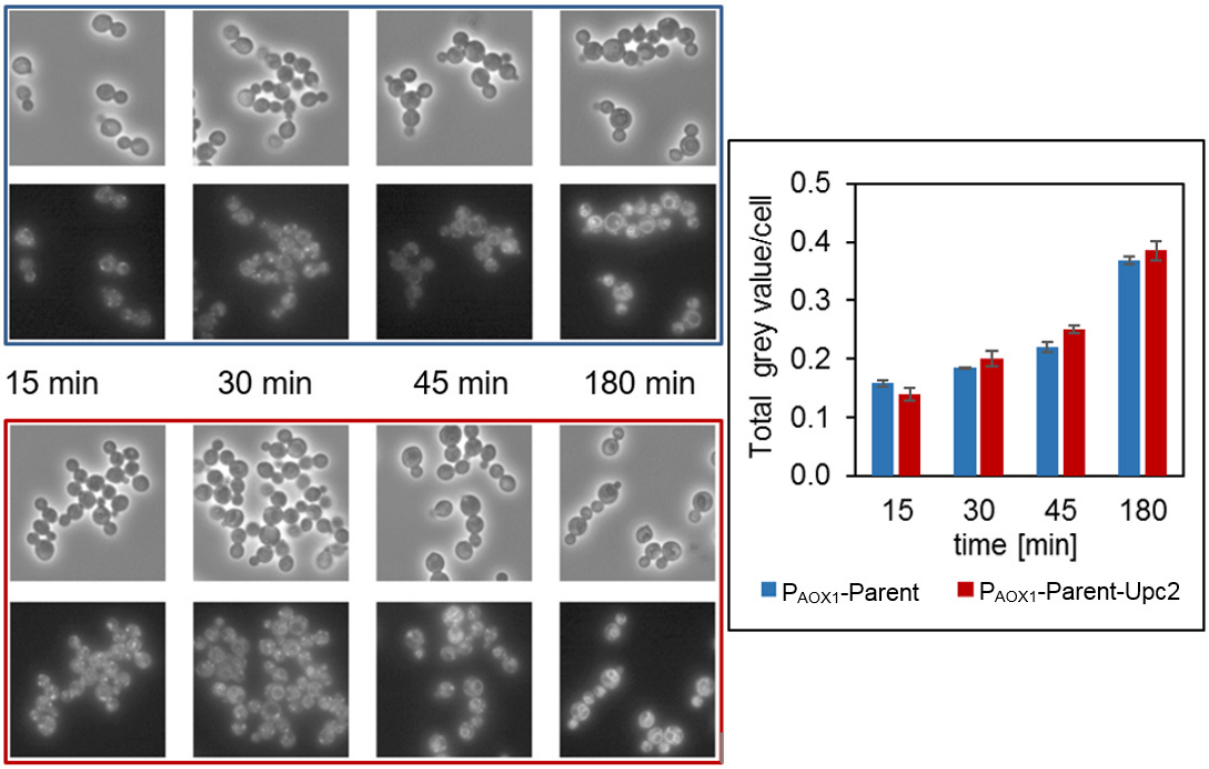


Figure 4.14 FM4-64 staining of *P. pastoris* P_{AOX1}-Parent and P_{AOX1}-Parent-Upc#5. 10 µL of 400 µM FM4-64 were provided to cells with an OD_{600} of approximately 0.4 for uptake. Left: The images show the staining development of the FM4-64 uptake by the cells over time for each used strain (blue – P_{AOX1}-Parent, red – P_{AOX1}-Parent-Upc2#5). Samples were taken after 15 min, 30 min, 45 min and 180 min and were examined under the microscope. Right: Development of the total grey value/cell over time. Two images with 30 cells have been used for calculation. The black lines illustrate the standard deviation.

Also, the strains P_{GAP}-Parent and P_{GAP}-Parent-Upc2#7 were studied, comparing enzyme secretion with enzyme secretion upon Upc2 overexpression. Figure 4. shows the uptake of FM4-64 into these strains. As before, fluorescent dots were observed at the cell wall which were later moving into the cytosol. After 180 min of incubation, nearly all vacuoles were stained with the dye. The quantification using ImageJ showed that already after 15 min the uptake of FM4-64 was 10 % increased in strain P_{GAP}-Parent –Upc2#7. This was further increased to nearly 46 % compared to the parent strain after 30 min of incubation and still 39% increase was observed after 45 min. After 180 min, the cells of both strains were saturated. Here, it was clearly demonstrated that enzyme secretion together with Upc2 overexpression significantly increased the uptake of FM4-64 compared to parent under these conditions. Again, indicated changes were connected to cell wall composition or endocytic effects.

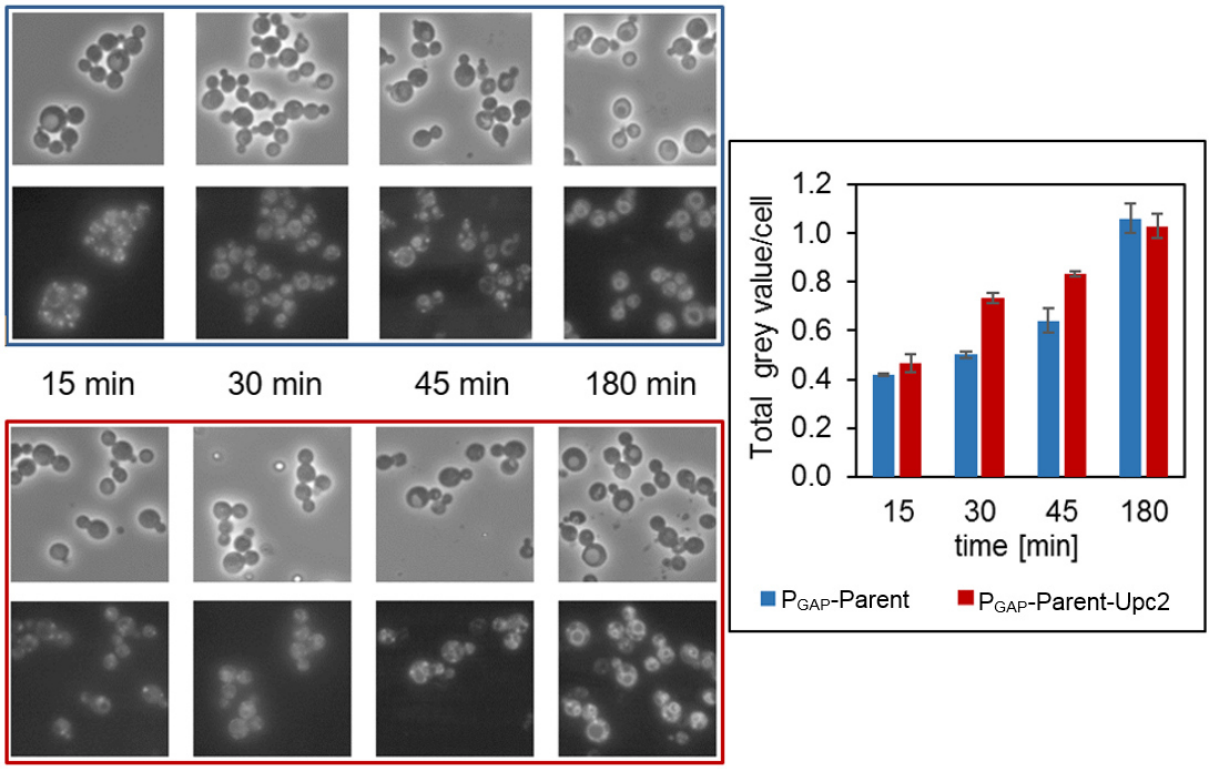


Figure 4.15 FM4-64 staining of *P. pastoris* P_{GAP}-Parent and P_{GAP}-Parent-Upc2#7. 10 µL of 400 µM FM4-64 were provided to cells with an OD_{600} of approximately 0.4 for uptake. Left: The pictures show the staining development of the FM4-64 uptake by the cells over time for each used strain (blue – P_{GAP}-Parent, red – P_{GAP}-Parent-Upc2#7). Samples were taken after 15 min, 30 min, 45 min and 180 min and examined under the microscope. Right: Development of the total grey value/cell over time. Two pictures with 30 cells were used for calculation. The black lines illustrate the standard deviation.

5 Conclusion and Outlook

Overexpression of Upc2 resulted in five markedly improved transformants showing between 2 and 8-fold improved enzyme activity (P_{AOX1}-Parent-Upc2#1, P_{AOX1}-Parent-Upc2#5 and P_{AOX1}-Parent-Upc2#10, P_{GAP}-Parent-Upc2#5 and P_{GAP}-Parent-Upc2#7). The good performance of these transformants was confirmed by SDS-PAGE. All culture supernatants of enzyme secreting and Upc2 overexpressing transformants studied in bioreactor cultivation showed a strong protein band of about 130 kDa on SDS gels. MS analysis discovered a lectin-like protein similar to Flo1, which might be detached from the cell wall upon Upc2 overexpression. Because of the membrane-related function of this protein combined with increased enzyme secretion, Upc2 might be involved in regulation of cell wall porosity/integrity. Additional experiments with membrane/cell-wall targeted toxins and FM4-64 dye supported this assumption. This is resulting in reduced growth on calcofluor white and Congo red containing media and increased uptake of lipophilic dye compared to the parent strains.

Overexpression of the transcription factor Mit1 resulted in fore improved transformants showing between 2- and 3-fold increased enzyme activity (P_{AOX1}-Parent-Mit1#20, P_{AOX1}-Parent-Mit1#16 and P_{AOX1}-Parent-Mit1#9, P_{GAP}-Parent-Mit1#18). However, the performance of some of these strains, such as P_{AOX1}-Parent-Mit1#20, could not be confirmed in a repetition experiment. Further, PCR amplification of the expression cassette with cassette-specific primers from gDNA of these transformants was unsuccessful. The reason for this observation remained unclear. Mit1 overexpression under P_{GAP} might have resulted in some genetic re-arrangement and loss of the Mit1 cassette. A control experiment with Upc2 and Rpn4 was successfully carried out.

Overexpression of Rpn4 resulted in two improved transformants with 3- and 4-fold increased enzyme activity (P_{AOX1}-Parent-Rpn4#1 and P_{AOX1}-Parent-Rpn4#22). Initial problems, i.e. loss of activity upon long cultivation times, was hardly observed during later cultivation experiments. This was most probably due to better and slightly adapted bioprocesses. Bioreactor cultivations with fluctuations in dissolved oxygen should be repeated in an environment allowing strict oxygen control.

Methanol-induced *P. pastoris* cultivations generally yielded higher enzyme titers (~ 60%) and improved transformants upon TF overexpression compared to the respective P_{GAP}-carboxylesterase experiments. Based on the problems with Mit1, it

might also be rational to test different other promoters for TF overexpression, such as weaker or inducible ones to prevent possible recombination effects.

Using M2 media in screening, 7 of 11 well-secreting transformants were confirmed in bioreactor cultivations. In comparison, a later performed screening on BM media showed that 80 % of the transformants had been chosen correctly under these conditions. Notably, M2 media showed up to 60 % lower cell densities compared to the BM media. In addition, the variability between yield and titer was significantly reduced on BM. Therefore, BM be preferred for enzyme screenings to gain more reliable results for scale-up, even if the composition of the M2 media is more similar to the defined bioreactor cultivation media.

6 Sources

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7 Appendix

Table 7.1 Sequences of transcription factors.

Upc2 (1359 bp) 5' → 3'

1 ATGGCTAACC TAAAGATCCC TGGATCAGAG CAGTTAACAA CAAATAAGGT GCTTAAGTCT
61 TCCAAGGGCA AGCGGAAATA CCATAACAAG AGCAGGAAAG GATGTACGAC TTGCAAAAAA
121 AGGCGAGTCA AGTGTGATGA AAATAAGCCC ATATGCAACA AATGTGAGCA CTTGGGCTTG
181 GACTGTATCT ATATGAGTCC ACCACCTAAG AGGCTCAGCT CCAGTACGAG TGATGGCAGT
241 CCAGCTATAA CAGTACCATT GTCGTCTGGG CCTCCTCTTG GGGCCACTTC TAACTCACCC
301 TCCAACCAGT ACACAGGAAA TCTGAACATG CTAGATCTAA GGCTAATGTA TCACTACATA
361 ACTAAAGTTT GGCATACGAT AACAGCAGCT GGTATATCTG ATGCCAAGAT CTGGTGTGAA
421 GACATTCCTA TGCTGGCTTT CAATTATCCG TTTTTGATGC ATTCAATTTT AGCCTTCAGT
481 GCTACACATC TTTCACGGAC TGAAAAGGGG TTGGACCAGT GTGTAACTTG CCATAGAGGC
541 GATGCTCTAC GTCTCCTACG TGAGGCCGTA CTTGAGATTT CACCAGCAAA TACTGATGCC
601 CTGGTGGCTT CAGCTTTAAT ACTAATAATG GACTCTCTGG CCAATGCATC GTTACCTACT
661 TCAACGTCTC CTAAATCACT TCCAGCTTCG GCCTGGATTT TCCACGTTAA GGGAGCTGCC
721 ACTATATTGA CAGCTGTCTG GCCATTGAAC GAAAGTTCCC GATTTCACAA ATTTATATCC
781 GTTGATTTAA GTGATCTGGG AGACGTTGAC CTGATGGGCG ATAATGCCAA TGCTAACCAC
841 CCACATGATG TGAAATATTC CCAGTTGTTA TGCTTTGATG AAGAGCTAAC CGACCTTTTC
901 CCCGTTACTT ATACGTCACC TTACCTTATT ACTCTGGCTT ACTTGAACAA ATTGCACAAT
961 GAGCGATACA AATCAGATTT TATCCTGAGA GTTTTTGCCT TTCCAGCTTT GTTGGATAAA
1021 ACGTTCCTGA CTTTGTTAAT CAATGGAGAC ATTTCTGCCA TGAGAATTAT GAGATCGTAC
1081 TACACGCTTC TCAGAAACTT CACTGACGAG ATGAAAGATA AGGTTTGGTT TTTGGAAGGC
1141 GTGTCAAAGG TTCTTCCAGT AGATGTTGAC GAGTATTCGG GAGGAGGAGG AATGCATATG
1201 ATGATGGACT TTTTAGGAGG TCCTTCTACT TTGAACGATA ATGACACAAA TGACATTGCT
1261 GACAATATAG CCCAGTCTGG CCTTTTGGAT ACCGACAATT TGCCAAGTTC CATAACAGAC
1321 AACTTAGATA TCATGCACAG TAGTATATAT GATGAGTAA

Mit1 (1158 bp) 5' → 3'

1 ATGCAGTCTT ATCACGGAGT TATAGTTACT CCAAAAGACG CCATTATACT GGTTGACGCC
61 GCCCTCAAGA AAATGATCCC CCAGGTTACA CGTAGGCTAA CTGAGTTTGA GAGGCAAACT
121 CAAATCGGCC ATGGGTCTGT GTTTGTGTGG GATGAGAAAG AAAGTGGTAT GAAGCGATGG
181 ACTGACGGAA GATCCTGGTC TGCTTCTAGA GTAAGCGGTT CGTTCTTGAC ATACAAAGAG
241 ATGGAAAATG CAAAGAGTGG CAATTCAAAC TCTTACATTT ACGGCAAACA GTCAGAGAGT
301 TACAGATACA AGGACAACGG CCTATTGAAG CAAAGTTTCT CAGTTACGCT GAAAAACGGA
361 AAGAAACTGC ACCTGATAAG CTATGTTTAT GCGACTTACC TCAAGACATT GTCAGGTCCC
421 AATTCGTCAA ACAAGTCAGT TTCTTCGAAC CCCATGTCCA ATTCCGTGGG AACACTGTCG
481 GGCTCTAGCT CTGATATGAC ACAGGACGGG CTGCTGAAAC GACCATCTGA AGATGACAGA
541 TTTCAATCTT TGGACCTTAA CTCCGACTTA TATCCGGAGA CTGTTTTGAA CGAAACATAC
601 ACATTACAAT ATCCTCCATC TTCTCCAATT TCAAGCACAA CATCCGCTAA CAGCGGTGGA
661 GTTAGCAAGC CAAAGAAAAG CAAACGTGTT GCAAGTAGGA TCACTGATGA ACGAGTGATT

721 AGTGTGCCAA AGTCGCCTTC GCCAGTGTCT TCCTCTACTA CACCCTCCAC TACTGCATTT
781 TCAACTATGG GAGGTGGAGC GTCCTCTGTT GGTTCACACC TGAGTACTGG TACCGCTGGT
841 GTCTTGCGCT TTCCTCCACA GAATCAGATG ATGATGAATC ACCAAGTGAT CACTCTACCT
901 GCATTGCACA CTCCGGCTTC GACGCTTCCT GCCTACAAGG TCCCTAGATC GTATCTCGAC
961 GATCCAACCC CAAAGAATAC CTTCCTGCTC CCCAGATCAC ACTCTATCAC TCCTATCCCA
1021 TACGTGCAGC AGCAACAGCA ACAATATTTC CCACCACAAC AACCAATGCA GCATTTAGCT
1081 CCACCAGTGA GAGCACCGGG TTACGTTTCG CATGAGGATG GACGAGCTCT AAGTGCTTTA
1141 GACAGAGCTT TTTGTTAA

Rpn4 (1386 bp) 5' → 3'

1 ATGACATTTC AAGTAGCCTT ACCGAAACTT AGACGAACTC TCACAGACAT AATGGAGGAT
61 GAGCTGTATC ATGTACCGGA TCTTCCAGCG ACCGCTGATG CTGATATGAC CGAACCATCT
121 ATGTACGCAA GCAACACGAA TATTGATACA GAAATGAACC TGTACCAGGA TTATTCCCGA
181 GCCCTCAACA GAATAAGCAG CGGATCAAAC AGCCCTGTCG TTTCCCATTC TGGCGCATAC
241 TGGTCAAATA CTAACTCCAT AAATGGGAGC CAGGAGTTCC TAACGATACC GGATACCATG
301 GATGAAGATA AAGATCAAGA TATGACAGGC ATGATGGGTT TGCAACAGAA TGATCCGGCT
361 CAACCCCCTT TGGAAGAAGA CAATGGTGAG GATGAAGACG ACGAATTTGA TGATGACGAG
421 GTAATTTATG ATTATGACTA CGAGGTCAAA CCATTCACCG CATATGCCGA TCGTAATGCT
481 CTTAGTTATG GATATGAACC ATTTCAAAAA GTTGAGGATT ATAACAAGAA TTACATGCTC
541 AGTTCTTTTA AGGGATTCCC TGAGAAAGCT GATCCACAAC TTAGCTTTCC CGATGACGAA
601 ATTTTAGGGC GTAATCCGTT TGATTTTGAA ATGAATGACA CATCCTCCAA ACTCTACATT
661 TATCCCGAGG AAGAAGACAA GGCCGAGATC AATCATTTCG ACCTCAAGAA AGAGTTCCTT
721 GAGGAAGATA TTTCTGAGGA TGAGAACGAT GATACCAACA GAACTAACAA CATTGAAAAC
781 AATACAACAA GCATGATTAA CATCAACCCT GGGTTGGCAG AAGCTGGCAA TTCTGTAATT
841 CCCCCACAAT TACTCCGAAG TTCCCCAGTC ATTTCCCCCA TCTCCAACCA AGCCCAGGAG
901 GCTACCGGTA CAAACTCCGT AAGATCCAAT GTTTCTGAGA ACAACACCCC CAAGCAGCAA
961 GAAATCAAGT TCAAGATTCC ATCGTCTCCT TCCGGGGAAG AAGACGTCCA CCAGTGTCAA
1021 CTTGTAAATC CTACCACCGG TCAGAAATGT TTCAAGCAGT TTTCTAGACC CTATGATTTA
1081 ATCAGACATC AAGAAACCAT CCATGCGGAA CGCAAGAAGA TTTTTCGATG TATTCTGTGT
1141 GAAACGGATG CTTTAAGACA TGAGAATAGA GTTCCCGCCT ACTACGATGG GTGCAAGTTC
1201 GTTAGTGTTC CTACGGAATC AGGGGAATTG GCCCGGGTCG TTCTTCCAGA TCAACCCCCT
1261 CGTATCTCCA AGAAGACTTT TAGCCGTGGA GATGCTCTTT CTCGACATGT TCGTGTAAAG
1321 CATGGACTTA CCGGTACATC TGCAACTGAT GCTATAAGAT ATGCAAAAGA TCATGTAGAA
1381 TACCTT

Table 7.2 Sequences of primers. The sequencing was done at Microsynth AG and the analyses was done with the CLC workbench.

Primer	Used for	Sequence (5' → 3')
Mit1_seq_fw_1	Sequencing	GATCCTGGTCTGCTTCTAGAGTAAGC
Mit1_seq_fw_2	Sequencing	GCACACTCCGGCTTCGACGCTTCC

Primer	Used for	Sequence (5' → 3')
Mit1_seq_rv_3	Sequencing	CACCTCCCATAGTTGAAAATGCAG
Mit1_seq_rv_4	Sequencing	CATTTTCTTGAGGGCGGCGTCAACC
Rpn4_seq_fw_1	Sequencing	GTCGTTTCCCATTCTGGCGCATAC
Rpn4_seq_fw_2	Sequencing	CTGAGAACAACACCCCCAAGCAGC
Rpn4_seq_rv_3	Sequencing, PCR	GACACTGGTGGACGTCTTCTTCC
Rpn4_seq_rv_4	Sequencing	CCATGGTATCCGGTATCGTTAGG
cyc_TT_seq_rv	Sequencing, PCR	CTGTCAAGGAGGGTATTCTGGG
pGAP_fw	Sequencing, PCR	AAAGGCGAACACCTTTCC
Upc2_seq5_rv	Sequencing, PCR	CGTAGGAGACGTAGAGCATCG
ARG4_RT_fw	RT-PCR	TCCTCCGGTGGCAGTTCTT
ARG4_RT_rv	RT-PCR	TCCATTGACTCCCGTTTTGAG
Kan_RT_fw	RT-PCR	GATGTTGGACGAGTCGGAATC
Kan_RT_rv	RT-PCR	CACCGAGGCAGTTCCATAGG

Table 7.3 Screening results for P_{AOX1}-Parent-Upc2. Triplicate measurements. Calculated concentrations according to enzyme standard curve with 4PL regression. FC related to P_{AOX1}-Parent with 712 ± 110 ng/mL and 12.9 ± 3.69 ng/mL/OD_{600}. Highlighted transformants chosen for bioreactor cultivation. FC- Fold-change.

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
Upc2#1	275	30.8	0.39	10.9	1.22	0.84
Upc2#2	496	81.8	0.70	27.1	4.47	2.09
Upc2#3	178	28.0	0.25	7.00	1.10	0.54
Upc2#4	356	104	0.50	14.7	4.27	1.13
Upc2#5	505	63.2	0.71	23.5	2.95	1.82
Upc2#6	333	91.0	0.47	14.5	3.96	1.12
Upc2#7	255	7.00	0.36	15.9	0.44	1.23
Upc2#8	179	51.6	0.25	8.77	2.52	0.68
Upc2#9	237	78.1	0.33	3.16	1.04	0.24
Upc2#10	509	14.1	0.71	8.21	0.04	0.63
Upc2#11	90	2.40	0.13	4.29	0.75	0.37
Upc2#12	287	9.30	0.40	7.88	0.25	0.61

Table 7.4 Screening results for P_{AOX1}-Parent-Mit1. Triplicate measurements. Calculated concentrations according to enzyme standard curve with 4PL regression. FC related to P_{AOX1}-Parent with 123 ± 22.5 ng/mL and 4.10 ± 0.51 ng/mL/OD_{600}. Highlighted transformants chosen for re-screening. FC- Fold-change.

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
Mit1#1	16.8	0.00	0.14	3.17	0.00	0.77
Mit1#2	81.7	29.3	0.66	5.46	1.60	1.33
Mit1#3	26.8	0.00	0.22	5.92	0.00	1.44
Mit1#4	57.7	30.8	0.47	6.84	2.98	1.67
Mit1#5	118	62.4	0.96	7.88	3.40	1.92
Mit1#6	32.5	28.0	0.26	7.42	4.52	1.81
Mit1#7	49.1	32.9	0.40	7.98	4.36	1.95
Mit1#8	16.4	0.00	0.13	4.51	0.00	1.1
Mit1#9	196	2.42	1.59	7.05	0.07	1.72
Mit1#10	44.0	15.8	0.36	3.81	1.12	0.93
Mit1#11	81.9	19.0	0.66	5.93	1.13	1.45
Mit1#12	50.3	23.1	0.41	4.36	1.64	1.06
Mit1#13	51.9	17.5	0.42	8.44	2.32	2.06
Mit1#14	100	10.2	0.81	7.21	0.60	1.76
Mit1#15	178	13.3	1.44	9.29	0.57	2.27
Mit1#16	211	65.4	1.71	11.2	2.45	2.73
Mit1#17	76.8	15.3	0.62	5.57	0.91	1.36
Mit1#18	128	77.3	1.03	10.9	4.67	1.77
Mit1#19	101	21.7	0.81	8.78	1.34	2.14
Mit1#20	103	30.8	0.84	9.07	1.91	2.21
Mit1#21	177	2.61	1.44	9.05	0.11	2.21
Mit1#22	144	8.86	1.16	8.51	0.43	2.07
Mit1#23	57.9	10.9	0.47	6.86	1.05	1.67
Mit1#24	93.5	12.9	0.76	1.58	0.18	0.38

Table 7.5 Screening results for P_{AOX1}-Parent-Rpn4. Triplicate measurements. Calculated concentrations according to enzyme standard curve with 4PL regression. FC related to P_{AOX1}-Parent with 712 ± 132 ng/mL and 12.9 ± 1.65 ng/mL/OD_{600}. Highlighted transformants chosen for re-screening. FC- Fold-change.

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
Rpn4#1	341	11.6	0.48	10.1	0.24	0.78
Rpn4#2	563	71.7	0.79	18.2	1.89	1.41
Rpn4#3	648	49.4	0.91	17.3	1.08	1.33

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
Rpn4#4	597	60.0	0.84	15.0	1.23	1.16
Rpn4#5	1185	140	1.66	30.4	2.93	2.35
Rpn4#6	853	8.13	1.20	20.3	0.14	1.57
Rpn4#7	953	39.8	1.34	24.2	0.72	1.87
Rpn4#8	250	102	0.35	3.38	0.98	0.26
Rpn4#9	654	28.5	0.92	19.1	0.59	1.48
Rpn4#10	464	65.9	0.65	11.8	1.37	0.91
Rpn4#11	916	92.1	1.29	21.5	1.53	1.66
Rpn4#12	323	108	0.45	12.3	2.89	0.95
Rpn4#13	1128	170	1.58	26.9	2.87	2.08
Rpn4#14	321	111	0.45	11.9	2.90	0.92
Rpn4#15	263	114	0.37	3.76	1.15	0.29
Rpn4#16	344	111	0.48	12.6	2.86	0.97
Rpn4#17	1138	17.7	1.60	28.4	0.31	2.19
Rpn4#18	278	100	0.39	10.8	2.75	0.84
Rpn4#19	304	94.7	0.43	12.4	2.74	0.96
Rpn4#20	248	111	0.35	3.48	1.10	0.27
Rpn4#21	488	182	0.69	8.12	2.14	0.63
Rpn4#22	994	18.2	1.40	26.6	0.35	2.06
Rpn4#23	246	113	0.34	3.82	1.24	0.30
Rpn4#24	307	104	0.43	11.0	2.64	0.85
Rpn4#25	34	105	0.49	15.2	3.24	1.18
Rpn4#26	315	104	0.44	13.2	3.09	1.02
Rpn4#27	317	99.6	0.45	13.2	2.93	1.02

Table 7.6 Screening results for P_{AOX1}-Parent-evc. Triplicate measurements. Calculated concentrations according to enzyme standard curve with 4PL regression. FC related to P_{AOX1}-Parent with 239 ± 19.8 ng/mL and 13.6 ± 1.12 ng/mL/OD_{600}. Highlighted transformants chosen for bioreactor cultivation**.** FC- Fold-change.

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
evc#1	163	3.15	0.68	9.34	0.18	0.68
evc#2	104	7.53	0.44	8.38	0.60	0.61
evc#3	112	0.50	0.47	7.97	0.04	0.58
evc#4	104	0.30	0.44	5.81	0.02	0.43
evc#5	101	1.48	0.42	9.19	0.13	0.67
evc#6	123	12.2	0.51	7.34	0.73	0.54
evc#7	157	13.2	0.66	9.72	0.81	0.71

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
evc#8	95.2	0.27	0.40	5.97	0.02	0.44
evc#9	187	11.7	0.78	10.5	0.65	0.77
evc#10	47.6	0.94	0.20	3.84	0.08	0.28
evc#11	192	14.8	0.80	9.62	0.74	0.71
evc#12	135	8.05	0.56	8.70	0.52	0.64

Table 7.7 Screening results for P_{GAP}-Parent-Upc2. Triplicate measurements. Calculated concentrations according to enzyme standard curve with 4PL regression. FC related to P_{GAP}-Parent with 545 ± 309 ng/mL and 8.16 ± 1.20 ng/mL/OD_{600}. Highlighted transformants chosen for bioreactor cultivation**.** FC- Fold-change.

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
Upc2#1	274	36.9	0.50	9.99	1.55	1.22
Upc2#2	125	42.8	0.23	5.73	1.97	0.70
Upc2#3	141	39.9	0.26	6.17	1.74	0.76
Upc2#4	139	43.4	0.26	6.13	1.91	0.75
Upc2#5	227	69.5	0.42	11.1	2.01	1.36
Upc2#6	148	15.5	0.27	5.66	1.55	0.69
Upc2#7	205	3.50	0.38	9.35	0.10	1.15
Upc2#8	159	19.7	0.29	5.80	0.59	0.71
Upc2#9	178	42.7	0.33	2.32	0.41	0.28
Upc2#10	136	39.0	0.25	6.95	1.32	0.85
Upc2#11	168	12.7	0.31	2.63	0.45	0.32

Table 7.8 Screening results for P_{GAP}-Parent-Mit1. Triplicate measurements. Calculated concentrations according to enzyme standard curve with 4PL regression. FC related to P_{GAP}-Parent with 25.6 ± 2.67 ng/mL and 0.83 ± 0.11 ng/mL/OD_{600}. Highlited transformants chosen for re-screening**.** FC-Fold-change.

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
Mit1#1	16.0	0.99	0.63	0.64	0.03	0.78
Mit1#2	12.0	1.48	0.47	0.59	0.06	0.71
Mit1#3	15.4	1.38	0.60	0.59	0.04	0.71
Mit1#4	16.7	1.47	0.65	0.58	0.04	0.70
Mit1#5	16.4	1.38	0.64	0.52	0.04	0.63
Mit1#6	16.6	2.02	0.65	0.50	0.05	0.61
Mit1#7	19.3	1.16	0.75	0.54	0.03	0.65
Mit1#8	18.4	2.04	0.72	0.45	0.04	0.54

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
Mit1#9	37.7	3.35	1.47	1.15	0.08	1.38
Mit1#10	16.7	1.58	0.65	0.51	0.04	0.62
Mit1#11	35.9	3.97	1.40	1.06	0.10	1.29
Mit1#12	15.2	1.10	0.60	0.52	0.03	0.62
Mit1#13	24.2	2.65	0.95	0.89	0.08	1.08
Mit1#14	31.7	5.30	1.24	0.97	0.13	1.18
Mit1#15	30.9	2.66	1.21	1.25	0.09	1.51
Mit1#16	19.3	1.86	0.76	0.57	0.04	0.69
Mit1#17	16.7	1.10	0.65	0.58	0.03	0.70
Mit1#18	41.6	4.24	1.63	1.41	0.12	1.70
Mit1#19	20.4	1.63	0.80	0.85	0.06	1.03
Mit1#20	19.4	1.91	0.76	0.70	0.06	0.85
Mit1#21	32.3	3.11	1.26	1.03	0.08	1.25
Mit1#22	17.8	1.08	0.69	0.73	0.04	0.88
Mit1#23	38.4	3.51	1.50	1.27	0.09	1.54
Mit1#24	18.0	1.83	0.70	0.73	0.06	0.88

Table 7.9 Screening results for P_{GAP}-Parent-Rpn4. Triplicate measurements. Calculated concentrations according to enzyme standard curve with 4PL regression. FC related to P_{GAP}-Parent with 544 ± 309 ng/mL and 8.16 ± 1.20 ng/mL/OD_{600}. Highlighted transformants chosen for re-screening. FC-Fold-change.

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
Rpn4#1	116	22.1	0.21	11.3	2.16	1.38
Rpn4#2	394	110	0.72	16.2	4.51	1.99
Rpn4#3	258	26.7	0.47	7.99	2.58	0.98
Rpn4#4	114	27.3	0.21	3.19	0.77	0.39
Rpn4#5	209	64.0	0.38	5.57	1.71	0.68
Rpn4#6	212	71.2	0.39	6.07	2.04	0.74
Rpn4#7	431	66.0	0.79	16.1	2.58	2.07
Rpn4#8	178	57.9	0.33	5.32	1.74	0.65
Rpn4#9	233	56.7	0.43	7.61	1.85	0.93
Rpn4#10	123	48.2	0.23	3.52	1.96	0.43
Rpn4#11	271	12.8	0.5	12.3	1.58	1.51
Rpn4#12	567	33.4	1.04	21.1	1.25	2.59
Rpn4#13	438	135	0.80	11.7	1.99	1.44
Rpn4#14	157	23.6	0.29	4.47	1.78	0.55

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
Rpn4#15	328	93.4	0.59	8.53	2.46	1.05
Rpn4#16	236	55.6	0.43	8.25	1.94	1.01
Rpn4#17	579	115.6	1.06	19.4	1.54	2.38
Rpn4#18	382	86.6	0.7	10.7	2.65	1.31
Rpn4#19	435	27.4	0.80	6.06	0.38	0.74
Rpn4#20	1141	268	2.10	15.6	2.65	1.91
Rpn4#21	536	144	0.98	8.67	2.11	1.06
Rpn4#22	426	56.4	0.78	5.62	0.74	0.69
Rpn4#23	261	59.9	0.48	3.94	0.85	0.48
Rpn4#24	299	67.9	0.55	10.3	1.09	1.26

Table 7.10 Screening results for P_{GAP}-Parent-evc. Triplicate measurement. Calculated concentrations according to enzyme standard curve with 4PL regression. FC related to P_{GAP}-Parent with 3.97 ± 0.70 ng/mL and 0.10 ± 0.01 ng/mL/OD_{600}. Highlighted transformants chosen for bioreactor cultivation**.** FC- Fold-change.

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
evc#1-1	4.50	0.11	1.14	0.12	0.00	1.18
evc#1-2	3.83	0.00	0.97	0.11	0.00	1.10
evc#2-1	5.07	1.18	1.28	0.13	0.03	1.30
evc#2-2	6.50	1.48	1.64	0.16	0.04	1.54
evc#3-1	3.43	0.06	0.87	0.07	0.00	0.68
evc#3-2	4.33	0.46	1.09	0.10	0.01	1.03
evc#4-1	4.77	0.16	1.20	0.07	0.00	0.73
evc#4-2	2.97	1.29	0.75	0.05	0.02	0.47
evc#5-1	3.47	2.84	0.87	0.08	0.07	0.80
evc#5-2	5.91	0.44	1.49	0.17	0.01	1.67
evc#6-1	4.17	0.35	1.05	0.12	0.00	1.16
evc#6-2	2.81	0.21	0.71	0.07	0.01	0.71

Table 7.11 Re-screening results for P_{AOX1}-Parent-Mit1. Triplicate measuremenst. Calculated concentrations according to enzyme standard curve with 4PL regression. FC related to P_{AOX1}-Parent with 30.41 ± 6.28 ng/mL and 1.00 ± 0.33 ng/mL/OD_{600}. FC- Fold-change.

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
Mit1#21	42.9	17.5	1.41	1.15	0.17	1.15
Mit1#9	38.8	14.1	1.28	1.59	0.37	1.58
Mit1#20	34.4	9.47	1.13	1.67	0.36	1.66
Mit1#16	28.7	11.7	0.94	1.33	0.05	1.32

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
Mit1#23	25.0	0.83	0.82	1.24	0.03	1.23
Mit1#3	10.5	0.43	0.35	0.76	0.03	0.76

Table 7.12 Re-screening results for P_{AOX1}-Parent-Rpn4. Triplicate measurements. Calculated concentrations according to enzyme standard curve with 4PL regression. FC related to P_{AOX1}-Parent with 605 ± 95.7 ng/mL and 21.80 ± 1.81 ng/mL/OD_{600}. FC- Fold-change.

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
Rpn4#5	835	84.2	1.38	33.9	3.62	1.56
Rpn4#17	38.0	36.4	0.63	24.7	2.38	1.14
Rpn4#22	1051	59.3	1.74	32.2	1.72	1.48
Rpn4#6	664	91.7	1.10	18.4	2.57	0.85
Rpn4#3	738	191	1.22	19.6	5.04	0.90
Rpn4#1	468	78.1	0.77	13.1	2.20	0.60

Table 7.13 Re-screening results for P_{GAP}-Parent-Mit1. Triplicate measurements. Calculated concentrations according to enzyme standard curve with 4PL regression. FC related to P_{GAP}-Parent with 12.0 ± 0.38 ng/mL and 0.76 ± 0.13 ng/mL/OD_{600}. FC- Fold-change.

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
Mit1#18	14.7	0.58	1.22	0.90	0.10	1.18
Mit1#23	17.3	1.26	1.44	1.09	0.12	1.42
Mit1#9	17.4	2.38	1.45	1.02	0.12	1.32
Mit1#13	10.3	0.23	0.85	0.70	0.06	0.91
Mit1#19	9.87	0.87	0.82	0.62	0.17	0.80
Mit1#3	8.71	1.49	0.72	0.71	0.08	0.93

Table 7.14 Re-screening results for P_{GAP}-Parent-Rpn4. Triplicate measurements. Calculated concentrations according to enzyme standard curve with 4PL regression. FC related to P_{GAP}-Parent with 147 ± 3.26 ng/mL and 7.27 ± 0.66 ng/mL/OD_{600}. FC- Fold-change.

No.	Concentration [ng/mL]	Deviation	FC titer	Concentration [ng/mL/OD600]	Deviation	FC yield
Rpn4#12	282	47.1	1.92	15.6	1.74	2.14
Rpn4#14	260	25.9	1.77	12.6	0.88	1.73
Rpn4#2	242	20.5	1.65	11.7	0.61	1.61
Rpn4#11	156	6.84	1.01	7.05	2.74	0.97
Rpn4#15	156	29.8	1.00	7.28	0.97	1.01
Rpn4#19	25.9	0.66	0.18	1.32	0.11	0.18

Table 7.15 BMM-Screening results for P_{AOX1} strains. Calculated concentrations according to enzyme standard curve with 4PL regression. FC related to P_{AOX1}-Parent with 182 µg/mL and 3.42 µg/mL/OD_{600}. FC- Fold-change.

No.	Concentration [µg/mL]	FC titer	Concentration [µg/mL/OD600]	FC yield
Rpn4#1	203	1.12	3.80	1.11
Rpn4#3	195	1.07	3.61	1.06
Rpn4#5	163	0.90	3.02	0.88
Rpn4#6	181	1.00	3.43	1.00
Rpn4#21	185	1.02	3.51	1.02
Rpn4#17	172	0.95	3.20	0.93
Mit1#3	105	0.58	1.90	0.55
Mit1#5	282	1.55	5.10	1.49
Mit1#9	391	2.15	7.27	2.12
Mit1#16	184	1.01	3.41	1.00
Mit1#17	176	0.97	3.08	0.90
Mit1#20	178	0.98	3.24	0.95
Mit1#21	166	0.91	3.05	0.89
Upc2#1	190	1.05	3.68	1.08
Upc2#3	163	0.89	3.01	0.88
Upc2#5	222	1.26	4.14	1.21
Upc2#7	305	1.68	5.90	1.72
Upc2#10	422	2.32	7.92	2.31
evc#1	191	1.05	3.74	1.09
evc#3	186	1.02	3.43	1.00
evc#7	174	0.96	3.35	0.98
evc#9	174	0.96	3.19	0.93

Table 7.16 BMM-Screening results for P_{GAP} strains. Calculated concentrations according to enzyme standard curve with 4PL regression. FC related to P_{GAP}-Parent with 126 µg/mL and 2.58 µg/mL/OD_{600}. FC- Fold-change.

No.	Concentration [µg/mL]	FC titer	Concentration [µg/mL/OD600]	FC yield
Rpn4#2	345	2.73	7.47	2.90
Rpn4#11	335	2.66	6.14	2.39
Rpn4#12	394	3.12	7.48	2.90
Rpn4#14	387	3.06	8.10	3.15
Rpn4#13	5.67	0.04	0.10	0.04
Rpn4#7	395	3.13	7.16	2.78
Mit1#3	182	1.44	3.20	1.24
Mit1#7	237	1.88	4.20	1.63

No.	Concentration [µg/mL]	FC titer	Concentration [µg/mL/OD600]	FC yield
Mit1#9	471	3.73	9.60	3.73
Mit1#13	144	1.14	2.64	1.02
Mit1#18	208	1.65	3.63	1.41
Mit1#19	122	0.97	2.42	0.94
Mit1#23	192	1.52	4.21	1.63
Upc2#1	122	0.97	2.18	0.85
Upc2#2	179	1.42	3.08	1.20
Upc2#4	232	1.84	4.55	1.77
Upc2#5	2.13	0.02	0.04	0.01
Upc2#7	454	3.60	9.05	3.51
Upc2#9	1.56	0.01	0.03	0.01
evc#3	118	0.93	1.99	0.77
evc#5	116	0.92	2.25	0.87
evc#6	107	0.85	1.97	0.76

Table 7.17 Bioreactor cultivation results for P_{AOX1} strains (No.1). The enzyme activity was calculated with Formula 3.2 using the pNSi-assay. The values are for the first time point at inoculation and the last one at harvest. FC - Fold-change. / - no values obtained because of a low enzyme concentration.

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
Parent	0.00	0.00	/	/	/
	17.5	28.6	/	/	/
	24.8	43.6	/	/	/
	39.4	75.9	/	/	/
	50.7	83.8	35.4	2.08	1.00
	65.1	97.8	38.2	1.09	1.00
	75.8	106	55.0	3.67	1.00
	83.3	107	58.0	3.33	1.00
	98.6	110	154	22.0	1.00
	112	116	187	24.3	1.00
Upc2#1	0.00	0.00	/	/	/
	17.5	26.8	/	/	/
	24.8	47.9	/	/	/
	39.4	78.3	/	/	/
	50.7	86.5	77.3	2.84	2.18
	65.1	92.5	97.5	0.24	2.55
	75.8	94.4	225	0.72	4.08
	83.3	104	234	0.35	4.02
	98.6	107	367	0.05	2.38

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
	112	111	327	40.5	1.75
Mit1#16	0.00	0.00	/	/	/
	17.5	28.2	/	/	/
	24.8	47.4	/	/	/
	39.4	75.4	/	/	/
	50.7	87.1	220	0.10	6.21
	65.1	96.9	373	17.7	9.76
	75.8	99.8	404	18.1	7.33
	83.3	110	448	40.8	7.72
	98.6	112	527	9.83	3.42
	112	118	673	1.80	3.60
Mit1#21	0.00	0.00	/	/	/
	17.5	28.1	/	/	/
	24.8	47.5	/	/	/
	39.4	79.3	/	/	/
	50.7	91.6	73.9	7.04	2.08
	65.1	103	80.0	2.33	2.09
	75.8	106	113	9.46	2.05
	83.3	114	113	10.0	1.95
	98.6	118	199	13.6	1.29
	112	128	187	12.9	1.00
Mit1#20	0.00	0.00	/	/	/
	17.5	27.4	/	/	/
	24.8	43.6	/	/	/
	39.4	80.1	/	/	/
	50.7	93.0	260	15.5	7.34
	65.1	107	386	0.67	10.1
	75.8	115	422	22.9	7.66
	83.3	121	457	9.16	7.88
	98.6	122	626	8.97	4.06
	112	132	815	0.55	4.36

Table 7.18 Bioreactor cultivation results for P_{AOX1} strains (No.2). The enzyme activity was calculated with Formula 3.2 using the pNSi-assay. The values are for the first time point at inoculation and the last one at harvest. FC - Fold-change. / - no values obtained because of a low enzyme concentration.

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
Parent	0.00	0.00	/	/	/
	17.0	31.4	/	/	/

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
	24.3	43.0	/	/	/
	41.0	64.6	/	/	/
	49.8	69.0	/	/	/
	64.5	79.3	27.0	5.45	1.00
	72.8	86.2	44.0	7.13	1.00
	90	97.5	62.5	7.42	1.00
	100	101	86.5	10.3	1.00
	115	107	125	9.13	1.00
	125	109	115	7.35	1.00
	137	112	134	9.24	1.00
Mit1#16	0.00	0.00	/	/	/
	17.0	29.8	/	/	/
	24.3	44.1	/	/	/
	41.0	67.8	/	/	/
	49.8	71.8	/	/	/
	64.5	84.7	25.3	4.76	0.94
	72.8	91.6	61.0	9.96	1.39
	90	106	138	0.00	2.21
	100	108	253	3.56	2.93
	115	112	354	19.6	2.83
	125	118	377	17.8	3.27
	137	119	367	23.6	2.74
Mit1#20	0.00	0.00	/	/	/
	17.0	28.7	/	/	/
	24.3	46.0	/	/	/
	41.0	69.5	/	/	/
	49.8	74.9	/	/	/
	64.5	87.4	27.2	5.66	1.01
	72.8	93.1	42.2	6.65	0.96
	90	109	59.6	6.85	0.95
	100	112	118	8.42	1.36
	115	116	138	20.9	1.10
	125	122	189	10.9	1.64
	137	123	182	4.33	1.36
Upc2#5	0.00	0.0	/	/	/
	17.0	31.0	/	/	/
	24.3	44.7	/	/	/
	41.0	67.1	/	/	/
	49.8	73.7	/	/	/

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
	64.5	85.6	27.9	5.10	1.03
	72.8	93.8	50.9	10.5	1.16
	90	107	73.5	9.78	1.18
	100	111	107	12.0	1.24
	115	116	119	8.68	0.95
	125	118	184	8.27	1.60
	137	123	237	8.45	1.77

Table 7.19 Bioreactor cultivation results for P_{AOX1} strains (No.3). The enzyme activity was calculated with Formula 3.2 using the pNSi-assay. The values are for the first time point at inoculation and the last one at harvest. FC - Fold-change. / - no values obtained because of a low enzyme concentration.

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
Parent	0.00	11.2	/	/	/
	17.4	29.2	/	/	/
	25.1	46.9	/	/	/
	40.7	57.0	187	2.00	1.00
	49.2	63.5	282	45.0	1.00
	64.2	72.4	293	2.45	1.00
	77.2	79.4	415	58.3	1.00
	88.6	85.7	289	53.3	1.00
	101	92.7	282	4.00	1.00
	113	92.7	85.9	24.2	1.00
Upc2#1	0.00	8.45	/	/	/
	17.4	31.4	/	/	/
	25.1	51.6	/	/	/
	40.7	58.1	202	15.2	1.09
	49.2	61.0	348	23.3	1.23
	64.2	64.6	82.7	9.80	0.28
	77.2	65.9	98.4	22.5	0.24
	88.6	66.9	86.9	2.74	0.30
	101	69.4	39.6	1.57	0.14
	113	72.1	34.2	1.53	0.40
Rpn4#5	0.00	7.25	/	/	/
	17.4	32.45	/	/	/
	25.1	54.75	/	/	/
	40.7	69.6	71.9	18.9	0.39
	49.2	78.9	465	34.3	1.65
	64.2	94.2	607	128	2.07

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
	77.2	105	132	16.3	0.32
	88.6	116	80.9	9.54	0.28
	101	122	41.8	17.3	0.15
	113	131	33.9	0.34	0.39
Rpn4#22	0.00	8.10	/	/	/
	17.4	32.2	/	/	/
	25.1	52.1	/	/	/
	40.7	62.9	265	0.65	1.42
	49.2	70.7	591	100	2.10
	64.2	83.0	758	164	2.59
	77.2	91.0	660	41.3	1.59
	88.6	104	196	2.40	0.68
	101	113	165	2.10	0.58
	113	116	68.2	2.35	0.79
Rpn4#6	0.00	8.80	/	/	/
	17.4	31.0	/	/	/
	25.1	49.4	/	/	/
	40.7	60.9	334	25.1	1.79
	49.2	67.7	512	51.4	1.81
	64.2	81.8	502	81.3	1.71
	77.2	89.9	231	37.8	0.56
	88.6	96.8	219	13.3	0.76
	101	104	279	48.9	0.99
	113	106	128	5.80	1.49

Table 7.20 Bioreactor cultivation results for P_{AOX1} strains (No.4). The enzyme activity was calculated with Formula 3.2 using the pNSi-assay. The values are for the first time point at inoculation and the last one at harvest. FC - Fold-change. / - no values obtained because of a low enzyme concentration.

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
Parent	0.00	0.0	/	/	/
	17.5	26.8	/	/	/
	29.3	36.6	/	/	/
	40.8	50.2	/	/	/
	51.5	54.4	73.2	12.9	1.00
	65.2	60.5	157	17.2	1.00
	71.5	61.3	163	15.8	1.00
	88.1	74.8	226	13.4	1.00
	98.8	83.0	208	19.1	1.00

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
	114	101	282	60.2	1.00
evc#1	0.00	0.0	/	/	/
	17.5	30.5	/	/	/
	29.3	37.8	/	/	/
	40.8	49.3	/	/	/
	51.5	55.3	138	23.9	1.89
	65.2	57.7	266	52.5	1.69
	71.5	61.8	276	45.8	1.69
	88.1	70.6	279	85.6	1.23
	98.8	81.9	317	47.0	1.52
	114	97.5	327	59.5	1.16
Rpn4#1	0.00	0.00	/	/	/
	17.5	33.2	/	/	/
	29.3	37.3	/	/	/
	40.8	50.9	/	/	/
	51.5	55.5	231	17.7	3.16
	65.2	62.0	388	15.9	2.46
	71.5	65.9	411	19.8	2.52
	88.1	79.4	798	113	3.53
	98.8	89.4	1127	82.4	5.42
	114	103	1337	217	4.74
Mit1#9	0.00	0.00	/	/	/
	17.5	29.2	/	/	/
	29.3	32.8	/	/	/
	40.8	46.4	/	/	/
	51.5	51.4	77.9	4.60	1.07
	65.2	54.0	213	7.78	1.36
	71.5	57.6	245	10.6	1.50
	88.1	65.8	440	43.4	1.95
	98.8	73.6	638	76.0	3.07
	114	83.5	655	67.6	2.32
Upc2#10	0.00	0.00	/	/	/
	17.5	31.8	/	/	/
	29.3	34.7	/	/	/
	40.8	47.1	/	/	/
	51.5	55.6	215	26.1	2.94
	65.2	59.9	492	30.2	3.13
	71.5	64.8	632	27.5	3.87

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
	88.1	78.0	1198	62.3	5.30
	98.8	88.4	1571	70.0	7.55
	114	101	2337	281	8.28

Table 7.21 Bioreactor cultivation results for P_{GAP} strains (No.5). The enzyme activity was calculated with Formula 3.2 using the pNSi-assay. The values are for the first time point at inoculation and the last one at harvest. FC - Fold-change. / - no values obtained because of a low enzyme concentration.

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
Parent	0.00	0	/	/	/
	16.9	30.8	/	/	/
	25.3	40.6	/	/	/
	40.5	64.0	/	/	/
	49.0	74.4	86.8	2.42	1.00
	66.0	98.5	115	2.57	1.00
	75.6	109	163	2.15	1.00
	90.2	122	175	1.65	1.00
	101	130	132	6.54	1.00
	113	133	109	14.7	1.00
Upc2#1	0.00	0.00	/	/	/
	16.9	21.9	/	/	/
	25.3	39.0	/	/	/
	40.5	62.6	/	/	/
	49.0	72.2	150	7.87	1.37
	66.0	97.7	151	13.0	1.39
	75.6	108	162	1.20	1.49
	90.2	119	175	13.4	1.61
	101	128	169	14.6	1.54
	113	134	154	6.48	1.41
Rpn4#12	0.00	0.00	/	/	/
	16.9	35.2	/	/	/
	25.3	42.0	/	/	/
	40.5	63.8	/	/	/
	49.0	75.3	90.9	2.41	0.84
	66.0	98.9	172	15.6	1.59
	75.6	105	174	16.5	1.60
	90.2	123	191	13.1	1.75
	101	129	173	22.7	1.59
	113	132	177	5.28	1.63

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
Rpn4#2	0.00	0.00	/	/	/
	16.9	32.1	/	/	/
	25.3	37.8	/	/	/
	40.5	63.3	/	/	/
	49.0	74.2	10.9	0.54	0.10
	66.0	96.1	15.3	0.71	0.14
	75.6	109	56.4	0.08	0.52
	90.2	120	34.0	5.71	0.31
	101	12	21.4	0.82	0.20
	113	135	13.9	19.9	0.13
Rpn4#7	0.00	0.00	/	/	/
	16.9	23.8	/	/	/
	25.3	39.2	/	/	/
	40.5	64.2	/	/	/
	49.0	78.5	32.4	9.97	0.30
	66.0	101	38.4	11.6	0.35
	75.6	115	118	14.0	1.09
	90.2	126	146	19.9	1.34
	101	137	76.3	3.29	0.70
	113	143	28.1	0.60	0.26

Table 7.22 Bioreactor cultivation results for P_{GAP} strains (No.6). The enzyme activity was calculated with Formula 3.2 using the pNSi-assay. The values are for the first time point at inoculation and the last one at harvest. FC - Fold-change. / - no values obtained because of a low enzyme concentration.

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
Parent	0.00	0.00	/	/	/
	17.2	24.7	/	/	/
	25.3	33.2	/	/	/
	41.0	59.0	/	/	/
	51.6	74.7	33.5	7.17	1.00
	66.0	95.4	34.3	5.18	1.00
	76.5	108	35.8	7.18	1.00
	89.2	119	33.4	10.3	1.00
	101	129	45.7	14.8	1.00
	113	138	58.8	7.12	1.00
Mit1#18	0.00	0.00	/	/	/
	17.2	26.0	/	/	/
	25.3	37.3	/	/	/

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
	41.0	61.7	/	/	/
	51.6	73.6	139	2.12	4.17
	66.0	93.3	244	8.59	7.10
	76.5	103	150	7.51	4.19
	89.2	115	173	11.7	5.18
	101	130	184	9.82	4.02
	113	133	201	5.33	3.41
Mit1#23	0.00	0.00	/	/	/
	17.2	24.8	/	/	/
	25.3	32.7	/	/	/
	41.0	54.3	/	/	/
	51.6	73.6	53.2	3.36	1.59
	66.0	90.1	53.8	3.04	1.57
	76.5	101	44.0	7.48	1.23
	89.2	112	59.7	33.5	1.79
	101	123	72.4	27.2	1.58
	113	127	90.8	8.25	1.54
Mit1#13	0.00	0.00	/	/	/
	17.2	27.4	/	/	/
	25.3	36.5	/	/	/
	41.0	67.7	/	/	/
	51.6	88.7	/	/	/
	66.0	110	/	/	/
	76.5	122	10.5	1.72	0.29
	89.2	135	21.7	0.77	0.65
	101	143	26.4	5.33	0.58
	113	144	31.8	2.24	0.54

Table 7.23 Bioreactor cultivation results for P_{GAP} strains (No.7). The enzyme activity was calculated with Formula 3.2 using the pNSi-assay. The values are for the first time point at inoculation and the last one at harvest. FC - Fold-change. / - no values obtained because of a low enzyme concentration.

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
Parent	0.00	0.00	/	/	/
	17.8	28.8	/	/	/
	24.0	31.7	/	/	/
	41.2	61.1	/	/	/
	46.5	73.6	14.6	2.35	1.00

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
	65.3	103	23.1	1.76	1.00
	75.3	117	22.9	3.90	1.00
	89.4	136	18.3	1.92	1.00
	99.6	142	16.4	2.25	1.00
	113	148	26.5	9.87	1.00
Mit1#19	0.00	0.00	/	/	/
	17.8	31.9	/	/	/
	24.0	32.4	/	/	/
	41.2	65.9	/	/	/
	46.5	73.0	29.8	5.15	2.04
	65.3	98.6	65.8	5.36	2.85
	75.3	106	84.8	0.64	3.71
	89.4	118	95.2	1.73	5.21
	99.6	126	123	8.44	7.51
	113	126	117	8.58	4.42
Mit1#3	0.00	0.00	/	/	/
	17.8	31.3	/	/	/
	24.0	32.5	/	/	/
	41.2	63.2	/	/	/
	46.5	78.7	23.6	3.75	1.61
	65.3	105	49.6	2.28	2.15
	75.3	119	97.7	1.77	4.27
	89.4	143	95.0	4.73	5.20
	99.6	141	56.8	2.50	3.46
	113	149	52.6	5.28	1.99
Upc2#7	0.00	0.00	/	/	/
	17.8	29.0	/	/	/
	24.0	31.5	/	/	/
	41.2	67.5	/	/	/
	46.5	72.9	120	10.9	8.18
	65.3	91.9	408	28.6	17.7
	75.3	101	430	24.0	18.8
	89.4	122	520	7.18	28.5
	99.6	131	668	0.00	40.8
	113	126	609	18.6	23.0

Table 7.24 Bioreactor cultivation results for P_{GAP} strains (No.8). The enzyme activity was calculated with Formula 3.2 using the pNSi-assay. The values are for the first time point at inoculation and the last one at harvest. FC - Fold-change. / - no values obtained because of a low enzyme concentration.

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
Parent	0.00	0.00	/	/	/
	15.5	25.4	/	/	/
	24.5	45.3	/	/	/
	39.5	70.2	/	/	/
	48.0	81.2	13.1	0.14	1.00
	64.5	104	30.2	0.18	1.00
	76.0	118	52.0	1.46	1.00
	89.5	127	63.7	6.75	1.00
	99.0	136	95.4	4.89	1.00
	111	142	62.2	5.59	1.00
evc#3	0.00	0.00	/	/	/
	15.5	32.2	/	/	/
	24.5	44.4	/	/	/
	39.5	69.1	/	/	/
	48.0	78.9	21.7	4.04	1.66
	64.5	103	32.8	6.12	1.09
	76.0	117	52.9	4.29	1.02
	89.5	120	49.4	5.16	0.78
	99.0	137	84.9	6.23	0.89
	111	141	64.4	4.95	1.04
Mit1#18	0.00	0.00	/	/	/
	15.5	16.9	/	/	/
	24.5	42.3	/	/	/
	39.5	67.1	/	/	/
	48.0	76.3	81.5	8.85	6.22
	64.5	95.9	161	0.05	5.34
	76.0	108	174	0.80	3.34
	89.5	120	188	17.6	2.95
	99.0	124	231	17.6	2.42
	111	127	258	1.47	4.15
Upc2#1	0.00	0.00	/	/	/
	15.5	13.9	/	/	/
	24.5	45.3	/	/	/
	39.5	71.8	/	/	/
	48.0	82.7	40.0	8.66	3.06
	64.5	102	43.6	5.74	1.45

	time point [h]	DCW [g/L]	Activity [U/L]	Deviation	FC
	76.0	118	49.2	8.89	0.95
	89.5	121	48.8	7.49	0.77
	99.0	130	87.1	12.2	0.91
	111	136	79.8	7.15	1.28
Upc2#5	0.00	0.00	/	/	/
	15.5	11.0	/	/	/
	24.5	45.1	/	/	/
	39.5	66.1	/	/	/
	48.0	75.4	74.3	0.10	5.67
	64.5	94.5	86.1	8.61	2.86
	76.0	105	102	1.75	1.96
	89.5	116	118	0.40	1.85
	99.0	124	188	3.94	1.97
	111	125	212	4.79	3.41