IMPROVEMENT OF *PICHIA PASTORIS* *AOX1* PROMOTER EXPRESSION SYSTEM

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Ich widme diese Doktorarbeit meinem Mann Juan Carlos Rosales Rodriguez und meinem ungeborenen Kind.
Ich liebe Euch von ganzem Herzen.

Le dedico esta tesis doctoral a mi esposo Juan Carlos Rosales Rodriguez y al ser que llevo en mi vientre.
Los amo desde lo más profundo de mi corazón.

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ABSTRACT

The methylotrophic yeast *Pichia pastoris* is a well established system for the high yield production of recombinant proteins as it combines a lot of advantageous features like easy to handle, growth to high cell densities on cheap media, possibility of product secretion into the fermentation broth mostly by use of the *Saccharomyces cerevisiae* alpha mating factor secretion signal, availability of the genome sequence of the commercially used host strain, well characterized and widely used in biochemical and genetic studies and the option of adaptation of pharmaceutically important properties like glycosylation to e.g. human patterns. An advantage and a drawback at the same time is the disposability of an extremely strong methanol inducible promoter, the alcohol oxidase 1 promoter. In this study several options for improvement of the AOX1 promoter expression system were investigated.

One important result was unraveling the genomic background of the *P. pastoris* wild type strain CBS 7435, which was broadly introduced for use as expression host strain. As one specific result of genome sequencing, the *P. pastoris* alpha mating factor secretion signal was identified and isolated.

In a second line strategies to improve the expression from the strong, tightly regulated AOX1 promoter were developed. The analysis of the integration status of a high level expression strain for production of a plant derived hydroxy-nitrile lyase (1) revealed a special AOX1 “head-to-head” promoter situation and the existence of three functional Hnl cassettes. Cloning of the “head-to-head” promoter fragment in front of a single copy of a mutant Hnl gene was able to reconstitute the same expression level as seen in the original high level expression strain. In addition, studies for improving the regulation options with this promoter were performed.

As secretion is always a bottleneck, a new surface display system in *P. pastoris* was established using newly identified and isolated potential *P. pastoris* anchors. By use of *B. subtilis* levanase (*sacC*) (2) and *P. pastoris* codon optimized EGFP as model proteins the suitability of two anchors for efficient surface expression of proteins in *P. pastoris* was demonstrated. To generally show the potential of surface display in *P. pastoris*, a very complex *Escherichia coli* protein was surface displayed with the well-established alpha-

agglutinin system originating from *Saccharomyces cerevisiae*. To my knowledge, an efficient expression of a functional hexamer on the surface of a cell has never been reported before in literature.

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KURZZUSAMMENFASSUNG

Die methylotrophe Hefe *Pichia pastoris* ist ein gut etabliertes System für die Produktion von rekombinanten Proteinen in hoher Ausbeute, da es viele vorteilhafte Eigenschaften wie leichte Handhabung, Wachstum zu hohen Zelldichten auf günstigen Medien, die Option, das gewünschte Produkt in den Fermentationsüberstand zu sekretieren, was meistens mit Hilfe des *Saccharomyces cerevisiae* „alpha mating factor" Sekretionssignals passiert, die Verfügbarkeit der Genomsequenz des kommerziell genutzten Wirtsstammes, gut charakterisiertes System mit weit verbreitetem Einsatz bei biochemischen und genetischen Studien, sowie die Möglichkeit der Anpassung von pharmazeutisch wichtigen Eigenschaften wie Glykosylierung an zum Beispiel humane Muster in sich vereint. Ein Vorteil, aber auch zugleich eine Einschränkung ist die Verfügbarkeit eines extrem starken, Methanol induzierbaren Promotors, des Alkoholoxidase 1 (AOX1) Promotors. In dieser Studie wurden mehrere Optionen zur Verbesserung des AOX1 Promotor Expressionssystems erforscht.

Ein wichtiges Ergebnis war die Entschlüsselung der genomischen Hintergrundinformation des *Pichia pastoris* Wildtypstammes CBS 7435, der inzwischen breite Verwendung als Expressionsstamm gefunden hat. Ein konkretes Resultat der Genomsequenzierung war die Identifizierung und Isolierung des *P. pastoris* „alpha mating factor" Sekretionssignals.

Eine zweite Linie, die in dieser Studie verfolgt wurde, war, Strategien für eine verbesserte Expression durch den starken, streng regulierten AOX1 Promotor zu entwickeln. Die Analyse des Integrationsstatus eines sehr ertragsstarken Expressionsstammes, der eine aus einer Pflanze stammende Hydroxinitrillyase (Hnl) (1) produziert, ergab eine besondere „Head-to-Head" Promotorsituation und das Vorhandensein dreier funktioneller Hnl-Expressionskassetten. Durch die Klonierung dieses „Head-to-Head" Promotors vor eine einzige Kopie eines mutierten Hnl-Gens konnte ein zum hohen Expressionslevel des ursprünglichen Expressionsstammes vergleichbares Expressionsniveau erreicht werden. Zusätzlich wurden Studien zur Verbesserung des regulatorischen Potentials dieses Promotors durchgeführt.

Da Sekretion immer einen Kapazitätsengpass bedeutet, wurde ein neuartiges System zur Expression von Proteinen an der Oberfläche von *P. pastoris* - Zellen etabliert, wofür neue,

Pichia - eigene Zellwandproteine zur Verankerung identifiziert und isoliert wurden. Durch die Verwendung der Modellproteine *B. subtilis* Levanase (*sacC*) (2) und *P. pastoris* – Codon - optimiertes EGFP konnte die Eignung zweier Anker-Proteine für die effiziente Oberflächenexpression in *P. pastoris* bewiesen werden. Um allgemein das Potential der Oberflächenexpression von Proteinen in *Pichia* zu zeigen, wurde unter Verwendung des bereits etablierten, aus *S. cerevisiae* stammenden Zellwandankers alpha - Agglutinin ein sehr komplexes Enzym aus *Escherichia coli* an der *P. pastoris*-Zelloberfläche immobilisiert. Meines Wissens wurde bisher noch nie in der Literatur über die effiziente und funktionelle Expression eines Hexamers an der Zelloberfläche berichtet.

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GENERAL INTRODUCTION TO *PICHIA PASTORIS*

Meanwhile since decades, the methylotrophic yeast *Pichia pastoris* has proved itself as a valuable host for recombinant protein production. It combines a lot of advantageous features like easy handling, growth to high cell densities on cheap media, possibility of product secretion into the fermentation broth mostly by use of the *Saccharomyces cerevisiae* alpha mating factor secretion signal, availability of the genome sequence of the commercially used host strains, well characterized and widely used in biochemical and genetic studies and the option of adaptation of pharmaceutically important properties like glycosylation to e.g. human patterns. Additionally is has reached GRAS (generally recognized as safe) status as awarded by the US food and drug administration (FDA) because it is virtually free of endotoxin production and oncogenic virus DNA. Therefore, the *Pichia* expression system is generally regarded as a straightforward, cost-effective, fast and versatile facility for high quality and high yield production of recombinant proteins.

HISTORICAL BACKGROUND

During the first half of the twentieth century, two separate isolates of wild yeast strains, one in California (1940s) and one in France (1919), were collected probably in order to find contaminating yeast strains battling against productive winegrowing strains. Due to restriction to 1940´s technology, Herman Phaff failed to distinguish between the old and the new strains and somehow the older species was transitioned to the newer one, yielding in two yeasts being classed as a single species.

In the 1960s, Koichi Ogata detected the capability of some yeast species to grow on methanol as sole carbon and energy source. In the 1970s, Phillips Petroleum Company initiated the production of methanol from naturally available methane gas in order to manufacture high protein animal feed (single cell protein) by yeast fermentation. Although there were several yeast strains known being capable of growth on methanol Phillips petroleum insisted on *Pichia pastoris* for commercial scale-up. Since process development was successful, Phillips applied for a patent and as part of it the two original *Pichia* strains

collected by Guilliermond and Phaff were consigned to Northern Regional Research Laboratory (NRRL) culture collection. Due to the oil-crisis in the 1970s the prices for natural gas rose enormously and the process became uneconomical. Molecular biology techniques to engineer yeast genomes progressed during the 1980s and Phillips Petroleum decided to develop tools for the employment of *Pichia pastoris* as host for the expression of recombinant proteins by funding research at the Salk Institute BioTechnology / Industrial Associates (SIBIA, spin-out of The Salk Institute in La Jolla, California). Scientists at SIBIA isolated *AOX1* expression cassette and developed all the basic vector systems, culture media and methods still known and applied for successful *Pichia pastoris* manipulation and cultivation (1)(2).

METHANOL UTILIZATION PATHWAY (MUT)

Methylotrophic yeasts are able to metabolize methanol as sole carbon and energy source and therefore they have something in common: the MUT pathway. It is strictly regulated at the transcriptional level (3)(4). Methanol metabolism partially occurs in the peroxisomes which expand tremendously upon methanol induction and in the cytosol (5)(6). First of all, methanol is oxidized to formaldehyde and hydrogen peroxide by alcohol oxidases (AOX) (see Figure 1 for details). Resulting H_2O_2 is cleaved by catalase (CAT) resulting in oxygen and water. Both reactions take place in peroxisomes. Formaldehyde is further metabolized by two different pathways: the dissimilatory and the assimilatory. In the dissimilation pathway, formaldehyde is broken down to carbondioxide by two consecutive steps taking place in the cytosol, namely the spontaneous reaction with glutathione to S-hydroxymethylglutathione followed by oxidation via NAD^+-dependent formaldehyde dehydrogenase (FLD) and NAD^+-dependent formate dehydrogenase (FDH). In the assimilation pathway, formaldehyde condensates with xylulose-5-phosphate (Xu_5P) catalyzed by dihydroxyacetone synthase (DAS), which converts Xu_5P and formaldehyde to dihydroxyacetone (DHA) and glyceraldehydes-3-phosphate (GAP) which are further metabolized in the cytosol (6).

Generally, AOX and DAS genes are repressed by glucose and strongly induced by methanol, whereas FLD and FDH induction was also achieved by e.g. methylamine (methylated nitrogen sources), even if glucose was present (7)(6).

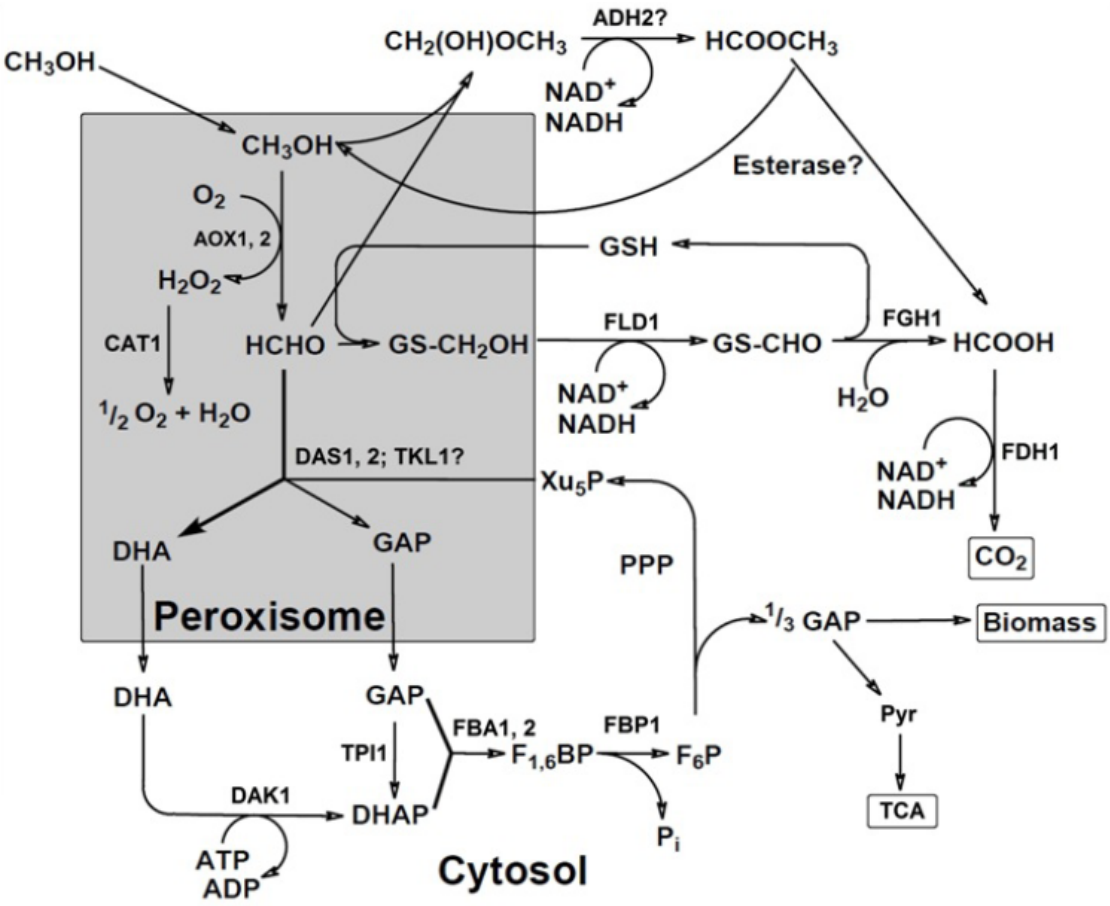

Fig. 1: Methanol utilization pathway in *Pichia pastoris* (8)**.** AOX1, 2: alcohol oxidases, CAT1: catalase, FLD1: formaldehyde dehydrogenase, FGH1: S-formylglutathione hydrolase, FDH1: formate dehydrogenase, DAS1, 2: dihydroxyacetone synthases, TKL1: transketolase, TPI1: triosephosphate isomerase, DAK1: dihydroxyacetone kinase, FBA1, 2: fructose 1,6-bisphosphate aldolase, FBP1: fructose 1,6-bisphosphatase, ADH: methylformate synthase, DHA: dihydroxyacetone, GAP: glyceraldehyde 3-phosphate, DHAP: dihydroxyacetonephosphate, $F_{1,6}BP$: fructose 1,6-bisphosphate, F_6P: fructose 6-phosphate, P_i: phosphate, Xu_5P: xylulose 5-phosphate, GSH: glutathione, Pyr: pyruvate; PPP: pentose phosphate pathway, TCA: tricarboxylic acid cycle

Recently, an improved recombinant protein production by engineering the methanol utilization pathway was reported. Thereby, co-overexpression of dihydroxyacetone synthase caused a 2- to 3-fold more efficient methanol conversion but finally achieved reduced volumetric productivity. In contrast, co-overexpression of formaldehyde dehydrogenase yielded in a 2-fold more efficient substrate conversion and at least similar volumetric productivities by contrast with strains without an engineered MUT pathway (8).

PICHIA PASTORIS EXPRESSION SYSTEM

1. PROMOTER

Most of the commonly used promoters for recombinant protein expression in *Pichia pastoris* originate from methanol metabolism and are generally strongly abundant in the cell. Although the positive features like strength and inducibility prevail, methanol as inducing agent creates safety problems and sometimes the strong promoter activity results in a conflict of the secretory system of the host cell (9). There is a need for alternative promoters that do not need toxic inducer.

Glyeraldehyde-3-phosphate dehydrogenase promoter (P_{GAP}):

The strong constitutive GAP promoter derives from glyceraldehyde-3-phosphate dehydrogenase, one of the key enzymes in glycolysis. As distinct from *AOX1* promoter cells are grown on glucose media, but under optimal conditions similar expression rates can be attempted as seen with *AOX1* promoter (10). For industrial applications, an advantage as opposed to *AOX1* promoter becomes evident: the waiver of methanol.

Translation elongation factor 1-α promoter (P_{TEF1}):

The constitutive TEF1 promoter activity strictly correlates with growth, showing an increased action under high glucose conditions. Comparable product yields to GAP promoter could be obtained (11).

Glycolytic enzyme-3-phosphoglycerate kinase promoter (P_{PGK1}):

The constitutive PGK1 promoter shows a carbon source dependent activity. Using glucose media, productivity is twice as high compared to productivity obtained with glycerol or methanol media (12).

YPT1 promoter (P_{YPT1}):

Moderate, constitutive promoter of a small GTPase involved in secretion which is induced on media containing glucose, methanol or mannitol as carbon source (13)(14).

Alcohol oxidase 1 promoter (P_{AOX1}):

P_{AOX1} is the most frequently used inducible promoter for heterologous protein production in *Pichia pastoris*. Transcription is strictly regulated and determined by a repression/derepression mechanism. It is strongly repressed by most of the carbon sources used for biomass generation like glucose or glycerol and can be easily turned on just by methanol addition.

Dihydroxyacetone synthase promoter (P_{DHAS}):

P_{DHAS} is also repressed on glucose and induced by methanol.

Formaldehyde dehydrogenase promoter (P_{FLD1}):

P_{FLD1} is also a tightly regulated promoter which is induced either on methanol as sole carbon source (with ammonium sulfate as nitrogen source) or on particular alkylated amines such as methyl amine as sole nitrogen source (with glucose as carbon source) (15).

Isocitrate lyase promoter (P_{ICL1}):

P_{ICL1} is repressed in the presence of glucose and induced in its absence or upon ethanol induction (16).

Peroxin 8 promoter (P_{PEX8}):

P_{PEX8} works weakly but to a certain level on glucose and is moderately induced on methanol (14).

(Na^+)-coupled phosphate symporter Pho89 promoter:

Recently described phosphate responsive promoter which is very active under phosphate limiting conditions (17).

Promoter libraries and synthetic promoters:

Hartner *et al.* were the first to report the design of an AOX1 promoter library as well as the synthesis of short artificial promoters to fine-tune gene expression in *Pichia pastoris*. Thereby providing promoters with expression levels that can be perfectly fitted to the needs of the respective recombinant proteins avoiding e.g. too high expression levels in case of cell

toxic components. Based on the deletion analysis of P_{AOX1} new synthetic promoter variants combining *cis*-acting elements with basal promoter were designed (18). A combination of this already described and analyzed artificial promoters and novel variants were compared in the study of Ruth *et al.* (19). Thus, the time of increasing product concentration (the production window of trypsinogen) varied substantially depending on the used promoter variant. Besides, the beginning of auto-proteolytic product degradation can be predefined by the promoter choice. In 2012, another application was reported combining codon optimized versions with the synthetic promoter variants for the expression of lignocellulolytic enzmes in *Pichia pastoris* (20). Moreover, Qin *et al.* published recently the creation of a GAP promoter library by error-prone mutagenesis (21).

2. HOST STRAINS

Pichia pastoris GS115 and X-33 strains which are the most commonly used host strains for *Pichia* expression derive from the parental strain NRRL Y-11430 being consigned as CBS 7435 (CBS, Centraalbureau voor Schimmelcultures) and classified as *Komagataella phaffii* (22). The draft genome sequence of *P.p.* GS115, which was generated by nitrosoguanidine mutagenenesis of the NRRL Y-11430, was published in 2009 (23) as well as the strain CBS 704 (= DSMZ 70382; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) being considered as the European *P. pastoria* type strain (24). Finally, in 2011, the genome sequence of the native strain CBS 7435 was reported by Küberl *et al.* including the identification and isolation of *Pichia pastoris* alpha mating factor (25).

Recently, the creation of a *Pichia pastoris ku70* deletion strain was reported showing significantly increased targeting efficiency to the aimed integration locus (26). Degradation by proteases is sometimes a big problem in secretion of heterologous proteins mostly occurring in fermenter cultures caused by high cell densities and cell lysis. Protease deficient host strains were developed to overcome the problem. They seem to be efficient concerning reduction of proteolytic degradation but create other drawbacks as they are not as productive as normal host strains, they are not that viable and trickier to handle. SMD1163 (*his4 pep4 prb1*), SMD1165 (*his4 prb1*) and SMD1168 (*his4 pep4*) are the most popular, commercially available host strains (1).

3. SECRETION SIGNAL

A versatile feature of protein production in *Pichia pastoris* is the possibility of secreting the recombinant product into the fermentation broth. This can be done by the application of the natural signal sequence of the protein or by the fusion with an alternative secretion signal. The most prominent secretion signal in case of *Pichia pastoris* is the alpha mating factor secretion signal originating from *Saccharomyces cerevisiae*. This well established system proved to be effective in many cases. Since 2011, the *Pichia* alpha mating factor is also available (25). Another *Pichia pastoris* secretion signal sequence that can be applied is the acid phosphatase (PHO1) secretion signal. Invitrogen™ (Life Technologies™) offers in its Pichia Pink Expression Kit® 8 alternative secretion signal sequences: *S. cerevisiae* alpha mating factor pre-peptide, *Aspergillus niger* α-amylase secretion signal, *Aspergillus awamori* glucoamylase secretion signal, *Homo sapiens* serum albumin signal sequence, *Kluyveromyces marxianus* inulinase signal sequence, *Saccharomyces cerevisiae* invertase signal sequence, *Saccharomyces cerevisiae* killer toxin signal sequence and lysozyme signal sequence from *Gallus gallus* (27).

4. GLYCOSYLATION

For the production of pharmaceutical proteins glycosylation is indispensable to assure biological activity of the protein and to avoid immunogenic effects. *P. pastoris* is able to carry out O- and N- linked glycosylation (28). O-glycosylation is a crucial post-translational modification on the serine / threonine residuals. O-linked N-acetylgalactosamine (GalNAc), fucose and glucose sugars are mostly abundant in mammals, whereas yeasts possess O-linked oligomannose. For yeast cell wall stability, O-mannosylation is indispensable. Not solely yeast cellular proteins comprise O-mannosylation but recombinant proteins do as well. Recombinant proteins expressed in *Pichia pastoris* have O-glycans containing dimeric to pentameric alpha-1, 2-linked mannose (29). N-glycosylation in *P. pastoris* and other fungi differs from that in higher eukaryots. But in all eukaryotes, the endoplasmatic reticulum is the starting point. A lipid-linked oligosaccharide unit $Glc_3Man_9GlcNAc_2$ (Glc = glucose; GlcNAc = N-acetylglucosamine) is transferred to asparagine at the recognition sequence Asn-X-

Ser/Thr followed by trimming of this core unit to $Man_8GlcNAc_2$. That´s were lower and higher eukaryotic glycosylation patterns start to vary (Fig. 2). The mammalian Golgi apparatus conducts a number of trimming and addition reactions that result in oligosaccharides containing either $Man_{5-6}GlcNAc_2$ (high-mannose type), a mixture of several different sugars (complex type) or a combination of both (hybrid type). The typical profile of the outer chain of a secreted protein of *Pichia pastoris* shows $Man_8GlcNAc_2$ or $Man_9GlcNAc_2$. Furthermore, *P. pastoris* oligosaccharides appear not to have any terminal α-1,3-linked mannosylation which makes recombinant proteins inappropriate for pharmaceutical use in humans (14). Anyway, in the 2000s GlycoSwitch and GlycoFi technologies made it possible to bring out recombinant proteins with humanized N-linked glycans (Fig. 3).

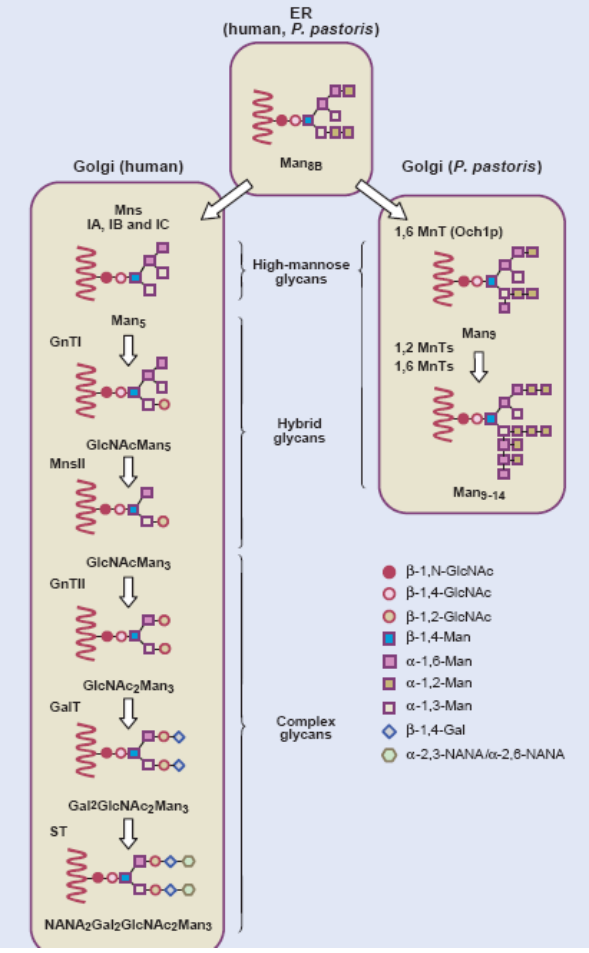

Fig. 2: Glycosylation pathways in humans and *Pichia pastoris*. Mns: α-1,2-mannosidase, Mns II: mannosidase II, GnTI: β-1, 2-N-acetylglucosaminyl-transferase I, GnTII: β-1, 2-N-acetylglucosaminyl-transferase II, GalT: β-1,4-galactosyltransferase, ST: sialyltransferase, MnT: mannosyltransferase (41)

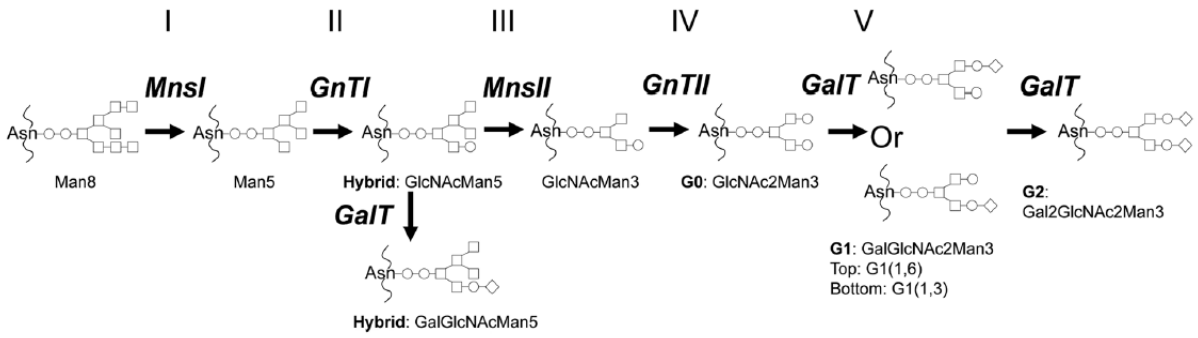

Figure 3. The schematic outline of N-glycan biosynthesis pathway in glycoengineered *Pichia pastoris* that mimics human N-glycan synthesis. MnsI: α-1,2-mannosidase; GnTI: β-1,2-N-acetylglucosaminyltransferase I; MnsII: mannosidase II; GnTII: β-1,2-N-acetylglucosaminyltransferase II; GalT: β-1,4-galactosyltransferase. ○: GlcNAc; □: Man; □ : Gal. To simplify nomenclature, the two GlcNAc sugars at the reducing end of all glycans are omitted (30).

5. AOX1 PROMOTER REGULATION

For the majority of heterologous proteins expressed in *P. pastoris* the *AOX1* promoter is used as it is highly inducible by methanol, but tightly repressed if glucose or glycerol are present (31). Under derepressing conditions *AOX1* transcriptional activity corresponds to about only 2% as compared to inducing conditions. Methanol-grown cells, on the other hand, show a 1000-fold higher transcriptional level than repressed ones (32). Therefore, regulated expression driven by the *AOX1* promoter is possible. Up to now little is known how methanol regulates the *AOX1* promoter. One transcription factor, Mxr1, has been found, which has a binding site in this promoter region. It has been shown that Mxr1p localizes to the nucleus upon methanol induction. In cells lacking functional *MXR1,* methanol induction of the *AOX1* promoter does not work (33). *mxr1* cells were initially identified in a screen for peroxisomal biogenesis-defective mutants (34).

Hartner *et al.* identified 7 regions in the P_{AOX1} where promoter activity was reduced to less than 50 % when smaller or larger parts thereof where removed designating potential transcription factor (TF) binding sites or *cis*-acting elements. They further postulate that Mxr1p additionally to its P_{AOX1} binding controls minimum one more TF protein associated with P_{AOX1} regulation (18). In 2009, Kranthi *et al.* determined 6 binding sites for Mxr1p in the P_{AOX1}. Their interpretation was that the elevated expression from the highly methanol

inducible P_{AOX1} is founded by a synergistic effect resulting from multiple Mxr1p molecules bound to the Mxr1p response elements (MXREs) (35). The same group also detected Mxr1p binding sites in DHAS and PEX8 promoters and extended the Mxr1 core binding motif by the addition of indispensable nucleotides to it (5´ CYCCNY 3´). Putative Mxr1p binding sites were also determined in FLD1 and PEX14 promoters by *in silico* analysis (36). Recently, a 14-3-3 family protein was detected in *Pichia pastoris* regulating Mxr1p activity contingent on the carbon source used for cultivation. Two mechanisms of Mxr1p regulation are hypothesized: an inhibition by the supplied carbon source mediated by 14-3-3 and an activation which is somehow triggered by methanol induction (37). In *Candida boidinii* two proteins were identified showing high homology to *P. pastoris* Mxr1p and *S. cerevisiae* Adr1p. Trm1p on the one hand is related to methanol induced activation of genes but doesn't seem to be connected to peroxisomal assembly or proliferation (38). It has a unique feature, a glutamine-rich region (Q-rich region) putatively acting as a transcriptional activation domain that has also been found in Mxr1p. Trm2p in contrast seems to be the regulatory feature responsible for derepression in *Candida*. In 2010, Sasano *et al.* suggested the following mechanism: methanol-induced gene expression is totally repressed by growth on glucose. When glucose is used up or methanol induction takes place Trm2p causes glucose repression thereby activating Trm1p which in turn activates methanol-specific genes (39). There is a second gene encoding an alcohol oxidase in *Pichia pastoris*, *AOX2*. *AOX1* and *AOX2* share a nearly identical coding sequence, but their promoter regions differ significantly (4). *AOX1* is responsible for greater than 90% of the enzyme activity in the cell (40). *Pichia pastoris* has three different methanol utilization phenotypes. Wild type cells carry two functional *AOX* genes and are thus defined as mut^+, i.e. methanol utilization plus. Deletion of *AOX1* causes a phenotype that shows slower growth on methanol as the sole carbon source, termed methanol utilization slow (mut^S) phenotype. Deletion of both alcohol oxidase genes leads to a methanol utilization minus, mut^-, phenotype. The latter strains are not able to grow on methanol any more (40)(6).

AIMS

Although the AOX1 promoter expression system is well established, the aim of this study picks out different starting points for a further improvement. Genome sequencing of the host strain *P. pastoris* CBS 7435 used for all kinds of genetic manipulations is an important prerequisite as it provides the researcher with a lot of "insider" information. The fact that an ordinary expression strain created for the production of *Hevea brasiliensis* hydroxy-nitrile lyase reached the extraordinary product yield of 22 g/l in a fed-batch fermentation excited our curiosity. An analysis of the genomic situation in the high level expression within the framework of a diploma thesis (S. Nussbaumer) and a doctoral thesis (B. Krammer) revealed the existence of a special head-to-head AOX1 promoter situation. Thus, the second goal of my work was to establish a new expression system with this novel promoter and to analyze the potential thereof. As there is not much known about AOX1 promoter regulation, M. Tscherner tried to find regulatory elements in the context of his diploma thesis. With the help of an AOX1/AOX2 double knock out strain expressing *B. subtilis* levanase under the control of AOX1 promoter he screened a *Pichia pastoris* gene library for genetic elements influencing expression driven by this promoter. Curiously enough, he found several clones with enhanced levanase expression under derepressing conditions and they all had one thing in common: they had integrated a complete and functional AOX1 expression cassette. Therefore a putative auto-regulatory function of AOX1 was hypothesized. My major aim was to continue his work and to find more evidences to underpin the hypothesis. In a further line, a solution for a major disadvantage of the methanol inducible AOX1 promoter system was supposed to be found. The idea of a methanol free induction of the AOX1 promoter system arose. I supervised the diploma thesis of T. Nudl dealing with the development of a new *Pichia* strain being able to induce AOX1 promoter expression without the addition of methanol. As secretion is always a bottleneck, another aim of my work was to establish a *Pichia pastoris* surface display system using newly identified and isolated cell wall anchors from *Pichia*. Finally, my last approach intended to show the general potential of *Pichia pastoris* as a whole cell biocatalyst. For that purpose, a complex *Escherichia coli* enzyme was chosen as surface display of such multimeric proteins has not been reported before in literature.

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Chapter 2

High-quality genome sequence of *Pichia pastoris* (Journal of Biotechnology)

My contribution to the paper is as follows:

- Gap closure (95 %)
- Identification and Isolation of the *Pichia* alpha factor (100 %)
- Writing of the research article (3 %)

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Chapter 3

High-level Expression of *Hevea brasiliensis* Hnl W128F Mutant in *Pichia pastoris* Using a Head-to-head AOX1 Promoter Fragment

My contribution to the paper draft is as follows:

- Conceptual formulation (30 %)
- Construction of the head-to-head plasmids (100 %)
- In situ Hnl assays (100 %)
- Hnl platereader activity assays (100 %)
- SDS PAGES (100 %)
- Head-to-head promoter analysis (100 %)
- Writing of the research article (60 %)

High-level expression of *Hevea brasiliensis* Hnl W128F mutant in *Pichia pastoris* using a head-to-head promoter fragment

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ABSTRACT

In 1997, Hasslacher *et al.* published the high yield expression of *Hevea brasiliensis* hydroxy-nitrile lyase in *Pichia pastoris* resulting from spheroplast transformation of pHILD2_HNL wild type plasmid into *P. pastoris* GS115 strain. To analyse the genomic background of the high expression capacity of this strain, southern blot experiments were made revealing the existence of three HNL expression cassettes, two of them driven by a special head-to-head AOX1 promoter. Cloning of the "head-to-head" promoter fragment in front of a single copy of a mutant Hnl gene was able to reconstitute the same expression level as seen in the original high level expression strain.

INTRODUCTION

Pichia pastoris has been, for many years, a suitable host for the expression of heterologous proteins, either intracellular or secretory. Many attempts have been made since now to optimize and adapt this host system, be it by metabolic engineering, design and synthesis of promoters for any purpose, by mimicking human glycosylation patterns for mostly pharmaceutical applications, or the improvement of secretion pathway for most efficient, correctly folded and functional protein production and release into the medium. It was already assumed from the very first attempts of foreign protein production in *Pichia pastoris* (1)(2)(3) that this methylotrophic yeast bears potential for high-yield expression, but nevertheless it was surprising to see that *Hevea brasiliensis* hydroxy-nitrile lyase (Hnl) expression attempts resulted in a volume yield of intracellular Hnl protein of about 22g/l constituting up to 30 % of the total soluble protein of a methanol-grown *P. pastoris* cell (4). Hydroxy-nitrile lyase, HNL1 (EC 4.1.2.39), catalyzes the cleavage of cyanohydrins to hydrocyanic acid plus the corresponding aldehyde or ketone. The release of HCN serves as a defense against herbivores and microbial attack for a variety of plants and may also serve as a nitrogen source for the biosynthesis of asparagine. Moreover, this enzyme is an interesting target for the detoxification of cyanogenic foodcrops forming a potential health risk to its consumers mainly in the third world (5). Although *Hevea brasiliensis* hydroxy-nitrile lyase is able to convert a broad range of aldehydes and ketones into cyanohydrins, the activity against bulky substrates was not satisfying. In 1999, DSM Fine Chemicals applied for a patent for S-hydroxy nitrile lyases with improved substrate acceptance deriving from *Hevea brasiliensis* and *Manihot esculenta* S-hydroxy nitrile lyases (6). They claimed a novel Hnl enzyme with less bulky amino acids replacing bulky amino acid residues in the hydrophobic channel leading to the active center. In case of *H. brasiliensis* Hnl, Trp128 with its aromatic ring constricts the active center. The patent particularly preferred mutants with alanine or phenylalanine replacing tryptophane 128. Therefore, *Hb* HNL W128A and W128F mutants, significating so-called tunnel mutants with improved accessibility of hydroxyl nitrile lyase for bulky substrates like m-phenoxybenzaldehyde-cyanohydrine, became interesting. Cloning and expression in *Pichia pastoris* using a conventional expression vector like pHILD2 was successful for both mutants, W128F and W128A, but the expression level was not as high as

with the GS115pHILD-HbHNL1.17 strain (E. Steiner, unpublished data). That was the reason for us to use the W128F mutant as the model protein for the construction of our new expression system. In this study we present the identification of a special head-to-head promoter situation, which is proposed to be responsible for the high-yield intracellular expression of a hydroxy nitrile lyase. Analysis of our head-to-head promoter fragment by Matinspector software showed no formation of novel transcription factor binding sites, but the loss or multiplication of possible positive or negative acting factor binding sites, e.g. Adr1 or some of the claimed Mxr1 response elements (7). We also hypothesize that the integration of the expression cassette next to another strong promoter might also enhance the transcription of the integrated one, which makes sense considering chromosome packaging and chromatin remodelling and the effect on the accessibility of the DNA for regulatory elements. First pinpointed by Schadt *et al.*, expression quantitative trait loci (eQTL) “hot spots”, i.e., transcription activity hot spots, defined as chromosome regions that contain more eQTL, that would have been detected by chance, have been points of research interest in almost all studies that search for genetic regulators for gene expression (8). Hot spots of gene regulation are most prominent in yeast (9; 10), where 8 have been detected, which were functionally subsumed under e.g. mating or mitochondrial. Nevertheless, the construction of new AOX1 head-to-head promoter expression vectors carrying HNL W128F mutant as model protein and some *Pichia pastoris* transformants thereof were able to reach expression and activity levels comparable to the *Hb* HNL expression level described by Hasslacher *et al.* in 1997.

MATERIALS AND METHODS

Strains and Materials

E. coli and *P. pastoris* strains used in this study are listed in Table 1. *E. coli* media components were purchased from Roth, Lactan (Graz, Austria), *Pichia* media components were purchased from BD Biosciences (Becton Dickinson Austria GmbH, Vienna, Austria). RNase A, Proteinase K and Tetra base [Di(4-dimethyl-aminophenyl)methane] (98%) was purchased from Sigma Aldrich Handels-GmbH (Vienna, Austria). NuPage 10 % Bis/Tris gels, 4 x LDS loading dye, MES buffer, SeeBlue Plus2 marker, Zeocin and the plasmids pGAPZ_alphaA, pGAPZ A, pPIC9, pHILD2 and pSE420 were purchased from Invitrogen (Life Tech Austria, Vienna, Austria). dNTPs and Pfu Taq Polymerase were ordered from Fermentas GmbH, St. Leon-Rot, Germany. HotStar Taq polymerase was ordered from Qiagen, Hilden, Germany. Y-PER was purchased from Fisher Scientific Austria GmbH (Vienna, Austria). Copper-(II) ethyl-acetoacetat was purchased from ABCR (Karlsruhe, Deutschland). meta-Phenoxybenzaldehydecyanohydrine and Mandelonitrile were a kind gift from DSM fine chemicals Austria. Mandelonitrile contained less than 0.5 % benzaldehyde. T4 DNA Ligase and the plasmid pGEM5Zf+ were purchased from Promega GmbH (Mannheim, Germany). 96 well PCR plates were ordered from Biozym (Hessisch Oldendorf, Germany). DIG labeling and detection kit was ordered from Roche Diagnostics GmbH (Vienna, Austria). Ederol filter paper was purchased from Binzer&Munktell (Bärenstein, Germany), Whatman filter paper was purchased from VWR International GmbH, Vienna, Austria. UV-Star microtiter plates were purchased from Greiner Bio-One GmbH, Kremsmünster, Austria. BioTrace NT membranes (pore size 0.45 μm) were purchased from PALL Austria GmbH, Vienna, Austria. All other chemical reagents used in this study were purchased from Roth / Lactan (Graz, Austria).

Media and cultivation conditions

P. pastoris cells were cultivated either in BYPD medium (1 % yeast extract, 2 % peptone, 2 % dextrose, 0.2 M potassium phosphate buffer pH 6 or 7), ½ BYPD (0.5 % yeast extract, 1 % peptone, 1 % dextrose, 0.2 M potassium phosphate buffer pH 7) or buffered minimal medium (1.34 % yeast nitrogen base without amino acids, 4 x 10^{-5} % biotin, 0.2 M potassium phosphate buffer pH 6 or 7) supplemented with either 2 % dextrose (BMD) or methanol (BMM 10 %, 2 % , 1 % or 0.5 %). For *P. pastoris* transformation cells were plated onto YPDS (0.5 % yeast extract, 1 % peptone, 1 % dextrose, 1 M Sorbitol) plates supplemented with a final concentration of 100 µg/ml zeocin. All growth of *Pichia pastoris* strains was done at 28° C. Transformations of *P. pastoris* were done according to the electro transformation method (11) or the condensed protocol (12). *Escherichia coli* cells were cultured in LB medium (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl) or SOC medium (2 % bactotryptone, 0.5 % yeast extract, 0.05 % NaCl, 0.2 % $MgCl_2$, 0.018 % KCl, 0.246 % $MgSO_4$, 0.346 % glucose) at 37° C for use with recombinant DNA techniques. Antibiotics were added to LB medium at the following concentrations: zeocin, 25 µg/ml; ampicillin, 100 µg/ml for plasmid selection. Transformation and other standard recombinant DNA techniques used in this study were performed as described previously (13).

Induction on carbon sources

For induction studies of *P. pastoris* strains in deep well plates (liquid media), cells were first grown 24 hours in ½ (B)YPD medium to stationary phase. 2 % BMM medium was added for a final concentration of 1 % methanol. Further induction to a final concentration of 0.5 % or 1 % methanol was done twice daily. Cells were grown with vigorous shaking at 28 °C and 80 % humidity. Cells were harvested after 24 to 72 hours.

Yeast chromosomal DNA isolation.

Cells from overnight cultures were pelleted by centrifugation and washed with sterile water. Cells were disrupted by grinding with liquid nitrogen. 1 g of cell powder was suspended with 7 ml of extraction buffer (10 mM Tris/HCl, 0.1 M EDTA, 0.5 % SDS, pH 8.0, 1 mg RNAse A) and

incubated at 37 °C. After Proteinase K addition, samples were incubated at 50 °C. Phenol / Chloroform (1:1) extraction was done three times and remaining phenol was removed by extracting once with Chloroform / Isoamylalcohol (24:1), followed by ethanol precipitation. DNA was dissolved in ddH_2O.

Southern Blotting

For the analysis of *P. pastoris* GS115 pHILD1.17-HNL, GS115 pHILD-HNL-W128F and GS115 strain, chromosomal DNA was fully digested with the following restriction enzymes: *Hin*dIII, *Eco*RI, *Eco*RV, *Pvu*II and *Nco*I. Probes were PCR labeled according to the DIG DNA labeling and detection instruction manual from Roche (14). The following PCR primers were used for labeled probe amplification: $pSE420_{for}$ for HNL probe 5´ CGCACTCGACCGGAATTATC 3´, $pSE420_{rev}$ for HNL probe 5´ CACTTCTGAGTTCGGCATGG 3´, 5'$AOX1_{for}$1 for 5'AOX1 probe 5´ GATCTAACATCCAAAGACGAAAG 3´ and 5'$AOX1_{rev}$1 for 5'AOX1 probe 5´ GATCTTCTCAAGTTGTCG 3´, "for" indicating forward and "rev" indicating reverse. Standard PCR conditions for the HNL-probe: 95° C 15´/ 94°C 1´, 60°C 1´, 72°C 1´/ 72°C 7´, 4°C ∞; 35 cycles. Standard PCR conditions for 5´AOX1 probe: 95° C 15´/ 94°C 1´, 50°C 1´, 72°C 1´/ 72°C 7´, 4°C ∞; 35 cycles. Southern blotting was performed as described elsewhere (15), pre-hybridization and hybridization were done at 65° C. For detection, anti-DIG-alkaline phosphatase and BCIP/NBT were applied.

PCR amplification of 5´Aox1 region and sequencing of the *Nco*I 1.4 kb fragment

Mixed plasmid DNA of the *Nco*I gene libraries was used as a template. The following primers were used for amplification: $M13_{For}$5´GTAAAACGACGGCCAG3´, $M13_{Rev}$5´ CAGGAAACAGCTATGAC3´, 5'$AOX1_{rev}$1 5´GATCTTCTCAAGTTGTCG3´, 5'$AOX1_{rev}$2 5´ GAGAATGGACCTGTGGATGT3´. PCR conditions: 95°C 15´/94°C 1´ 55°C 1´ 72°C 1´/72°C 7´, 4°C ∞. PCR fragments were subjected to sequencing. For the sequencing of the pGEM5Zf(+) cloned 1.4 kb *Nco*I fragment and the subcloned *Sac*I fragment containing the head-to-head promoter the following primers were used: 5'$AOX1_{rev}$1 5´GATCTTCTCAAGTTGTCG 3´,

5’AOX1$_{rev}$2 5´GAGAATGGACCTGTGGATGT 3´, 5’AOX1$_{rev}$3 5´ GTGTTGAGGAGAAGAGGAGT 3´, 5’AOX1$_{rev}$4 5´ GACCAACTGGCCGTTAGCAT 3´.

P. pastoris head-to-head AOX1 promoter strain construction

pPIC9 vector was digested with *Bgl*II and the 5.6 kb band carrying HIS4 auxotrophy marker was gel purified using Qiagen Gel Extraction Kit. pGAPZalphaA vector was digested with *Bgl*II and *Bam*HI and the 1.9 kb band comprising ColE1 origin of replication was gel purified, dephosphorylated and heat inactivated. Fragments were ligated using T4 DNA Ligase. Chemo-competent *E.coli* XL1 Blue cells were transformed with the ligation mix using SEM transformation protocol. Cells were spread on LB zeocin plates. Colonies were picked and streaked for plasmid DNA isolation using Qiaprep spin miniprep Kit. Plasmid DNA was digested with *Nco*I or *Nsi*I, respectively, for confirmation and insert orientation. Positive clone was named phhBasic. pSE420 and pGEM5Zf(+)1.4kb hhaox1 plasmids were digested with *Nco*I and *Sac*I (Fermentas). 4.3 kb fragment from pSE420 and 0.75 kb fragment from pGEM5Zf(+)1.4kb hhaox1 were gel purified via Qiagen Gel Extraction Kit and ligated using T4 DNA ligase. Ligation mix was transformed into chemo-competent *E.coli* XL1 Blue cells via SEM transformation. Cells were spread on LB ampicillin plates. Colonies were picked and streaked for plasmid DNA isolation using Qiaprep spin miniprep Kit. Plasmid DNA was digested with *Eco*RV/*Eco*O109I and *Nco*I/*Sac*I, respectively, for insert verification. Positive clones each with one half of the head-to-head promoter fragment. Cloning of the head-to-head promoter fragment into phhBasic vector was done by PCR amplification of both halves from pSE420 subclones using the following primers: pSE420hhaox1forBglII 5´CACAGATCT-CCTCGTTTCGAATAATTAGTTG 3´, pSE420hhaox1forEcoRI 5´CAGAATTC-CCTCGTTTCGAATAATTAGTTG 3´ and pSE420rev(SacI) 5´ACCGCTTCTGCGTTCTGATT 3´. phhBasic was restricted with *Bgl*II and *Eco*RI, PCR fragments were restricted with *Bgl*II/*Sac*I or *Eco*RI/*Sac*I, respectively. Fragments were gel purified and joined by three-point-ligation using T4 DNA ligase. Chemo-competent *E.coli* XL1 Blue cells were transformed with the ligation mix using SEM transformation protocol. Cells were spread on LB zeocin plates. Colonies were picked and streaked for plasmid DNA isolation using Qiaprep spin miniprep Kit. Plasmid DNA was digested with 1) *Sac*I/*Ssp*I, 2) *Eco*O109I, 3) *Sfu*I and 4) *Bgl*I for confirmation (data not shown). Positive clones were named phhAOX915 and phhAOX561.

pHILD2 vector was used as PCR template for the amplification of the two truncated promoter fragments corresponding to the particular halves of the head-to-head promoter. The following primers were used: 5´aox1longBglIIfor 5´GCCAGATCT-ATGAAACCTTTTTGCCATCCGAC 3´, 5´aox1kurzBglIIfor 5´GCCAGATCT-GTTCCCAAATGGCCCAAAACTG 3´, 5´aox1EcoRIrev 5´GAATTCCTCGTTTCGAATAATTAG 3´. PCR products were digested with *Bgl*II and *Eco*RI, gel purified and the two fragments ("short" meaning P_{Aox1}Δ383bp and "long" meaning P_{Aox1}Δ29bp) were ligated with *Bgl*II/*Sac*I opened phhBasic vector. Chemo-competent *E.coli* XL1 Blue cells were transformed with the ligation mix using SEM transformation protocol. Cells were spread on LB zeocin plates. Colonies were picked and streaked for plasmid DNA isolation using Qiaprep spin miniprep Kit. Plasmid DNA was digested with 1) *Bgl*I, 2) *Eco*RV and 3) *Nco*I for confirmation (data not shown). Positive clones were named pAOXshort (P_{Aox1}Δ381 bp) and pAOXlong (P_{Aox1}Δ28 bp). For insertion of *Hb*HNL W128F gene, the four basic plasmids phhAOX915, phhAOX561, pAOXshort and pAOXlong were restricted with *Eco*RI and dephosphorylated. *Eco*RI cut and gel purified PCR product (*Hb*HNL W128F DNA) and the four purified basic plasmids were ligated via T4 DNA ligase, transformed into chemo-competent *E.coli* XL1 Blue cells and cells were spread on LB zeocin plates. Colonies were picked and streaked for plasmid DNA isolation using Qiaprep spin miniprep Kit. Plasmid DNA was digested with 1) *Nco*I and 2) *Ssp*I for confirmation (data not shown). The four resulting plasmids were named phhAOX915_*Hb*HNL W128F, phhAOX561_*Hb*HNL W128F, pAOXshort_*Hb*HNL W128F and pAOXlong_*Hb*HNL W128F. Plasmids were sequenced for verification. *Pichia pastoris* transformation was done according to the electro-transformation protocol (11). Cells were spread on YPDS zeocin plates and incubated for 2-3 days at 30° C.

in situ HNL activity assay

This assay is based on the Prussian-Blue reaction described by Feigl and Anger (16). The detection paper is prepared by soaking Ederolfilter disks (No. 14, ∅90 mm, Binzer&Munktell) or Whatman filter paper with a chloroform solution, containing 1 % (w/v) copper-(II) ethyl-acetoacetate and 1 % (w/v) tetra base [Di(4-dimethyl-aminophenyl)methane] (98 %). Dried, almost colourless test-paper can be stored in a dark and dry place for two weeks. For preparation of the substrate solutions 20 µl racemic

mandelonitrile was diluted in 10 ml citrate-phosphate-buffer (pH 6.5, 0.03 mM) or 20 µl meta-Phenoxybenzaldehydecyanohydrine (m-PBCH) was diluted in 3 ml dimethylformamide to overcome solubility problems prior to the addition of 7 ml citrate-phosphate-buffer (pH 6.5, 0.03 mM). Purified racemic mandelonitrile and m-Phenoxybenzaldehydecyanohydrine were a gift from DSM fine chemicals Austria and contained less than 0.5 % benzaldehyde. For determination of Hnl activity, *P. pastoris* strains transformed with the different HNL constructs were precultured in YPD medium in deep well plates. After 24 hours, cultures were diluted 1:100 with 0.9 % NaCl, spotted onto nitrocellulose BioTraceNT membranes (pore size 0.2 µm) lying on BMD plates, which were incubated for 24 hours at 28° C. Filters were transferred to 1 % BMM plates and incubated for 18 or 24 hours of induction. For 42 or 48 hours of induction, filters were laid onto fresh BMM plates after 24 hours. In case of crude lysates, 3 µl of each preparation were spotted onto Ederol filter disks. The membranes with colonies were pre-incubated for 25 minutes on a moisture filter paper soaked with a citrate-phosphate-buffer with the pH of interest and molarities varying between 0.5 M and 5 M. Membranes were placed on a filter paper soaked with substrate solution with colonies upside down. A nylon tissue was placed above and covered with the detection paper which was fixed with a Petri dish top and was not allowed to move during the developing time, until blue spots emerged on the detection paper (17).

Pichia cell disruption for photometric HNL activity assay

Deep well cultures were centrifuged for 10 minutes at 4000 rpm and 4 °C in an Eppendorf 5810R centrifuge. Supernatants were abolished. 15 µl of Y-PER reagent were added per well and pellets were incubated for 30 minutes softly shaking at 200 rpm. Suspensions were transferred to 96 well PCR plates and centrifuged for 40 minutes at 4000 rpm and room temperature. Subsequently, 20 µl of the supernatant were taken for HNL platereader activity measurement.

Photometric measurement of HNL activity

Hydroxy-nitrile lyase activity was measured at pH 5.0 by following the cleavage of racemic mandelonitrile into benzaldehyde and HCN at 25 °C. The formation of benzaldehyde was measured spectrophotometrically at 280 nm. At this wave length the absorption of mandelonitrile can be neglected, while this is not possible at the maximum of 250 nm. To 50 mM pH 5.0 potassium phosphate/citrate buffer (130 µl) in a UV microtiter plate, 20µl of the crude enzyme preparation was added. 50µl of a 60 mM mandelonitrile solution (in a 3 mM pH 3.5 potassium phosphate/citrate buffer) was added to start the reaction. Kinetics over 5 minutes were done.

SDS-PAGE

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was applied for separation of denatured proteins (18). NuPAGE 10 % Bis-tris gels were used. 3 µl from each crude lysate were loaded onto the gel. Samples were prepared according to the NuPAGE technical guide (19), using Invitrogen 4 x LDS sample buffer. 10 µl of SeeBluePlus2 were loaded as protein standard. Gels were run in 1 x MES buffer (50 mM MES, 50 mM Tris base, 0.1 % SDS, 1 mM EDTA, pH 7.3) at a constant voltage of 200 V and stained with Coomassie blue R-250. A destaining solution of 10 % acetic acid was used.

Miscellaneous methods

Recombinant DNA methods were performed essentially as described previously by Sambrook *et al.* (13). Plasmid-DNA digested with restriction enzymes and used for restriction mapping and cloning of fragments, hybridization probes, PCR products were separated on Tris-acetate-EDTA agarose gels, DNA fragments were purified from agarose gels using Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany) or Wizard SV Gel and PCR Clean-up Kit (Promega, Mannheim, Germany). Restriction enzymes were purchased from New England Biolabs GmbH (Frankfurt am Main, Germany) or Thermo Fisher Scientific Inc. (Fermentas GmbH, St. Leon-Rot, Germany). Sequencing was carried out at the Institute of Molecular Biotechnology, Graz University of Technology, Austria, according to the dideoxy chain termination procedure (Sanger *et al.*, 1977) using an ABI 373 DNA sequencer, the Dye Deoxy

Terminator Sequencing Kit (Applied Biosystems Inc., Faster, CA, USA) and Amply*Taq*DNA polymerase (Perkin-Elmer Corporation, Norwalk, USA). Gene-specific primers were synthesized by ABI 392 DNA/RNA synthesizer (Institute of Molecular Biotechnology, Graz University of Technology, Austria) and by Invitrogen Life tech, Vienna, Austria. DNA alignments were done with Contig Xpress (VNTI, Invitrogen) or SeqMan (DNAStar Inc., Madison, WI, USA).

RESULTS AND DISCUSSION

Southern Blot analysis of high level expression strain *P. pastoris* strain GS115 pHILD1.17-HNL.

In 1997, Hasslacher *et al.* reported about a high level HNL expressing strain, reaching a volume yield of HNL protein of 22g/l culture volume in high cell density fermentation (4). The strain resulted from a spheroplast transformation of *P. pastoris* GS115 strain with pHILD2-HNL plasmid, targeting *AOX1* locus for integration and replacement of *aox1* gene. First attempts to utilize the ability of high level expression also for heterologous expression of other recombinant proteins by replacement of the Hnl expression cassette in the strain *P. pastoris* pHILD1.17-HNL failed (B. Krammer, unpublished data). Interpretation of preliminary southern blot experiments (M. Tschemmernegg, unpublished data) suggest, that pHILD2-HNL expression cassette integrated once into the *AOX1* locus by *aox1* gene replacement as it was targeted to. Moreover, a deletion of about 7kb upstream of P_{Aox1} was assumed to be a likely reason for the enormously increased intracellular HNL expression level (H. Schwab, personal communication). Although *P. pastoris* GS115 was transformed with *Not*I digested plasmid DNA, creating 5´and 3´ homologous ends to *AOX1* locus and theoretically favoring single-copy integration – dependent from the amount of plasmid DNA in the transformation reaction – a multi-copy integration event became more likely. Southern blot procedure was optimized for clearer results to elucidate the copy number and the locus of integration of either the whole plasmid or the HNL expression cassette, respectively. Three different strains were applied to southern blotting: first of all *P. pastoris* GS115 pHILD1.17-HNL, second *P. pastoris* GS115, which was transformed with pHILD2-HNL plasmid for the construction of GS115 pHILD1.17-HNL, third, *P. pastoris* GS115 pHILD-HNL-W128F, expression strain for *Hb*_Hnl_W128F mutant. $Mut^{S}HIS^{+}Hnl^{+}$ phenotype probably resulted from single copy integration via gene replacement at the *AOX1* locus. Genomic DNAs of all the strains were digested with different restriction enzymes (see Materials and Methods for details) and Southern hybridization was performed using two different DNA probes, a HNL probe and a 5'AOX1 probe. The restriction fragment patterns obtainable in Southern analysis for each of the integration events are summarized in Table 2 and 3. The blots detected with the 5'AOX1 or Hnl specific probe respectively are shown in Fig. 1 and Fig. 2. In addition to

the predicted single fragments of *Hb_hnl* expression cassette as released by digestion with *Eco*RI, *Eco*RV or *Pvu*II, the Southern blot revealed the presence of additional fragments. Some of these additional fragments do not appear to be due to partial digestion of chromosomal DNA, but to multiple integration of the *Hb_hnl* expression cassette into the *P. pastoris* genome. Southern blot analysis revealed that *P. pastoris* GS115 pHILD-HNL-W128F has one expression cassette integrated at the *AOX1* locus. The detected fragments for the respective restriction enzymes and DNA probes agree with the obtainable restriction fragment patterns in the case of a single integration of the *Hb*HNL expression cassette at the *AOX1* locus. Additionally, the fragment sizes detectable with the *AOX1* probe correspond to the fragment sizes of chromosomal DNA of the untransformed strain *P. pastoris* GS115, detectable with the same probe incorporating differences in restriction sites within the *aox1* and the *hnl* coding region. For *P. pastoris* GS115 pHILD1.17-HNL a head-to-tail tandem repeat integration could be clearly ruled out, because this would result in a 6.1 kb fragment detectable with the *hnl* and the *AOX1* probe if chromosomal DNA is cut with *Eco*RI. Integration of the whole uncut vector is also not likely, because this would result in a fragment larger than 7.4 kb thus easily detectable with the *AOX1* probe if the chromosomal DNA is digested with *Eco*RI. The Southern blot data also clearly show that an integration of the expression cassette couldn't have occurred at the *HIS4* locus, which should result in a distinct 2.9 kb *Nco*I and a 2.3 kb *Eco*RV fragment detectable with the *AOX1* probe (Table 3). Another interesting fact found with this technique was that the pHILD2-HNL derived expression cassette has integrated more than once. One integration event occurred by replacement of the *AOX1* gene by correct double cross over at the homologous *5'AOX1* and 3*'AOX1* regions. Some of the additional fragments seen on Southern blots appear to be due to partial digestion of chromosomal DNA, e.g. the 7.9 kb *PvuII* fragments by detection with the hnl specific probe. But some small and faint bands that could be detected with the *5'AOX1* probe still could initially not be explained. Further integrations could have occurred next to the *AOX1 locus*, or somewhere else in the genome by non-homologous recombination. By digesting the chromosomal DNA with *NcoI* and hybridisation with the *AOX1* probe a second specific band of 1.4 kb could be detected. We hypothesized that this fragment most probably consists of the *AOX1* promoter fragment with a size of ca. 0.9 kb and an unknown chromosomal *Pichia pastoris* sequence part of 0.5 kb upstream of the

promoter fragment. To get more information about the sequence of this specific 1.4 kb fragment attempts were made to isolate and further characterize it.

Identification, isolation and sequencing of P_{Aox1} head-to-head promoter fragment.

A gene library was made containing the AOX1 promoter fragments which was screened by colony blots with a 5´AOX1 probe (see Supplementary data for details). 4 clones could be isolated and sequenced, but astonishingly they contained only 5´AOX1 sequence. In parallel, a PCR approach was followed. The isolated plasmid pool of the *E.coli* Top10F´ pGEM5Zf(+)1.17_1.4 kb gene library was used as template. PCR amplifications with either plasmid specific or 5´AOX1 primers were performed (Materials and Methods), but the results were confusing. Although distinct bands showed up on the agarose gel, the sizes of the fragments were smaller than expected (data not shown). A combination of PCR fragment sequencing and sequencing of the positive clones from the colony blots led to the assumption that the *Nco*I 1.4 kb fragment comprises two identical, but truncated 5´AOX1 regions positioned in a head-to-head manner. Restriction mapping with three restriction enzymes *SacI, BstXI* and *Bpu1102I* (data not shown) showed that the region around one of the two *Sac*I sites in the 1.4kb fragment was affected by the approximately 400bp deletion (Fig. 3). Due to the inverted repeat structure, sequencing of the head-to-head promoter fragment was challenging. To overcome the problem, subcloning experiments were undertaken to finally end up with the correct sequence of the 1.4 kb 5´*AOX1* promoter fragment (Fig. 4). This particular head-to-head situation gave reason to speculations about possible enhancing effects towards transcription ascribed to a loss of repressor binding sites or the creation of additional binding sites for an activator. An *in silico* analysis of the head-to-head promoter fragment was undertaken, analysing putative transcription factor binding sites of the rearranged head-to-head fragment using Matinspector software. One copy of the *AOX1* promoter has just a small deletion of 28 base pairs at the very 5´ end of the promoter, which should not influence expression level as no potential TF binding sites are affected. In case of the shorter fragment lacking 381 base pairs, some of the predicted TF binding sites are eliminated, amongst others Adr1p binding site, four of the six putative Mxr1 response elements containing the Mxr1p binding sites (7) and also Region D which was described to be a positive *cis*-acting element leading to a severe decrease in AOX1 promoter

activity upon deletion (20). No potential new binding sites were created at the fusion region according to Matinspector prediction (Fig. 5). The next step was now to create vectors and transform them into *Pichia pastoris* to possibly reconstitute the high level expression of 1.17 strain.

Analysis of HNL activity level in head-to-head promoter *Hb*HNL W128F constructs.

HNL W128F vectors with the head-to-head promoters were constructed as described in detail in Materials and Methods. Transformation in *P. pastoris* was followed by deep well fermentation under methanol induction conditions and cell disruption by Y-PER reagent. Crude lysates were used for plate reader activity assays, filter activity assays and SDS PAGE. 88 transformants per construct were analyzed. GS115pHILD2-HbHNL1.17 and GS115pHILD2-*Hb*HNLW128F strains were used as controls. Best results were obtained with phhAOX915-*Hb*HNL W128F construct transformed into the wild type strain *P. pastoris* GS115 or *P. pastoris* GS115pHILD2-*Hb*HNL W128F harbouring already a single copy of HbHNL W128F in its genome (data not shown). 3 clones, named phhAOX915_HbHNL W128F-C11, -D3 and -D12, showed HNL activity towards mandelonitrile that was comparable to the activity detected with *P. pastoris* GS115pHILD2-HbHNL1.17. Recent results from our lab (data not shown) show that depending from the amount of DNA used for *Pichia* transformation, it is possible to strictly conduct the transformation event towards single or multi copy integration. This has not been considered in this study. Transformations were done using 2 µg of restricted DNA which probably already favoured multi copy integrations relying on our results. But as the main goal was to reach Hnl expression level of 1.17 strain, copy number of the 2 high level expression strains was not determined and primarily not interesting. SDS-PAGE was performed to visualize HNL expression on protein level (Fig. 6 A and B). Therefore, crude lysates of the most promising clones as determined by platereader activity assay were loaded onto 10 % NuPAGE gels together with crude lysate preparations of control strains *P. pastoris* GS115pHILD2-*Hb*HNL1.17 and GS115pHILD2-*Hb*HNLW128F. For the verification of W128F mutation respectively HNL W128F expression, the crude lysates of the most promising clones were subjected to HNL filter activity assay using the bulky substrate m-phenoxybenzaldehyde-cyanohydrine (Fig. 7). This substrate is only well converted by the HNLW128F mutant, whereas the wild type HNL shows much less activity towards m-PBCH.

Results of the filter assay give a clear indication, that real W128F mutants could be isolated from the upper described screening assays. Clones D12, D3 and C11 were sequenced and HNLW128F mutation could be finally confirmed in each strain.

CONCLUSION

In this study, we were able to unravel the secret of the high level expression of the GS115pHILD2-HbHNL1.17 strain. Southern Blot experiments showed the existence of an additional 1.4 kb AOX1 fragment which later on turned out to be 2 AOX1 promoters which fused probably because of an unusual recombination event caused by spheroplast transformation and the presence of an inverted repeat structure in the AOX1 promoter. Sequencing of this newly detected promoter fragment showed, additionally to a complete HNL expression cassette, a head-to-head arrangement of 2 copies of the original AOX1 promoter both lacking 28 bp or 381 bp at the N-terminal end, respectively, each driving a copy of HNL. Cloning of this head-to-head promoter fragment after one copy of a mutant HNL gene was able to reconstitute the level of expression reached with the original 1.17 strain.

TABLES AND FIGURES

Table 1: Strains used in this study.

E. coli strains	Genotype	Reference
XL1 Blue	endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB$^+$ lacI^q Δ(lacZ)M15] hsdR17(r_K^- m_K^+)	Invitrogen Corporation Carlsbad, CA, USA
Top10 F´	F'[lacI^q Tn10(tet^R)] mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str^R) endA1 λ$^-$	Agilent Technologies, Inc. (former Stratagene) Santa Clara, CA, USA
P. pastoris strains	Genotype	Reference
GS115	His$^-$, Mut$^+$	Invitrogen Corp. (Life Technologies), Carlsbad, CA, USA
GS115 pHILD2-HNL W128F	Mut^S, His$^+$, Hnl$^+$	IMBT TU Graz
GS115 pHILD-HNL 1.17	Mut^S, His$^+$, Hnl$^+$	IMBT TU Graz
GS115 phhAox915-HNL W128F C11	Mut?, His$^+$, Hnl$^+$ (Mut phenotype not determined)	IMBT TU Graz
GS115 phhAox915-HNL W128F D3	Mut?, His$^+$, Hnl$^+$ (Mut phenotype not determined)	IMBT TU Graz
GS115 phhAox915-HNL W128F D12	Mut?, His$^+$, Hnl$^+$ (Mut phenotype not determined)	IMBT TU Graz

Table 2: Putative integration events in *Pichia pastoris* and calculated (a) as well as experimentally determined (b) fragment sizes of chromosomal DNA detected with the *HNL*-probe. bp: base pairs

	HindIII	***NcoI***	***PvuII***	***EcoRI***	***EcoRV***
(a)					
Single integration at AOX1 locus	1.201 bp	3.180 bp	> 1.241 bp + 718 bp	> 1.740 bp	> 2.244 bp
Head to head repeat	1.201 bp	3.180 bp	2.480 bp + 718 bp	3.482 bp	4486 bp
Head to tail repeat	1.201 bp	3.180 bp	> 1.241 bp +718 bp	6.117 bp + > 1.740 bp	2.292 bp + >2.244 bp
Single integration at HIS locus	1.205 bp	3.180 bp	2.462 bp + 718 bp	800 bp	2.304 bp
Whole vector integrated	1.205 bp	3.180 bp	5.342 bp + 718 bp	800 bp	5.184 bp
(b)					
GS115 pHILD1.17-HNL	~1.4 kb	~3.4 kb	~7.9 kb, 5.4 kb + 2.2 kb	~3 kb, 2.6 kb + 1.5 kb	~5.8 kb, 4.1 kb + 2.1 kb
GS115 pHILD2-HNL-W128F	~1.4 kb	~3.4 kb	~7.9 kb + 5.4 kb	~ 0.9 kb	~5.8 kb
GS115	-	-	-	-	-

Table 3: Putative integration events in *Pichia pastoris* and calculated (a) as well as experimentally determined (b) fragment sizes of chromosomal DNA detected with the *5'AOX1*-probe. bp: base pairs

	HindIII	***NcoI***	***PvuII***	***EcoRI***	***EcoRV***
(a)					
Single integration at AOX1 locus	> 873 bp	> 945 bp	> 1.241 bp	> 1.740 bp	> 2.244 bp
Head to head repeat	1.748 bp	1.892 bp	2.480 bp	3.482 bp	4.486 bp
Head to tail repeat	> 873 bp + 4.904 bp	> 945 bp + 2.937 bp	> 1.241 bp + 2.450 bp	> 1.740 bp + 6.117 bp	> 2.244 bp + 2.292 bp
Single integration at HIS locus	> 4.527 bp	2.949 bp	2.462 bp	> 4.589 bp	2.304 bp
Whole vector integrated	> 7.407 bp	5.829 bp	5.342 bp	> 7.478 bp	5.184 bp
(b)					
GS115 pHILD1.17-HNL	~ 1.2 kb	~ 10.9 kb + 1.4 kb	~ 10 kb, 6 kb + 2.1 kb	~ 3.1 kb, 2.6 kb + 1.4 kb	~ 6 kb, 4.1 kb + 1.9 kb
GS115 pHILD2-HNL-W128F	~ 1.2 kb	~ 10.9 kb	~ 10 kb, 6 kb	~ 1.8 kb	~ 6 kb
GS115	~ 1.2 kb	~ 10.9 kb	~ 12.5 kb, 10 kb + 8.9 kb	~ 6.3 kb	~ 4.3 kb

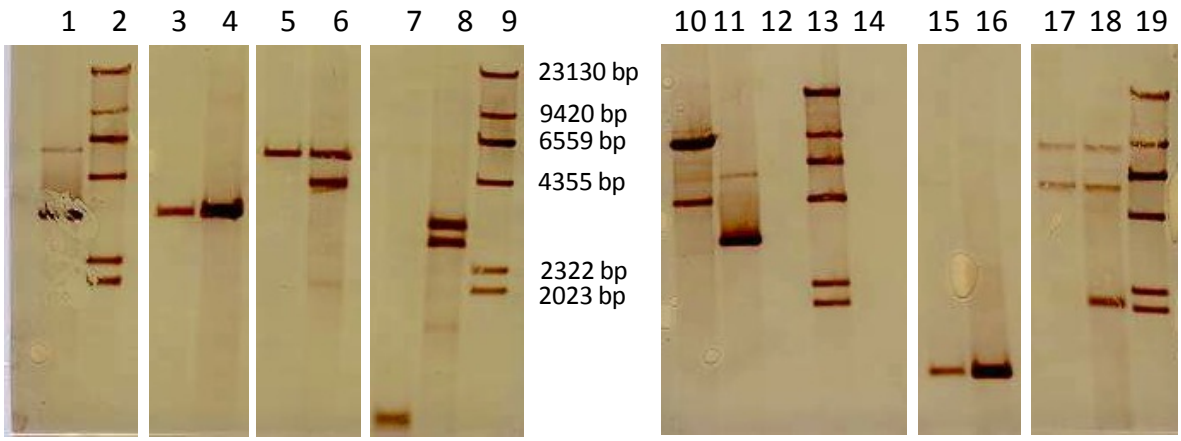

Figure 1: Southern blot analysis of genomic DNA from different *Pichia pastoris* strains and plasmid pHILD2-HNL W128F digested with either *Eco*RI, *Eco*RV, *Nco*I, *Hin*dIII or *Pvu*II. Detection was done with *hnl* specific probe. **1:** pHILD2-HNL-mut *Nco*I cut **2:** DIG labelled lambda *Hin*dIII standard, **3:** GS115 pHILD-HNL-W128F *Nco*I cut, **4:** GS115 pHILD1.17-HNL *Nco*I cut, **5:** GS115 pHILD-HNL-W128F *Eco*RV cut, **6:** GS115 pHILD1.17-HNL *Eco*RV cut, **7:** GS115 pHILD-HNL-W128F *Eco*RI cut, **8:** GS115 pHILD1.17-HNL *Hind*III cut, **9:** DIG labelled lambda *Hin*dIII standard, **10:** pHILD2-HNL-W128F *Eco*RI cut **11:** pHILD2-HNL-W128F *Nco*I cut **12:** empty lane, **13:** DIG labelled lambda *Hin*dIII standard, **14:** GS115 *Hin*dIII cut, **15:** GS115 pHILD-HNL-W128F *Hin*dIII cut, **16:** GS115 pHILD1.17-HNL *Hin*dIII cut, **17:** GS115 pHILD-HNL-W128F *Pvu*II cut, **18:**GS115 pHILD1.17-HNL *Pvu*II cut, **19:** DIG labelled lambda *Hin*dIII standard

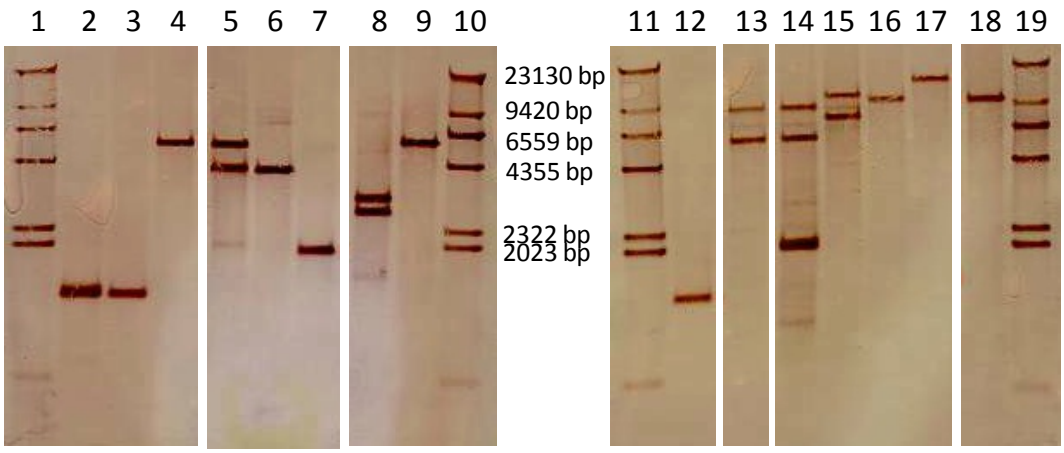

Figure 2: Southern blot analysis of genomic DNA from different *Pichia pastoris* strains digested with either *Eco*RI, *Eco*RV, *Nco*I, *Hin*dIII or *Pvu*II. Detection was done with *5'AOX1* specific probe. **1:** DIG labelled lambda *Hin*dIII standard **2:** GS115 pHILD1.17-HNL *Hin*dIII cut, **3:** GS115 *Hin*dIII cut, **4:** GS115 pHILD-HNL-W128F *Eco*RV cut, **5:** GS115 pHILD1.17-HNL *Eco*RV cut, **6:** GS115 *Eco*RV cut, **7:** GS115 pHILD-HNL-W128F *Eco*RI cut, **8:** GS115 pHILD1.17-HNL *Eco*RI cut, **9:** GS115 *Eco*RI cut, **10:** DIG labelled lambda *Hin*dIII standard **11:** DIG labelled lambda *Hin*dIII standard **12:***GS115* pHILD-HNL-W128F *Hin*dIII cut, **13:** GS115 pHILD-HNL-W128F *Pvu*II cut, **14:** GS115 pHILD1.17-HNL *Pvu*II cut, **15:** GS115 *Pvu*II cut, **16**: GS115 pHILD-HNL-W128F *Nco*I cut, **17:** GS115 pHILD1.17-HNL *Nco*I cut, **18:** GS115 *Nco*I cut, **19:** DIG labelled lambda *Hin*dIII standard

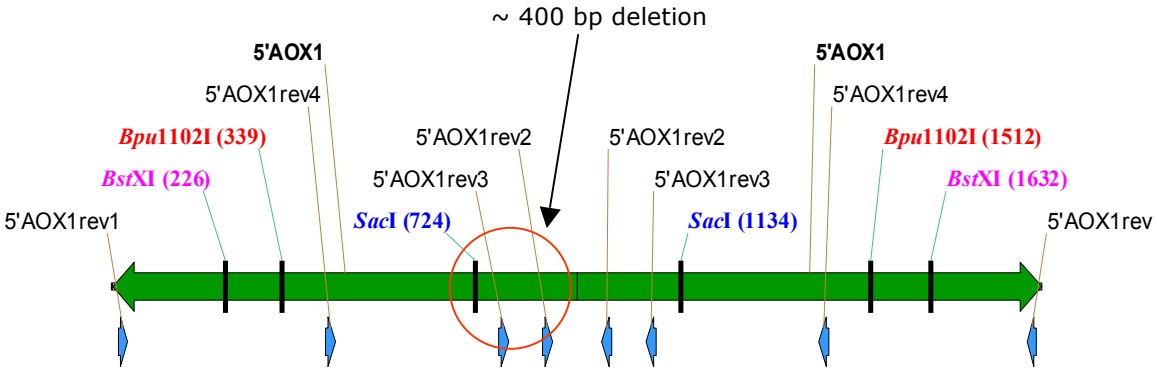

Figure 3: Restriction mapping with the enzymes *Sac*I, *Bst*XI and *Bpu*1102I revealed a deletion of about 400 bp affecting the second *Sac*I site in the 1.4 kb fragment. Blue arrows mark the primer binding sites of sequencing primers (see Materials and Methods for primer sequences).

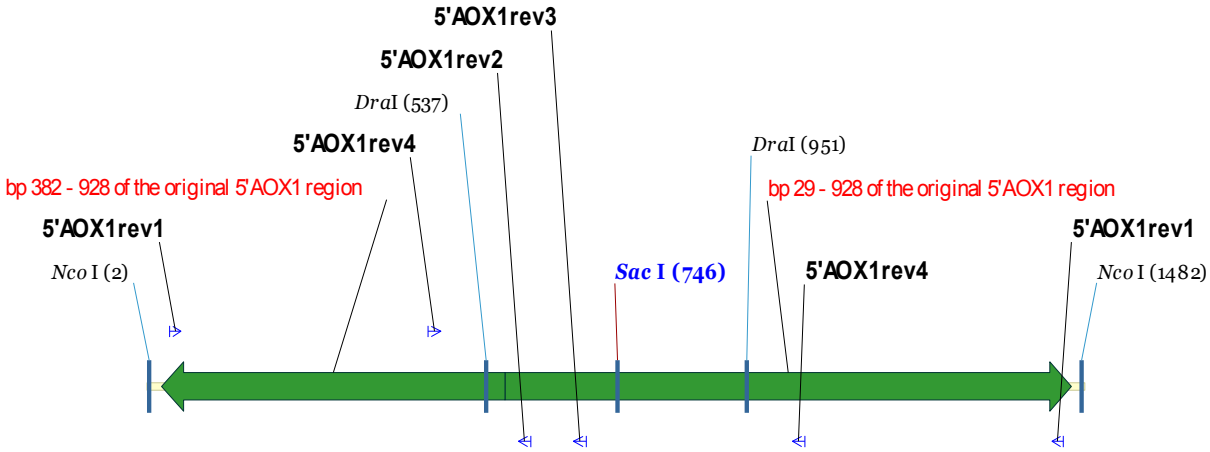

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Figure 4: Map of the GS115 pHILD1.17-HNL 1.4 kb head-to-head promoter fragment. A deletion of the first 381 bp of the one promoter fragment and the deletion of the first 28 bp of the other promoter fragment could be clearly figured out. Blue arrows mark the primer binding sites of sequencing primers (see Materials and Methods for sequencing primer sequences).

Fig. 5: Head-to-head P_{Aox1} analysis. See legend on next page for details.

Fig. 5: Analysis of the head-to-head AOX1 promoter fragment. Brown sequence: Δ 383 bp AOX1 promoter (P_{AOX1}) fragment; black sequence: Δ 29 bp AOX1 promoter fragment; blue sequence: 5´ untranslated (UTR) region; violet sequence: *Nco*I restriction site; magenta dashed boxes: inverted repeat structure that putatively led to a fusion promoter fragment consisting of two truncated copies of original P_{AOX1}; magenta highlighted regions: differences between the pHILD2 P_{AOX1} and *P.p.* GS115 or CBS 7534 P_{AOX1}; light orange boxes: Mxr1p response elements (MXRE) according to Kranthi *et al.* (7), the conserved motif 5´ CYCC/GGRG 3´ is shown in bold, light orange letters; black brackets: Region D; a putative positive *cis*-acting element identified by Xuan *et al.* (20); black broken lines and arrows: promoter deletions affecting P_{AOX1} function *in vivo* reported by Hartner *et al.* (21); red underlined and lettered regions mark the transcription factor binding sites determined by Matinspector ® software with the highest matrix similarity (1.000); dark green underlined and lettered regions mark the transcription factor binding sites determined by Matinspector ® software with a matrix similarity of < 0.975; light green underlined and lettered regions mark the transcription factor binding sites determined by Matinspector ® software with a matrix similarity of < 0.95; letters of conserved motifs are given in the respective color.

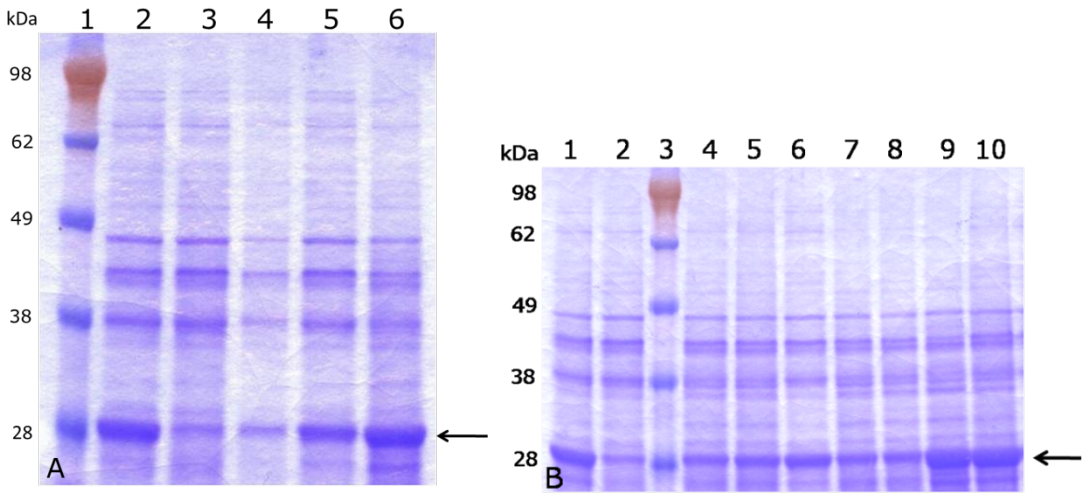

Figure 6: NuPAGE 10% BisTris PAGE with equal amounts of crude lysates applied. SDS PAGE was stained with Coomassie brilliant blue. HNL protein band (29 kDa) is marked with an arrow. **A:** GS115 phhAOX915- HNL W128F transformants. Lane 1: SeeBluePlus2 (Invitrogen) molecular weight standard; lane 2: *P. pastoris* GS115 [pHILD2-HNL1.17]; lane 3: *P. pastoris* GS115 [pHILD2-HNL-W128F] single copy; lane 4: *P. pastoris* GS115[phhAOX915-HNL W128F C11]; lane 5: *P. pastoris* GS115 [phhAOX915-HNL W128F D3]; lane 6: *P. pastoris* GS115[phhAOX915-HNL W128F D12]; **B:** GS115 pHILD2-HNL W128F[phhAOX915- HNL W128F] transformants. Lane 1: *P. pastoris* GS115 [pHILD2-HNL1.17]; lane 2: *P. pastoris* GS115 [pHILD2-HNL-W128F] single copy; lane 3: SeeBluePlus2 (Invitrogen) molecular weight standard; lane 4 to lane 10: different GS115 pHILD2-HNL W128F [phhAOX915- HNL W128F] transformants.

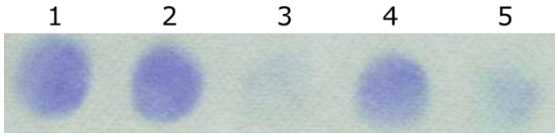

Figure 7: HNL activity filter assay for the confirmation of HNL W128F mutation. Equal amounts of crude lysates of the three best transformants and two control strains were spotted onto Ederol filter disk. mPBCH (meta-Phenoxybenzaldehyde cyanohydrine) was used as substrate. 1: *P. pastoris* GS115 phhAOX915-HNL W128F D3; 2: *P. pastoris* GS115 phhAOX915-HNL W128F D12; 3: *P. pastoris* GS115 phhAOX915-HNL W128F C11; 4: *P. pastoris* GS115 pHILD2-HNL-W128F; 5: *P. pastoris* GS115 pHILD2-HNL1.17

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Chapter 4

Identification of Genetic Elements Enhancing Methanol-free Expression from the AOX1 Promoter in *Pichia pastoris* Revealed a Putative Auto-regulatory Function of AOX1 Protein

My contribution to the paper draft is as follows:

- Conceptual formulation (50 %)
- Supervision of S. Kraßnig during Project Lab and project employment (100 %)
- Construction of the AOX1 mutant strains (25 %)
- Western Blotting for mutant AOX1 expression (10 %)
- Growth plate assays to test for the functionality of mutant AOX1 strains (30 %)
- Growth plate assays with formaldehyde and DHA (100 %)
- Levanase assays to test for the possible role of formaldehyde as inducer (100 %)
- Growth plate assays for AOX1 N616A mutant strain confirmation (100 %)
- Writing of the research article (50 %)

Identification of genetic elements enhancing methanol-free expression from the *AOX1* promoter in *Pichia pastoris* revealed a putative auto-regulatory function of *AOX1* gene

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ABSTRACT

Bacillus subtilis levanase was established as a reporter for a screening system in the methylotrophic yeast *Pichia pastoris* by placing it under the control of the methanol inducible *AOX1* promoter. A *Pichia pastoris* gene library was screened for genetic elements influencing expression driven by this promoter. An initial screening directly on transformation plates yielded several clones with enhanced levanase activity under derepressing conditions, i.e. glucose and glycerol deprivation. Adaptation to a 96-well format and kinetic measurements confirmed increased expression in the identified transformants. Thus, the developed system allowed finding clones that showed enhanced, methanol-free reporter expression. Characterization, re-transformation and mutagenesis experiments demonstrated that integration of a complete and functional *AOX1* gene was responsible for enhanced levanase expression under derepressing conditions.

INTRODUCTION

Over the last decades, the methylotrophic yeast *Pichia pastoris* has become an important host for heterologous protein expression. One of the key features of the *Pichia* expression system is the promoter of the *AOX1* gene encoding an alcohol oxidase. This enzyme catalyzes the first reaction in the methanol utilization pathway, the oxidation of methanol to formaldehyde and hydrogen peroxide (1). For the majority of heterologous proteins expressed in *P. pastoris* the *AOX1* promoter is used as it is highly inducible by methanol, but tightly repressed if glucose or glycerol are present (2). Under derepressing conditions *AOX1* transcriptional activity corresponds to about only 2% as compared to inducing conditions. Methanol-grown cells, on the other hand, show a 1000-fold higher transcriptional level than repressed ones (3). Therefore, regulated expression driven by the *AOX1* promoter is possible. Up to now little is known how methanol regulates the *AOX1* promoter. One transcription factor, Mxr1, has been found, which has a binding site in this promoter region. It has been shown that Mxr1p localizes to the nucleus upon methanol induction. In cells lacking functional *MXR1,* methanol induction of the *AOX1* promoter does not work (4). *mxr1* cells were initially identified in a screen for peroxisomal biogenesis-defective mutants (5). The exact activation mechanism is still unknown (4).

Hartner *et al.* identified 7 regions in the P_{AOX1} where promoter activity was reduced to less than 50 % when smaller or larger parts thereof where removed designating potential transcription factor (TF) binding sites or *cis*-acting elements. They further postulate that Mxr1p additionally to its P_{AOX1} binding controls minimum one more TF protein associated with P_{AOX1} regulation (6). In 2009, Kranthi *et al.* determined 6 binding sites for Mxr1p in the P_{AOX1}. Their interpretation was that the elevated expression from the highly methanol inducible P_{AOX1} is founded by a synergistic effect resulting from multiple Mxr1p molecules bound to the Mxr1p response elements (MXREs) (7). The same group also detected Mxr1p binding sites in DHAS and PEX8 promoters and extended the Mxr1 core binding motif by the addition of indispensable nucleotides to it (5´ CYCCNY 3´). Putative Mxr1p binding sites were also determined in FLD1 and PEX14 promoters by *in silico* analysis (8). Recently, a 14-3-3 family protein was detected in *Pichia pastoris* regulating Mxr1p activity contingent on the carbon source used for cultivation. Two mechanisms of Mxr1p regulation are hypothesized: an inhibition by the supplied carbon source mediated by 14-3-3 and an activation which is somehow triggered by methanol induction (9). In *Candida boidinii* two proteins were identified showing high homology to *P. pastoris* Mxr1p and *S. cerevisiae* Adr1p. Trm1p on the one hand is related to methanol induced activation of genes but doesn't seem to be connected to peroxisomal assembly or proliferation (10).

It has a unique feature, a glutamine-rich region (Q-rich region) putatively acting as a transcriptional activation domain, which has also been found in Mxr1p. Trm2p, in contrast seems to be the regulatory feature responsible for derepression in *Candida*. In 2010, Sasano *et al.* suggested the following mechanism: methanol-induced gene expression is totally repressed by growth on glucose. When glucose is used up or methanol induction takes place Trm2p causes glucose repression thereby activating Trm1p which in turn activates methanol-specific genes (11). There is a second gene encoding an alcohol oxidase in *Pichia pastoris*, *AOX2*. *AOX1* and *AOX2* share a nearly identical coding sequence, but their promoter regions differ significantly (12). *AOX1* is responsible for greater than 90% of the enzyme activity in the cell (13). *Pichia pastoris* has three different methanol utilization phenotypes. Wild type cells carry two functional *AOX* genes and are thus defined as mut^+, i.e. methanol utilization plus. Deletion of *AOX1* causes a phenotype that shows slower growth on methanol as the sole carbon source, termed methanol utilization slow (mut^S) phenotype. Deletion of both alcohol oxidase genes leads to a methanol utilization minus, mut^-, phenotype. The latter strains are not able to grow on methanol any more (13)(14).

Bacillus subtilis levanase is one of three enzymes in this bacterium involved in the hydrolysis of sucrose and is a member of the family of β-D-fructofuranosidases. Those proteins are named according to the substrates they hydrolyze, which are the polyfructans levan (levanases) and inulin (inulinases), respectively, and the disaccharide sucrose (sucrases and invertases). However, many of these β-D-fructofuranosidases are promiscuous in their substrate acceptance. *Bacillus subtilis* levanase hydrolyzes not only levan, but also inulin and sucrose. It is also known as *sacC* and *sacL* and is secreted by the bacterium (15). The enzyme first described by Kunst *et al*. (16), has been successfully expressed in *Escherichia coli*, *Saccharomyces cerevisiae*, *Lactobacillus plantarum* and *Lactobacillus casei* (17)(18). Usually, *S. cerevisiae* is not able to utilize inulin as carbon source. Strains expressing *B. subtilis* levanase grow on inulin plates (15). The levanase was also successfully expressed in *Pichia pastoris* using the constitutive *GAP* promoter (Sandra Majer and Helmut Schwab, unpublished results). The bacterial signal sequence had been replaced by *S. cerevisiae* alpha-factor signal sequence. *Pichia* strains expressing levanase were able to grow on media containing sucrose as the sole carbon source, whereas wild type cells were not. Inulin can be obtained from different Compositae and consists of a series of oligo- and polysaccharides of various lengths made up of β-1,2-linked anhydrofructofuranosyl units. They are often terminated by an anhydroglucopyranosyl residue, which is linked to the fructan chain via an α-1,2 bond (19).

One aim of this study was to develop a screening system based on *B. subtilis* levanase as a reporter, which should allow growth selection of positive clones. Furthermore, we screened a *P. pastoris* gene library for elements influencing the expression level from the *AOX1* promoter under

derepressing conditions. Thereby, we identified transformants containing gene library fragments encoding *AOX1* that showed methanol-free activation of the *AOX1* promoter. To test whether an active AOX1 is necessary for this effect a stop mutant and 4 mutants with mutagenized putative active site were made. Western Blots confirmed the expression of non-functional AOX1 in the active site mutants. Growth plate assays showed that a functional AOX1 is essential for an auto-regulatory function. Levanase activity assays in liquid media revealed an interesting feature of the N616A mutant strain. This AOX1/AOX2 double knock-out strain with a non-functional mutagenized AOX1 reaches higher levanase activity levels than the double knock-out strain transformed with a functional AOX1 cassette. These results are contradictory to the results presented earlier in this study. The exact mechanism remains to be elucidated.

MATERIALS AND METHODS

Strains and media

For cloning purposes, *E. coli* TOP 10F´ (Invitrogen Corporation, Carlsbad, CA, USA) was used. *E. coli* was cultivated in LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl). Antibiotics, if needed, where added after autoclaving to a final concentration of 100 µg/ml ampicillin and 25 µg/ml zeocin, respectively. LB medium plates were prepared by adding 20 g/l agar before autoclaving.

A mut$^-$ *Pichia pastoris* strain was constructed in the GS200 (*his4, arg4*) background (20). As control strains, *P. pastoris* CBS 7435 (wild type, mut$^+$, CBS, Utrecht, the Netherlands) and *P. pastoris* KM71H (*arg4*, *aox1*Δ::*ARG4* mut^S, Invitrogen Corporation, Carlsbad, CA, USA) were used. Genomic DNA for library preparation was isolated from *P. pastoris* X-33 (wild type, Invitrogen Corporation, Carlsbad, CA, USA). *Pichia pastoris* was cultivated either in YPD medium (10 g/l yeast extract, 20 g/l peptone, 20g/l glucose) or in buffered minimal dextrose (BMD) medium (13.4 g/l yeast nitrogen base without amino acids (YNB), 10 g/l glucose, 4 x 10^{-5} % biotin and 200 mM potassium phosphate, pH 6.0). Plates contained 20 g/l agar and 100 mM potassium phosphate, pH 6.0. Levanase activity screening plates contained buffered minimal inulin (BMI) medium (13.4 g/l YNB, 5 g/l inulin from *Dahlia* tubers (Fluka BioChemika, Sigma-Aldrich Chemie GmbH, Buchs, CH), 4 x 10^{-5} % biotin and 100 mM potassium phosphate, pH 6.0), buffered minimal inulin methanol (BMIMe) medium (BMI with 0.5% methanol), buffered minimal sucrose medium (BMS), i.e. inulin replaced by 0.5 % sucrose, buffered minimal dihydroxyacetone (DHA) (Sigma Aldrich Handels-GmbH, Vienna, Austria) medium (BMDHA), i.e. inulin replaced by 0.5 % dihydroxyacetone or buffered minimal sucrose, inulin, DHA or methanol formaldehyde (37 %; Lactan/Roth, Graz, Austria) medium (BMSFA, BMIFA, BMDHAFA, BMMeFA). In case of minimal formaldehyde plates, formaldehyde was supplied in the gaseous phase by the preparation of a humid chamber containing 0.1 od 1 % formaldehyde in the atmosphere. Buffered minimal methanol (BMM) plates (13,4 g/l YNB, 0.5 % methanol, 4 x 10^{-5} % biotin and 100 mM potassium phosphate, pH 6.0) were used for mut phenotype determination. If needed, L-histidine and L-arginine, respectively, were added to the media after autoclaving to a final concentration of 40 mg/l. Likewise, zeocin was added after autoclaving to a final concentration of 100 µg/ml. Yeast strains were streaked on sterile nylon membranes that had been carefully placed on top of agar plates to maintain methanol induction by transfer onto new plates after 3 days incubation.

Isolation of genomic DNA from *P. pastoris*

Isolation of genomic DNA from *P. pastoris* was done with the Easy-DNA™ Kit (Invitrogen Corporation, Carlsbad, CA, USA) as described in the manual and by a protocol from Hoffman and Winston (21). DNA was isolated from 10 ml of YPD culture grown to an OD_{600} of 8-12.

PCR

Colony PCRs and knock-out verifications were done using HotStarTaq DNA Polymerase (QIAGEN GmbH, Hilden, Germany). For other applications, PCRs were performed with Phusion™ Polymerase (Finnzymes Oy, Espoo, Finland) according to the polymerase manuals. One fresh colony was resuspended in 50 µl H_2O for *Pichia pastoris* colony PCR, incubated at 95°C for 10 min and centrifuged at maximum speed in an Eppendorf 5415R centrifuge for one min. 10 µl of the supernatant were used as template. Overlap extension PCRs were carried out with 10 ng of each template.

Transformation of *Pichia pastoris*

A single colony was inoculated in 50 ml YPD media and grown over night to an OD_{600} of 0.8-2 in a 250 ml baffled wide-mouthed flask at 30°C and 110 rpm. Preparation of competent cells and transformation of *P. pastoris* was done exactly as described in the condensed protocol of Lin-Cereghino (22). Roughly 2-5 µg of DNA were mixed with 100 µl of competent cells and the suspension was incubated on ice for 2 min. Upon electroporation at 1.5 kV, 200 Ω, 25 µF, cells were regenerated for 2-3 h at 30° C without shaking before plating on selective media. If a zeocin resistance cassette was transformed, cells were regenerated for 1 h in 500 µl 1 M sorbitol and then 500 µl YPD were added and cells regenerated for another hour. For the transformation of an auxotrophic marker only 1 ml 1 M sorbitol was added for regeneration.

Reporter strain construction

Standard molecular biology procedures were performed as described (23). The *Bacillus subtilis* levanase gene without its natural secretion signal was amplified from pKF3 plasmid (24) and cloned into pPIC9 vector (Invitrogen Corporation, Carlsbad, CA, USA) via *Xho*I and *Not*I. The obtained vector, pPIC9-LevS, was linearized by *Bgl*II and transformed into *P. pastoris* GS200 to knock out *AOX1*. Histidine prototrophic transformants were streaked on BMM plates to determine their mut phenotype. Knock-out confirmation was done by PCR using primer pairs 5´AOX1_fwd / alpha-col_rev and downstr._3´AOX1_rev / AOX1TT_fwd for confirming knock-out at the 5' and 3' regions, respectively.

To delete the *AOX2* gene, a cassette with *AOX2* promoter and terminator sequences flanking the *P. pastoris ARG4* gene was constructed via overlap extension PCR. *AOX2* promoter and terminator fragments were amplified from genomic DNA of *P. pastoris* CBS 7435 using primer pairs fpAOX2Pr / rpAOX2Pr and fwdAOX2Te2 / rpAOX2Te, respectively. The *ARG4* gene was amplified from pBLARG-IX plasmid (25) with the primers fwdARG4FC2 and rpARG4FC introducing 24 and 25 bp homologous regions to the *AOX2* promoter and terminator fragments, respectively. *AOX2* promoter and *ARG4* coding sequence were fused via overlap extension PCR. In a second reaction the product was fused to the *AOX2* terminator fragment. The whole construct was cloned into pBluescript II SK(-) vector (Fermentas Inc, Glen Burnie, MA, USA) via *Pst*I and *Not*I and transformed into *E. coli* yielding plasmid pBSII SK(-) AOX2KO. The plasmid was linearized by *Eco*RV and transformed into *P. pastoris* GS200 *aox1*Δ::Lev*SHIS4* to delete the *AOX2* gene. Arginine prototrophic transformants were streaked onto BMM plates to determine their mut phenotype. Knock-out confirmation was done by colony PCR using primer pairs colony1 / rev4 and colony2 / fwd7 for confirming knock-out at the 5' and 3' regions, respectively.

Gene library construction

P. pastoris X-33 genomic DNA was partially digested with *Bsp*143I (15 mU/µg DNA) for 1 h at 37° C. After gel purification the recessed 3´ termini of the fragments were partially filled-in with dATP and dGTP using Klenow Fragment (Fermentas Inc, Glen Burnie, MA, USA). The ends of *Xho*I digested pGAPZ A vector (Invitrogen Corporation, Carlsbad, CA, USA) were also partially filled-in with dCTP and dTTP using the same enzyme. Ligation of the fragments into the vector was followed by

transformation into *E. coli* by electroporation. Altogether 10,500 transformants were picked using a QPixII robot (Genetix, Queensway, UK) and conserved as glycerol stocks in 384-well microplates. Cells were pinned onto LB plates containing zeocin with a 384 pin replicator and incubated at 37°C for 24 h before washing off colonies with 5 ml of sterile H_2O. The suspension was used for plasmid isolation with QIAprep® Spin Miniprep Kit (QIAGEN GmbH, Hilden, Germany). After linearization with *Bgl*II, *Pag*I and *Avr*II, respectively, the gene library was transformed into *P. pastoris* GS200 *aox1*Δ::Lev*SHIS4* *aox2*Δ::*ARG4* plating on BMI plates containing 50 µg/ml zeocin.

Retransformation experiments

The integrated gene library fragment with the zeocin resistance gene of one identified clone was amplified using primers pTEF1start_fwd and A2int_rev. *AOX1* upstream region was amplified using the primers Seq5_rev and CYC1TT_rev. Silent point mutations were introduced into the *AOX1* coding sequence with the primers AOX CDSmut_fwd and AOX CDSmut_rev. AOX CDSmut_fwd and A2int_rev were used to amplify the 5' part of the integrated gene library fragment. Applying AOX CDSmut_rev and pTEF1start_fwd the 3' part was amplified. The obtained PCR products were fused via overlap extension PCR. Two stop codons in the *AOX1* coding sequence were inserted with the primers AOX CDSmut2_fwd and AOX CDSmut2_rev following the same procedure. To create the AOX1 mutants showing normal, but non-functional AOX1 expression, mutations were inserted into the AOX1 coding sequence following the upper described procedure using the following primers: H567A_for, H567A_rev, H567N_for, H567N_rev, H567Q_for, H567Q_rev, N616A_for, N616A_rev.

Semi-quantitative determination of levanase expression

Cells were cultivated in a 96-well footprint deep well plate (Bel-Art Products, Pequannock, NJ, USA) containing 300 µl BMD media per well. After incubation for 48 h at 28°C, 320 rpm and 80% humidity in a Multitron II shaker (Infors AG, Bottmingen-Basel, Switzerland) stationary phase cells were diluted 10- and 100-fold in H_2O. Dilutions were either pinned with a 96 pin replicator or were spotted as 5 µl aliquots onto BMI, BMIMe, BMM, BMS 0.1/1 % FA, BMI 0.1/1 % FA, BMDHA 0.5/1 % FA, BMMe 0.1/1 % FA plates. After incubation for 3-4 days at 28°C or room temperature spots were quantified in a G:Box (SynGene, Synoptics Ltd., Beacon House, Nuffield Road, Cambridge, England). An image of

each plate was taken using upper white light without any filter. The 3D feature of the GeneSnap software allowed a three-dimensional view of the spots on the plate. For quantification, the images were inverted and the spots were quantified using GeneTools software. Spot densities were normalized for the mean spot density of the reference strain, which was set to one.

Kinetic measurement of levanase activity

For kinetic measurements of levanase activity cells were cultivated in a 96-well deep well plate as described above. After incubation for 48 h, stationary phase cells were pelleted in 96-well deep-well plates by centrifugation at 3,200 x g in an Eppendorf 5810R centrifuge for 20 min. Two hundred µl of the supernatants were mixed with 225 µl H_2O and 475 µl "Glucose UV" reagent (Dipromed, Weigelsdorf, Austria) in half-micro cuvettes (Greiner Bio-One GmbH, Frickenhausen, Germany). Absorption at 340 nm was recorded every 10 s over 10 min. After 1 min 100 µl 10% sucrose solution was added to the cuvettes. Sucrose hydrolysis by levanase liberates glucose which is detected by the hexokinase method and leads to an increased absorption at 340 nm.

Formaldehyde fermentation and levanase assay of AOX1 mutants

Strains were streaked freshly before use. For precultures, 10 ml ½ BYPD (1 % peptone, 0.5 % yeast extract, 1 % glucose, 200 mM potassium phosphate buffer pH 7.0) was inoculated with a single colony and incubated in an Infors shaker at 28°C and 150 rpm for 24 hours. For the main culture, OD_{600} was measured and 30 ml of BYP0.1%D (1 % peptone, 0.5 % yeast extract, 0.1 % glucose, 200 mM potassium phosphate buffer pH 7.0) medium (100 ml flask) was inoculated to a start OD_{600} of 0.2. Cultures were shaken at 28 °C and 120 rpm for 24 hours. Subsequently, cultures were centrifuged in 50 ml tubes at 2500 rpm for 5 min. and then resuspended in either BYP (1 % peptone, 0.5 % yeast extract, 200 mM potassium phosphate buffer pH 7.0) or BYP0.1%D. OD_{600} was measured. Cultures were aliquoted (200 µl per well) into deep well plates (1 row per clone = 8 replicates). Substrate was added to the wished concentration. In case of formaldehyde deep well plates were sealed with sealing foil. Deep well plates were shaken on a Titrimax 1000 shaker (Heidolph) at 28 °C and 900 rpm for 12 or 24 hours. After that, OD600 was measured. Cultures were transferred from the deep well plate into v-bottom micro titer plates (Greiner Bio-One GmbH, Kremsmünster, Austria),

centrifuged at 3000 rpm in an Eppendorf 5810R centrifuge for 15 min. at 4 °C. Centrifuged plates were stored at 4 °C until all plates were spinned down as they should be proceeded at the same time. Meanwhile, another v-bottom plate was provided with 20 µl 1 % sucrose per well. 20 µl of supernatant was added to the provided sucrose per well, mixed by pipetting up and down and the mixture was incubated for 20 min. at room temperature. Meanwhile 190 µl / well glucose UV reagent was aliquotted into a UV micro titer plate. 10 µl per well of the sucrose-supernatant-mixture was added to the UV reagent, mixed rapidly by pipetting up and down and incubated at room temperature. Finally OD_{340} of the samples was measured (endpoint measurement) in a Synergy-Mx Biotek plate reader (Biotek GmbH, Germany).

Western Blot

For western blots for the determination of *AOX1* expression, different deep well fermentation protocols were applied according to the needs. Temperature, humidity and rpm were always the same, 28° C, 80 % and 320 rpm. For derepressed conditions, strains were inoculated in 600 µl ½ BYPD (pH6). 100 µl samples were taken after 28, 37 and 48 hours. Two different induction conditions were analyzed: buffered minimal methanol and buffered minimal sorbitol methanol. For BMM conditions, strains were inoculated in 300 µl ½ BYPD and cultivated for 24 hours before adding 300 µl BMM to a final concentration of 0.5 % (8 hours interval) or 1 % (16 hours interval) respectively. Samples (100 µl) were taken after 4, 9 and 24 hours of induction. For BMSM conditions, strains were inoculated in 300 µl ½ BYPD and cultivated for 24 hours before adding 300 µl BMS to a final concentration of 1 % sorbitol. After another 24 hours of incubation, methanol as well as sorbitol were added to a final concentration of 0.1 % (MeOH) and 1 % (sorbitol). The same was repeated 8 hours later. Samples were taken after 4, 9 and 24 hours of methanol induction. Samples (100 µl) were centrifuged at 4° C and 13200 rpm in an Eppendorf 5415R centrifuge, supernatants were withdrawn. Pellets were stored at -20° C. For SDS-PAGE, pellets were resuspended in 100 µl 1M KP_i buffer pH7. SDS PAGE samples were mixed with NaOH, DTT and sample buffer and heated to 95° C for 10 minutes before being loaded onto a 4-12 % BisTris NuPage gel (Invitrogen LifeTech Austria, Vienna, Austria). Western blotting was performed in a Hoefer TE 22 mini tank-blotter (Hoefer, VWR International GmbH, Vienna, Austria) using a standard blotting buffer containing methanol. Western blot procedure was performed as previously described (26). Anti-aox1/aox2 primary antibody (rabbit IgG; kindly provided by G. Daum, Institute of Biochemistry, University of Technology, Graz) was diluted 1:5000 and

secondary antibody (goat anti-rabbit IgG, Sigma Aldrich Handels-GmbH Vienna, Austria) was diluted 1:40000 in blocking buffer.

RESULTS

Setup of a levanase-based selection system

We set out to identify genetic elements increasing methanol-free expression from the *AOX1* promoter, because methanol is considered a risk factor in industrial-scale application. Therefore, we first had to establish a screening system capable of detecting increases in the expression level driven by the *AOX1* promoter. We chose a growth-based approach and placed *B. subtilis* levanase under *AOX1* promoter control. As carbon source, inulin was used which cannot be utilized by *P. pastoris* unless levanase is functionally expressed. Therefore, growth of yeast is directly dependent on the expression level from our promoter of interest.

To replace the *AOX1* by the levanase coding sequence, *Bgl*II linearized pPIC9-LevS was transformed into *P. pastoris* GS200. Several transformants grew comparable to KM71H on BMM plates and thus had a mut^{S} phenotype (data not shown). One of the mut^{S} strains was chosen for isolation of genomic DNA which was taken as template for two PCR reactions confirming correct integration of the transformed fragment on both sides. The strain was named *P. pastoris* LevSmut^{S}. Subsequently, an *aox1Δ aox2Δ* double knock-out strain was created. Therefore, *Eco*RV digested pBSII SK(-) AOX2KO was transformed into *P.p.* LevSmut^{S}. Several arginine prototrophic transformants did not grow on BMM plates indicating the expected mut^{-} phenotype for an *aox1Δ::*Lev*SHIS4 aox2Δ::ARG4* strain (Fig.1A). Upon confirmation of *aox2Δ* knock-out by colony-PCR, *P.p.* LevSmut^{-} was chosen as the target strain for gene library screening (strain 3, Fig.1).

The next step was to find suitable conditions for screening transformants carrying a *P. pastoris* gene library for methanol-free activation of the *AOX1* promoter. To simulate induction by the transformed gene library fragments screening conditions were optimized by the addition of methanol to the media. Assuming that activation triggered by the gene library fragments might not be as obvious as upon addition of methanol, it was necessary to find conditions that permitted a clear differentiation between basal and elevated levanase expression. To avoid the time consuming step of picking and cultivating many thousands of transformants in ordered arrays, the screenings were done directly on the transformation plates. In the end, plates containing 5 g/l inulin as the sole carbon source (BMI plates) were chosen for the gene library screening as under these conditions growth of transformants strictly depended on elevated levanase expression. Yeast cells lacking the levanase gene (wild-type) or having basal, uninduced levanase expression from the *AOX1* promoter

did not grow significantly (Fig.1C). *aox1Δ::*Lev*SHIS4 aox2Δ::ARG4* strains grew very well on inulin plates if levanase expression was enhanced by adding methanol, but not on methanol plates indicating that growth on BMIMe plates was due to inulin catabolism (Fig. 1B).

Identification of genetic elements enhancing levanase expression

A *Pichia pastoris* gene library consisting of fragments of an average length of 4.4 kbp in pGAPZ A was transformed into *P. pastoris* LevSmut$^-$ in 5 independent experiments. Each time an even mixture of plasmids linearized by *Bgl*II, *Pag*I and *Avr*II, respectively, was transformed. Linearization of vectors was performed with three different enzymes to minimize the risk that the desired gene library fragments would not be found as they might have recognition sites for the one or other endonuclease. After 5 days of incubation at 28°C about 20,000 transformants were obtained and among those 45 faster growing colonies were identified and restreaked for further characterization. For re-screening, we switched to an ordered 96-well format. Two consecutive re-screenings were performed to confirm enhanced levanase expression in the selected transformants. Of the initial 45 transformants 27 showed significantly increased growth on inulin as sole carbon source and thus elevated levanase expression in the first re-screening. All of those 27 transformants showed again a higher levanase expression, i.e. growth on inulin, in the second re-screening as compared to *P. pastoris* LevSmut$^-$ (Fig.2). As a control experiment empty pGAPZ A vector was linearized with *Bgl*II and transformed into the screening strain. No elevated growth on BMI plates was observed for these transformants (data not shown).

Ten randomly selected transformants from the second re-screening were chosen for a kinetic measurement of levanase activity in the culture supernatant. Our assay was based on levanase cleavage of sucrose and determining NADH formation as the by-product of hexokinase-based glucose determination. All of the ten transformants we checked lead to significantly more NADH signal than *P.p.* LevSmut$^-$ suggesting that the genomic library transformants had expressed a higher level of levanase (Fig.3). Higher NADH signal correlated very well with increased growth on inulin plates (Fig.2) underscoring that the transformants carried genetic elements enhancing levanase expression from the *AOX1* promoter. Consequently, we isolated the ten genomic library fragments by PCR and sequenced the products. To our surprise, nine of the ten transformants we had randomly selected contained a complete *AOX1* gene including coding, promoter and terminator sequences inserted

immediately upstream of the levanase expression cassette. The functional *AOX1* gene rendered transformants capable of metabolizing methanol as they had a mut$^+$ phenotype upon cultivation on BMM plates (data not shown). Thus we had unveiled an autoregulatory feature of the *AOX1* locus.

Autoregulatory feature of the *AOX1* locus

In order to confirm that the *AOX1* gene integration events were responsible for the enhanced levanase expression re-transformation experiments were done. Therefore, a fragment containing the *AOX1* gene with 2 kbp upstream region and the zeocin resistance cassette was amplified from genomic DNA isolated from transformant B4 (Fig.2) as template. The product was transformed into the original screening strain *P.p.* LevSmut$^-$. After selection for zeocin resistance a couple of transformants were screened for elevated levanase expression in the same way as the gene library transformants. Practically all clones showed enhanced growth on BMI plates (Fig.4A,C). They also had reacquired a mut$^+$ phenotype like the original gene library transformants (Fig.4B). On the other hand, transformation of a PCR product containing only the 2 kbp upstream region and the Zeocin resistance gene yielded no transformants with enhanced levanase expression (data not shown). Collectively, our data suggested that the genetic element eliciting enhanced levanase expression from the *AOX1* promoter was the *AOX1* coding sequence.

A putative open reading was identified on the complementary strand within the *AOX1* coding sequence. To exclude a possible role in P_{AOX1} regulation two point mutations were introduced into the ORF on the complementary strand by an overlap-extension strategy. These mutations were silent regarding *AOX1* but created two stop codons in the putative open reading frame. Therefore, no functional gene product should be built. Except for the two point mutations, the transformation cassette was the identical to the first re-transformation experiment. Resulting transformants were mut$^+$ indicating that the transformed *AOX1* gene with silent mutations was functional. Sequencing of colony PCR fragments confirmed the presence of the two mutations. The majority of the transformants in this experiment had similarly elevated levanase expression as in the previous experiments (data not shown).

The same procedure was repeated introducing two stop codons into the *AOX1* coding sequence to test whether a functional *AOX1* gene was required for enhancing levanase expression from the *AOX1* promoter. The PCR product with these two mutations was transformed into the original screening strain and the resulting transformants were screened for levanase expression.

Colony PCRs with upstream, downstream and *AOX1* internal primers and subsequent sequencing confirmed integration of the whole, but non-functional *AOX1* gene in the tested transformants. Thus, no functional alcohol oxidase should be expressed and the transformants should keep a mut$^-$ phenotype. This time sucrose was used as the sole carbon source in growth tests to assay levanase expression, because a new batch of inulin turned out to contain significant amounts of glucose. Glucose contamination of inulin prohibited reliable analysis of levanase expression as even wild type cells lacking a levanase gene grew on the new batch of inulin (data not shown). While an original gene library transformant grew on sucrose plates like it had grown on inulin plates, none of the transformants carrying inactivated *AOX1* showed enhanced growth on sucrose plates as compared to the screening strain (Fig.5A,C). No growth on methanol plates confirmed the expected mut$^-$ phenotype (Fig.5B). Thus, only functional alcohol oxidase seems to upregulate levanase expression driven by P_{AOX1} under derepressing and methanol-free conditions. These results hint at an autoregulatory function of the *AOX1* locus.

For a more profound analysis, four *AOX1* mutants were made creating an amino acid exchange at two positions of the propagated active triade (K. Gruber personal communication). Amino acids Histidin 567 and Asparagine 616 were chosen and mutated to alanine, asparagine and glutamine in case of His567 and to alanine in case of N616. Growth assays on inulin and sucrose were made showing the same results as obtained with the AOX1 stop mutant. A verification of *AOX1* expression in the H567 mutants was performed by western blotting experiments. The appendent fermentations were performed under three different conditions: glucose derepression, methanol induction and sorbitol methanol induction. Table 2 shows a summary of the western blot results. It was clearly shown that AOX1 H567 mutants were expressed normally under methanol induction and sorbitol/methanol induction but obviously in an inactive form. That leeds to the assumption that firstly His567 and N616 play an essential role at the active site of *AOX1* and secondly that an active *AOX1* is necessary for the autoregulatory function of AOX1.

Alternative inducers of the *AOX1* promoter

There is no proof so far that methanol is the inducer for the AOX1 promoter. Alternatives could be a secondary metabolite like formaldehyde or e.g. DHA (dihydroxyacetone). We performed growth assays with different strains based on the GS200 aox1/aox2 double knockout strain levanase expression to test for these possibilities. Table 3 shows a summary of the results. While data for methanol induction were like expected, formaldehyde seemed to have a positive or inducing effect.

DHA in contrast merely served as carbon source. Growth results on sucrose and inulin, respectively, were inconsistent. While the double knock out strain with a functional AOX1 cassette was able to grow on sucrose, no growth on inulin could be detected. The growth of the strains carrying a functional AOX1 cassette on sucrose could be another proof for an auto-induction effect.

The effect of formaldehyde was further investigated by levanase activity assays based on levanase secretion. Therefore the aox1/aox2 knockout reporter strain transformants each harboring the four different mutated AOX1 variants, the AOX1 stop mutant strain and several control strains were fermented in deep well plates (8 replicates per construct). Different induction conditions were tested (methanol, formaledyhde). The results were quite interesting. Whereas formaldehyde addition seems to have no effect at all, differences in the levanase expression/secretion level of the AOX1 mutant strains became apparent. The strains with the H567 mutations, the AOX1 stop mutant and the reproduced gene library strain carrying the functional AOX1 cassette showed more or less the same activity levels as the aox1/aox2 double knockout. In contrast, the N616A mutant strain and the original gene library retransformant strain H5 with the functional AOX1 cassette showed significantly increased levanase activity levels (Fig. 6). Results were reproduced by another eight replicates. Streaks on different agar plates were made for a confirmation of levanase activity assays. N616A strain was not able to grow on minimal methanol plates, showed weak growth on sucrose and strong growth on minimal sucrose methanol plates. In contrast and as expected, the original gene library transformant strain with the functional AOX1 grew strongly on minimal methanol and minimal methanol sucrose plates and weakly on minimal sucrose plates (Table 4).

Future experiments will show but it would be of great benefit for industrial application to have an expression strain that needs minimal amounts of methanol for a high yield production.

DISCUSSION

A screening system was developed placing *Bacillus subtilis* levanase under the control of the chromosomal copy of the *Pichia pastoris AOX1* promoter and eliminating at the same time the major alcohol oxidase, *AOX1*, coding sequence. Thereby, *Pichia pastoris* acquired the capacity to cleave inulin and grow on this unusual carbon source, which otherwise cannot be utilized by yeast. As methanol induction of the *AOX1* promoter was of particular importance in establishing the levanase-based, growth-dependent screening system and methanol should not interfere as potential carbon source, we additionally knocked out the second alcohol oxidase gene of *P. pastoris, AOX2*. Thus, the final screening strain could not metabolize methanol, but methanol could activate levanase expression, which was monitored by growth on inulin. We established conditions which allowed us to screen for genetic elements that upregulated *AOX1* promoter based levanase expression by testing the system with methanol as inducer (Fig.1). A clear difference was visible between methanol induced and uninduced conditions. In the latter case, there was no significant growth of yeast on inulin plates. Thus, this system was suitable to screen a *Pichia pastoris* gene library for elements enhancing the expression driven by the *AOX1* promoter. This was done by transforming a genomic library of *Pichia pastoris* into the screening strain and plating the cells on inulin containing media. Due to the enhanced growth of transformants with increased levanase expression a screening directly on the transformation plates was possible. Screening directly on the transformation plates represents a considerable advantage over other screening systems, where lots of transformants have to be picked randomly prior to the first screening. In our system faster growing colonies can be easily identified and picked for further characterization.

To confirm enhanced levanase expression observed in the first screening we re-screened the best transformants, i.e. the biggest colonies, of the first screening. Therefore, the system was adapted to a 96-well format. For the majority of transformants the enhanced reporter expression under de-repressing conditions could be verified in two successive re-screenings (Fig.2). Kinetic measurements with the culture supernatants of ten randomly selected transformants confirmed a higher levanase activity therein (Fig.3). Thus, our approach identified gene library transformants with enhanced levanase expression driven by the *AOX1* promoter under methanol-free conditions. Surprisingly, for the majority of selected gene library transformants an integration of the whole *AOX1* gene was determined. Re-transformation of a gene library fragment containing *AOX1* confirmed that this region was responsible for enhanced P_{AOX1} activity under derepressed conditions (Fig.4). Transformation of a fragment containing an inactivated *AOX1* gene with two stop codons in the

coding region did not lead to transformants with enhanced levanase expression (Fig.5). These results suggest that the alcohol oxidase influences expression from its own promoter. Our system showed that the derepression level of the *AOX1* promoter in cells containing a functional enzyme was significantly higher than in the knock-out. The exact reasons for this effect and if the presence of the enzyme also influences the induction level of its own promoter remain to be elucidated.

Transformation of fragments containing mutagenized AOX1 genes, which lead to the expression of a mutagenized AOX1 protein as proven by western blot experiments, resulted in strains that didn´t show enhanced levanase expression as well. These results supported the hypothesis that an auto-regulatory feature of alcohole oxidase 1 needs a functional enzyme. Furthermore, the two mutagenized amino acids are obviously essential for the active site of AOX1 protein. Interestingly, differences between growth on sucrose or inulin became obvious. Whereas strains with a functional AOX1 protein were able to grow on agar plates containing sucrose, they didn´t grow on inulin. This might as well be another hint or proof for an auto-induction effect of AOX1. Another possible explanation could be that levanase, although theoretically secreted via *S. cerevisiae* alpha factor, is expressed but gets stuck in the periplasm. Sucrose, much less complex than inulin, diffuses to periplasm, where it was cleaved by levanase. Additionally, an acidification of the media surrounding the emerging colonies caused by cell growth could have a supportive effect. We also noticed that inulin is a very sensitive substrate to work with, even the use of the same product but different charges influenced the results. Another explanation could be that *Pichia pastoris* might have an intrinsic source of tiny amounts of methanol that induced levanase expression.

It´s not proven so far that methanol is the inducer of the P_{AOX1}. The inducer might be a secondary metabolite like formaldehyde or for example dihydroxyacetone (DHA) formed by dihydroxyacetone synthase. The growth assays that were performed on plates grown under formaldehyde atmosphere led to the assumption that formaldehyde could be a possible inducer as an enhancing growth effect on sucrose and inulin plates was reached, even for the wild type strain *P.p.* CBS 7435 that should not be able to grow on sucrose or inulin. These results supported the fact that growth under formaldehyde atmosphere probably has a toxic effect on the cells. Liquid levanase assays were performed under methanol and formaldehyde inducing conditions whereas formaldehyde has definitely no inductive effect. A comparison of the four different AOX1 mutants in contrast showed that the N616A strain possesses an interesting feature. The strain cannot use methanol but under methanol inducing conditions levanase expression respectively activity reaches the same or even a higher level compared to the original gene library transformant strain having a functional AOX cassette (Fig. 6). The other mutants, no matter if carrying the H567 or the stop mutations, show almost the same level of levanase activity as well as the double knock-out strain. This is not

astonishing as all these strains do not have a functional AOX1 but P_{AOX1} is still induced by methanol and leads to levanase expression. Growth plate assays showed that the N616A strain doesn´t grow on methanol, but grows weakly on sucrose and strongly on methanol sucrose plates, whereas the retransformant strain grows strongly on methanol and methanol sucrose, but slightly on sucrose plates. These final results are contradictory to the former results as we postulated a functional AOX1 to be essential for the autoregulatory function. The exact reasons for this effect remain to be elucidated but it is of great benefit for a lot of biotechnological applications to have a strain in hand that needs just tiny amounts of methanol for high effective high yield production.

FIGURES AND TABLES

Table 1: Primers used in this study.

Restriction sites and point mutations are written in bold, homologous regions for overlap extension PCR in italic.

Name	Sequence (5´to 3´)
5´AOX1_fwd	AATTCCCGCTTTGATGCCTGAAATC
alpha-col_rev	GCAGCAATGCTGGCAATAGTAG
downstr._3´AOX1_rev	AGATCGTTGACGATATGTTGG
AOX1TT_fwd	TCAAGAGGATGTCAGAATGCC
fpAOX2Pr	ATTAACTGCAGGATATCAACCGTCCAGCCTTTCTC
rpAOX2Pr	TTTCTCAGTTGATTTGTTTGTGG
fwdAOX2Te2	GCTCACGGTTCTTGGACTCAGC
rpAOX2Te	AATATGCGGCCGCGATATCATCGGCCAATTCGGTAGC
fwdARG4FC2	*GCTGAGTCCAAGAACCGTGAGC*GCTTCTAGTGGTAGGAATTAATTCTG
rpARG4FC	*CCACAAACAAATCAACTGAGAAAA*TCTGCCCTCACGGTGGTTAC
colony1	TCCTTTAGCTTGTCGAAGG
rev4	CAAAATCTCTGTCTGAAACAGCG
colony2	TACTGGAGCAACCAATGAGG
fwd7	TATGTCTATTGTCACTGTGG
pTEF1start_fwd	ATCCCCCACACACCATAGC
A2int_rev	CTAGACATCGAAGACAGTGG
Seq5_rev	CGTTAGCATTTCAACGAACC
CYC1TT_rev	AGCTTGCAAATTAAAGCCTTCG
AOX CDSmut_fwd	*CCTCTTGAGAATGGGTATTCTAAGAAGTGG*AACATAGTC
AOX CDSmut_rev	*CCACTTCTTAGAATACCCATTCTCAAGAGG*TTCCATTCAC
AOX CDSmut2_fwd	*CGAAGACGGATGAGCTGCTGCTGTTTGAACC*GTTCCAAGC
AOX CDSmut2_rev	*GGTTCAAACAGCAGCAGCTCATCCGTCTTCG*ACAATAATTTTGTCG
aox1mutH567Afor	*ACACTGAGACCACATGGGCTTGTCTGGGAACCT*GTTCCATCG
Aox1mutH567Arev	*AGGTTCCCAGACAAGCCCATGTGGTCTCAGTGT*GCTCACG
Aox1mutH567Nfor	*ACACTGAGACCACATGGAACTGTCTGGGAACCT*GTTCCATCG
Aox1mutH567Nrev	*AGGTTCCCAGACAGTTCCATGTGGTCTCAGTGT*GCTCACG
Aox1mutH567Qfor	*ACACTGAGACCACATGGCAATGTCTGGGAACCT*GTTCCATCG
Aox1mutH567Qrev	*AGGTTCCCAGACATTGCCATGTGGTCTCAGTGT*GCTCACG
Aox1mutN616Afor	*ACAATGTTGGTTGTGCTACCTACACCACCGCTC*TTTTGATCG
Aox1mutN616Arev	*GAGCGGTGGTGTAGGTAGCACAACCAACATTGT*CTGGGCA

Table 2: Summarized western blot results.

Aox1 expression was detected with anti-Aox1/Aox2 rabbit IgG. + Aox1 expression; — no Aox1 expression; ctr. control; repr. repressed; BMM: buffered minimal methanol; BMS: buffered minimal sorbitol; double KO: *P. pastoris* GS200 *aox1*Δ::Lev*SHIS4 aox2*Δ::*ARG4;* CBS 7435: *P. pastoris* CBS 7435 wild type*;* B4: gene library transformant GL B4; GL H5 was also part of the experiment and behaved as GL B4 (data not shown). H567A, N, Q: *P. pastoris* GS200 *aox1*Δ::Lev*SHIS4 aox2*Δ::*ARG4* transformed with a PCR fragment carrying a base triplet exchange in the *aox1* CDS that leads to an active site Aox1 mutant at the given position.

	Double KO Glucose repr.	Double KO Induced BMM	Double KO Induced BMS	CBS 7435 Glucose repr.	CBS 7435 Induced BMM	CBS 7435 Induced BMS	B4 Glucose repr.	B4 Induced BMM	B4 Induced BMS
Ctr. 4 h	—	—	—	—	+	+	—	—	+
Ctr. 9 h	—	—	—	—	+	+	—	+	+
Ctr. 24 h	—	—	+	—	+	—	—	+	+

	H567A Glucose repr.	H567A Induced BMM	H567A Induced BMS	H567N Glucose repr.	H567N Induced BMM	H567N Induced BMS	H567Q Glucose repr.	H567Q Induced BMM	H567Q Induced BMS
Sample 4 h	—	+	+	—	+	+	—	+	+
Sample 9 h	—	—	—	—	+	+	+	+	+
Sample 24h	—	+	+	—	+	+	—	+	+

Table 3: Summarized growth assay data with formaldehyde (FA) and dihydroxyacetone (DHA) using inulin and sucrose, respectively, as C-source.

Different strains expressing a functional, non-functional or no Aox1 were grown to stationary phase and spotted onto buffered minimal agar plates containing different carbon sources or (possible) inducers as given below. Formaldehyde experiments were performed in a humid chamber containing formaldehyde in the gas phase in the given concentrations. All other components were directly added to the media. Spots were scanned after four days of growth and a 3D-image was generated using a G:box (SynGene). Growth intensity was rated from + (almost no growth) to ++++ (very strong growth). C-source: carbon source; Aox1 funct.: transformant carrying the re-transformed gene library fragment of strain GL B4 containing a functional *AOX1*; Aox stop: transformant carrying a non-functional *AOX1* allele; aoxH567A: transformant carrying a mutated *AOX1* allele (amino acid exchange at position His 567); Mut-: *P. pastoris* GS200 *aox1*Δ::Lev*SHIS4 aox2*Δ::*ARG4;* GL H5: gene library retransformant H5 carrying a functional *AOX1*; CBS 7435: *P. pastoris* CBS 7435 wild type

C-source/"inducer"	Aox1 funct.	Aox stop	aoxH567A	Mut-	GL H5	CBS7435
MeOH	++	no growth	no growth	no growth	+++	++
MeOH / 0.1 % FA	++	no growth	no growth	no growth	++++	++
MeOH / 1 % FA	++	no growth	no growth	no growth	++++	++
Sucrose	++	no growth	no growth	no growth	+++	no growth
Sucrose / 0.1 % FA	+++	+++	++++	++	+++	++
Sucrose / 1 % FA	+++	+++	+++	++	+++	++
Inulin	no growth	no growth	no growth	no growth	no growth	no growth
Inulin / 0.1 % FA	++	no growth	no growth	no growth	++	+
Inulin / 1 % FA	++	no growth	no growth	no growth	+++	+
DHA	++++	++++	++++	++++	++++	++++
DHA / 0.1 % FA	++++	++++	++++	++++	++++	++++
DHA / 1 % FA	++++	++++	++++	++++	++++	++++

Table 4: Summarized growth assay results for N616A verification.

Funct. AOX1: transformant carrying the re-transformed gene library fragment of strain GL B4 containing a functional *AOX1*; Aox1 stop: transformant carrying a non-functional *AOX1* allele; H567A: transformant carrying a mutated *AOX1* allele (amino acid exchange at position His 567); H567N: transformant carrying a mutated *AOX1* allele (amino acid exchange at position His 567); H567Q: transformant carrying a mutated *AOX1* allele (amino acid exchange at position His 567); N616A: transformant carrying a mutated *AOX1* allele (amino acid exchange at position Asp616); double KO: *P. pastoris* GS200 *aox1*Δ::Lev*SHIS4 aox2*Δ::*ARG4;* Retransf. H5: gene library retransformant H5 carrying a functional *AOX1*; CBS 7435: *P. pastoris* CBS 7435 wild type; BMM: buffered minimal methanol; BMSuc: buffered minimal sucrose; BMMSuc: buffered minimal methanol sucrose; BMD: buffered minimal dextrose; Growth intensity was rated from +/- (almost no growth) to +++ (very strong growth).

	BMM	BMSuc	BMMSuc	BMD
Funct. AOX1	++	+	++	+++
Aox1 stop	-	+-	+	+++
H567A	-	+-	+	+++
H567N	-	+-	+	+++
H567Q	-	+-	+	+++
N616A	-	+-	++	+++
Retransform. H5	++	+-	++	+++
Double KO	-	-	++	+++
CBS7435	+++	-	++	+++

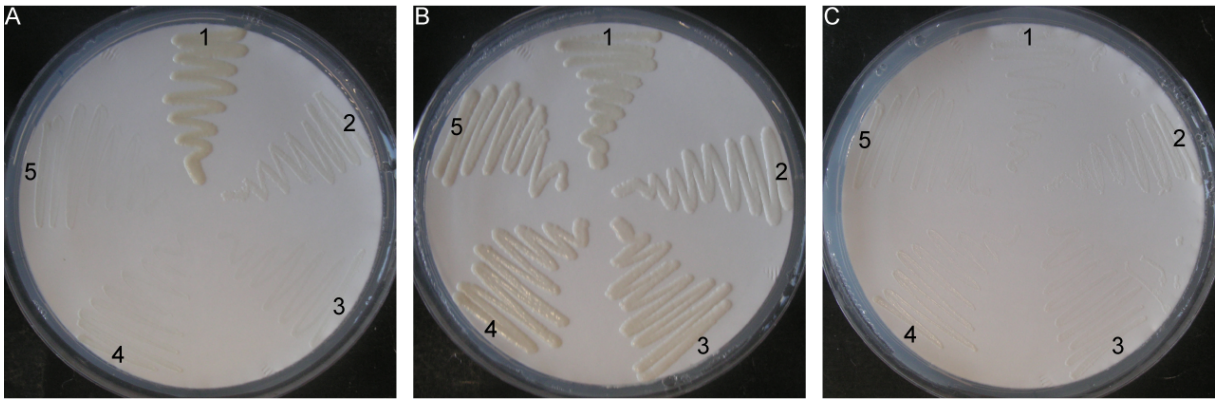

Fig. 1: *Pichia pastoris* growth tests on different carbon sources.

Pichia pastoris wild type strain CBS 7435 (1), GS200 *aox1*Δ::Lev*SHIS4* (mut^{S}) (2) and GS200 *aox1*Δ::Lev*SHIS4* *aox2*Δ::*ARG4* (mut^{-}) (3-5) were grown on buffered minimal methanol (A), inulin & methanol (B) and inulin plates (C), respectively. *P. pastoris* wild-type grows well on methanol as carbon source, but not at all on inulin. Functionality of at least one alcohol oxidase, Aox1 or Aox2, is essential for growth on methanol. Strains containing the *Bacillus subtilis* levanase under the control of the *AOX1* promoter were able to metabolize inulin efficiently only when the promoter was induced by methanol.

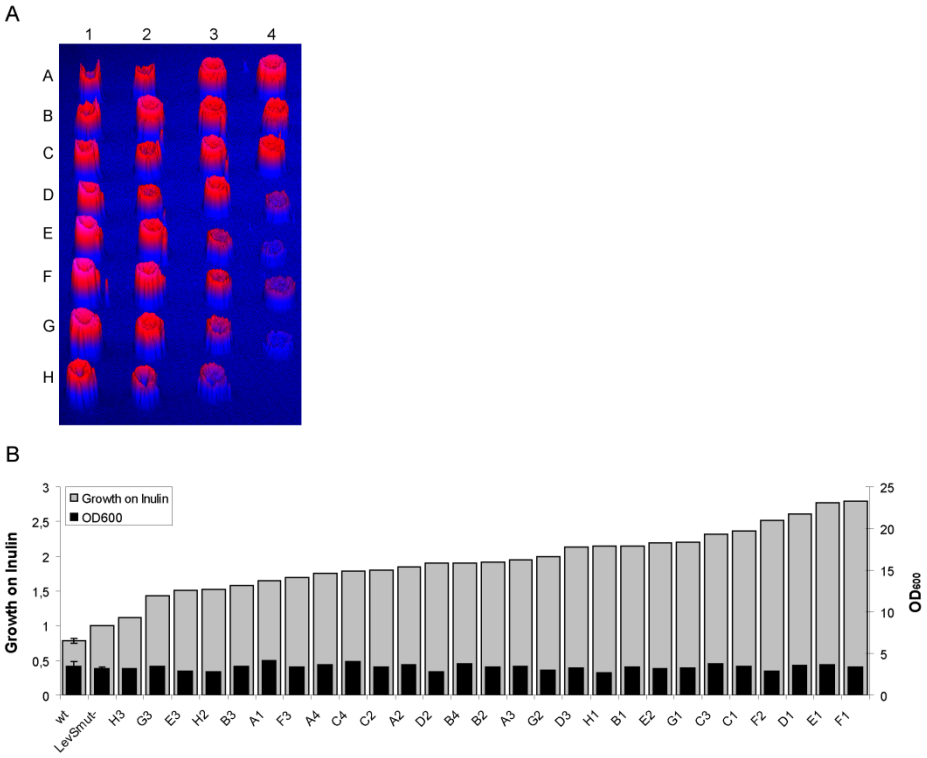

Fig. 2: Re-screening of gene library transformants.

Gene library transformants were grown to stationary phase and spotted onto a buffered minimal inulin plate to determine levanase expression. Spots of gene library transformants (columns 1-3, positions 4A-4C), GS200 *aox1*Δ::Lev*SHIS4* *aox2*Δ::*ARG4* (LevSmut$^-$; positions 4D & 4F) and CBS 7435 (wt; positions 4E & 4G) were scanned in a G:box, converted into a 3D image (A) and quantified using the GeneTools software (SynGene) after four days of growth (B). In the second rescreening all gene library transformants (H3-B4) showed enhanced growth and therefore increased levanase expression. Growth on inulin was normalized for the LevSmut$^-$ strain. For CBS 7435 data are given as means ± SD. Stationary deep-well cultures had reached comparable OD_{600} values allowing an unbiased growth test on inulin plates.

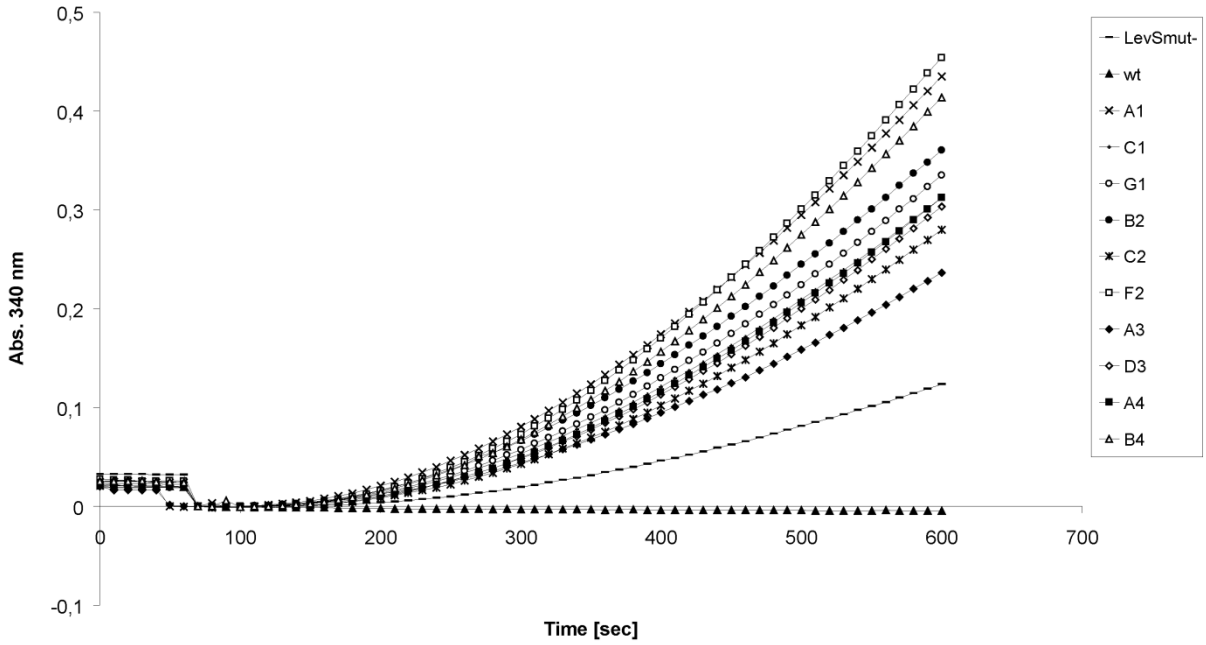

Fig. 3: Determination of levanase activity in culture supernatants.

Culture supernatants of several gene library transformants (A1-B4), GS200 *aox1*Δ::Lev*SHIS4* *aox2*Δ::*ARG4* (LevSmut$^-$) and wild type CBS 7435 (wt) were tested for levanase activity by assessing NADH formation at 340 nm over 10 min

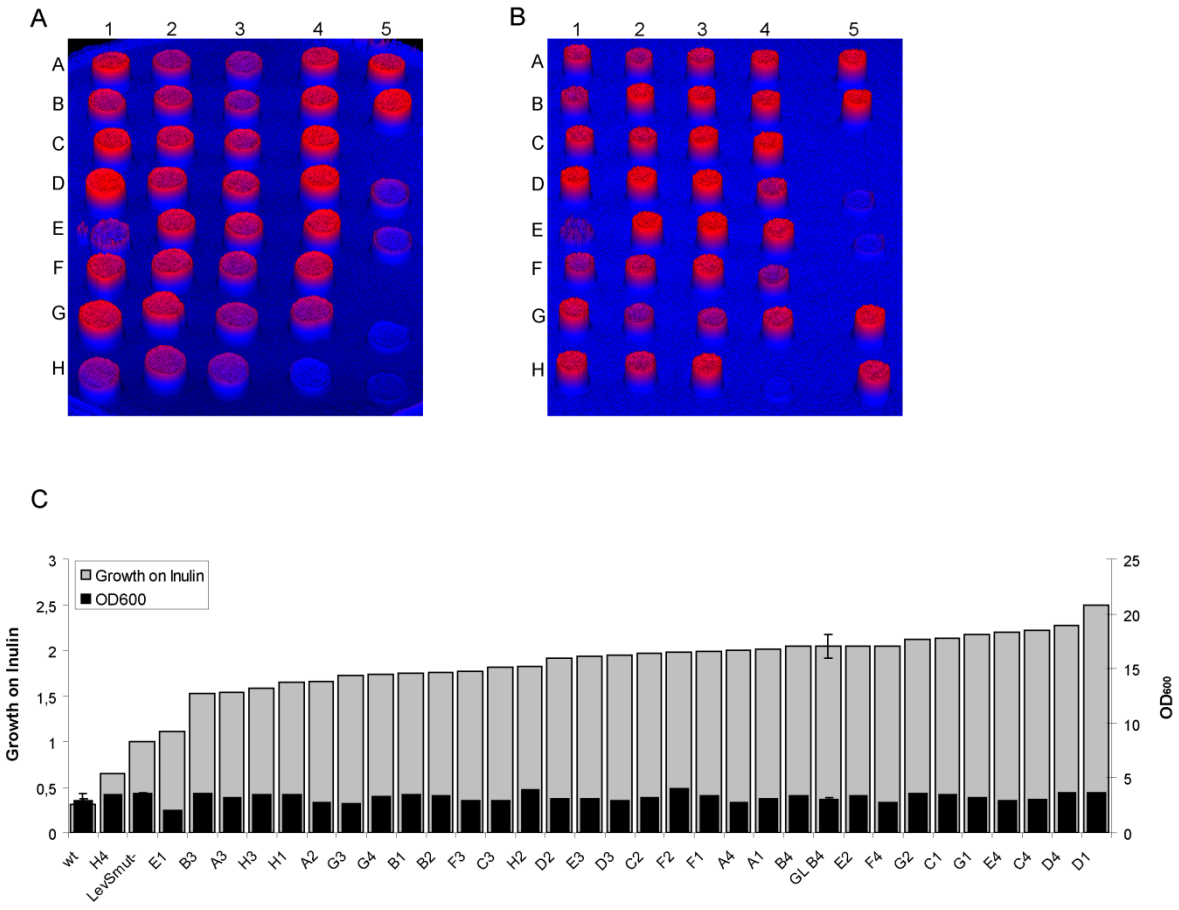

Fig. 4: Transformation of gene library fragment containing functional *AOX1*.

A couple of transformants carrying the re-transformed gene library fragment of strain GL B4 were grown to stationary phase and spotted onto buffered minimal inulin (A) or methanol (B) plates. Spots were scanned after four days of growth and a 3D-image was generated using a G:box (SynGene). Quantification of the spots of re-transformants (columns 1-4), initial gene library transformant GL B4 (positions 5A & 5B), GS200 *aox1*Δ::Lev*SHIS4 aox2*Δ::*ARG4* (LevSmut$^-$, positions 5D & 5E) and wild type CBS 7435 (wt, positions 5G & 5H) using the GeneTools software (SynGene) is shown (C). Growth on inulin was normalized for the LevSmut$^-$ strain. For GL B4 and CBS 7435 data are given as means ± SD. Stationary deep-well cultures had reached comparable OD_{600} values allowing an unbiased growth test on inulin plates.

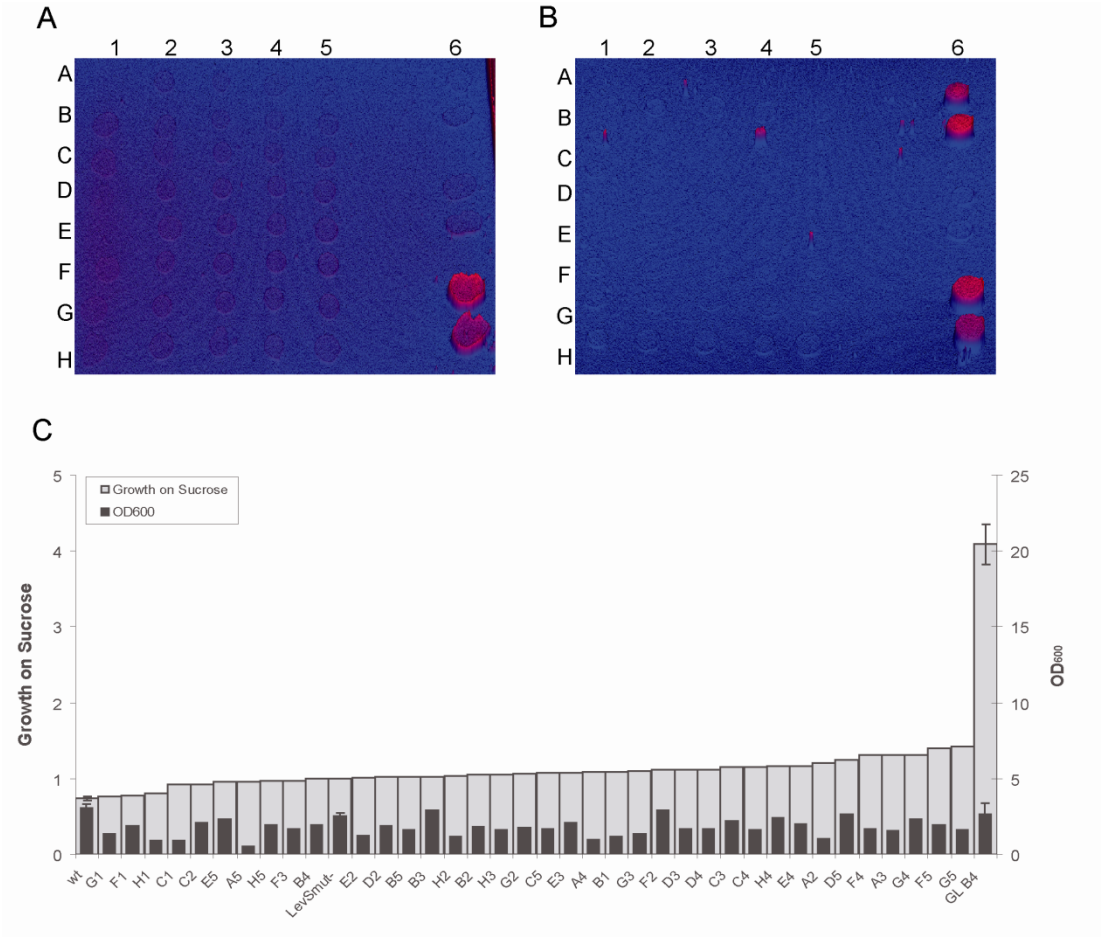

Fig. 5: Transformation of a non-functional allele of *AOX1*.

Transformants containing a non-functional *AOX1* allele (columns 1-5), wild type strain CBS 7435 (wt, positions 6A & 6B), GS200 *aox1*Δ::Lev*SHIS4* *aox2*Δ::*ARG4* (LevSmut$^-$, position 6D & 6E) and original gene library transformant GL B4 (position 6G & 6H) were grown to stationary phase and spotted onto buffered minimal sucrose (A) or methanol (B) plates. Spots were scanned in a G:box (SynGene) after four days of growth, converted into a 3D-image and quantified using the GeneTools software (SynGene) (C). No enhanced levanase expression was detected after transformation of the non-functional *AOX1* allele. Growth on sucrose was normalized for the LevSmut$^-$ strain. For GL B4 and CBS 7435 data are given as means ± SD. Growth on sucrose plates was not biased by the density of the stationary liquid cultures given as OD_{600} values.

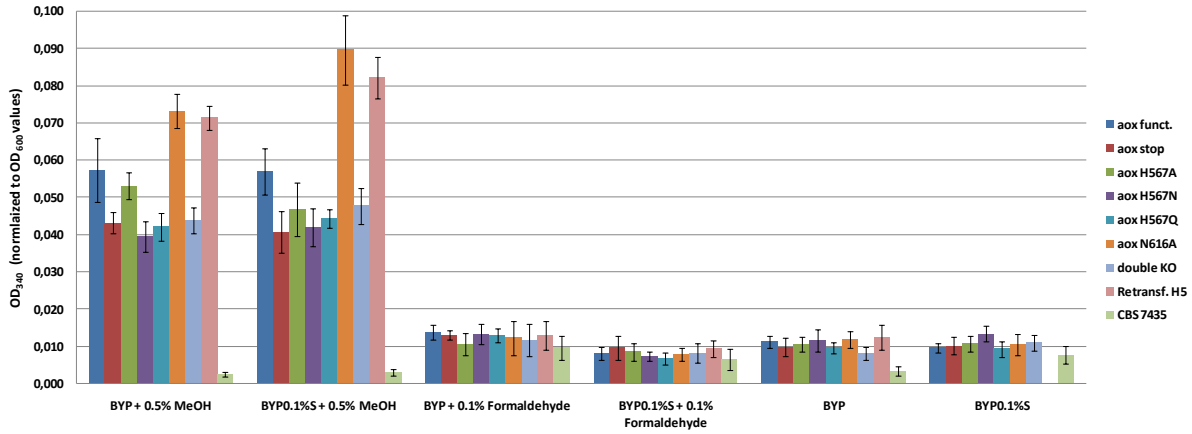

Fig. 6: Determination of levanase activity in culture supernatants to investigate the possible role of formaldehyde as inducer of $_{PAOX1}$.

Culture supernatants of the different AOX1 mutants, gene library fragment retransformants (aox funct., Retransf. H5), GS200 *aox1*Δ::Lev*SHIS4 aox2*Δ::*ARG4* (LevSmut$^-$) and wild type CBS 7435 (wt) were tested for levanase activity by assessing NADH formation at 340 nm after 20 min. of conversion (end point measurement). Data were normalized to OD_{600} and are given as means +/- standard deviation.

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Chapter 5

Constitutive Over-expression of an Autologous Methyl-carboxyl-esterase in *Pichia pastoris* Circumvents Direct Methanol Induction of *AOX1* Promoter

My contribution to the paper draft is as follows:

- Conceptual formulation (80 %)
- Supervision of diploma thesis of Tamara Nudl (95 %)
- Writing of the research article (70 %)

Constitutive overexpression of an autologous methyl-carboxyl-esterase in *Pichia pastoris* circumvents direct methanol induction

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ABSTRACT

Pichia pastoris AOX1 promoter expression system has been for decades a versatile tool for high yield protein production. A major drawback is the use of methanol for industrial applications as it is toxic and explosive. This study describes the circumvention of direct methanol induction by the constitutive expression of different *Pichia pastoris* esterase / lipase constructs and its secretion into the media. Esterase / lipase expression was driven by glyceraldehyde-3-phosphate dehydrogenase promoter. *Hevea brasiliensis* hydroxynitrile lyase was used as model protein in the form of a characterized high level Hnl expression strain which was transformed with the esterase / lipase constructs. Shake flask fermentations under biodiesel or methyl oleate induction conditions followed by the detection of Hnl protein via western blot revealed that the expression of an autologous methyl carboxyl esterase led to the indirect production of methanol by the cleavage of alternative long chain fatty acid methyl esters like biodiesel. Thereby, an equal Hnl expression level could be achieved already after 13 hours of induction compared to the expression level of the methanol induced reporter strain after an induction time of 49 hours.

INTRODUCTION

Pichia pastoris has been for many years now an excellent expression system regarding high product yields, cheap media, high cell densities, easy product availability by secretion, versatile expression systems, the possibility of adaptation to higher eukaryotic glycosylation patterns and much more.

The two most commonly used recombinant protein production systems are based on either constitutive expression by use of the e.g. glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter or the highly inducible, tightly regulated alcohol oxidase 1 (AOX1) promoter. The strong constitutive GAP promoter derives from glyceraldehyde-3-phosphate dehydrogenase, one of the key enzymes in glycolysis. As distinct from AOX1 promoter cells are grown on glucose media, but under optimal conditions similar expression rates can be attempted as seen with AOX1 promoter (1). For industrial applications, an advantage as opposed to AOX1 promoter becomes evident: the waiver of methanol. Recently, Qin *et al.* reported the creation of a functional GAP promoter library for fine-tuned, constitutive gene expression (2), promising distinct control of gene expression and its evaluation avoiding an overstress of the physiological capacities of the cell.

AOX1 promoter, as several other commonly used inducible promoters in *Pichia* like formaldehyde dehydrogenase (FLD1) (3) or dihydroxacetone synthase (DHAS) (4) promoter, is involved into the methanol utilization pathway (5). Due to the vast amounts of methanol, a toxic and highly explosive component, necessary for induction purposes large scale recombinant protein production in *Pichia* entails certain risks. Additionally, the enormous expression capacity of AOX1 promoters can lead to an overstrain of the secretion machinery. Several attempts have been made to avoid methanol induction by the design of (synthetic) promoters to reach similar or even higher product amounts or to optimize the expression cassette individually for a particular protein (6)(7)(8).

In this study we present a versatile new method to avoid methanol induction by the co-expression of an autologous methyl carboxyl esterase and thereby ensuring the supply with methanol by cleavage of methyl-ester rich compounds like e.g. biodiesel which is cheaper, less toxic and able to exploit waste products like deep frying oil. Induction of the construct

harboring the methyl carboxyl esterase with biodiesel lead to an equal HNL expression level compared to the high yield expression level of the methanol induced reporter strain (9) after an induction time of 49 hours. Remarkably, the expression level had already been reached after 13 hours of induction with biodiesel. These results bear a big potential for the development of an alternative strong expression system.

MATERIALS AND METHODS:

Strains and materials

E.coli Top 10F´[lacI^{q} Tn10(tet^{R})] mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str^{R}) endA1 λ^{-}] was used as host for recombinant DNA manipulations. *P. pastoris* CBS7435 wild type strain was used as the host for the newly created esterase/lipase expression plasmids for preliminary screenings. HNL high level expression strain *P. pastoris* GS115[pHILD 1.17-HNL] (9) was used as a reporter strain for esterase/lipase co-expression. *E.coli* media components were purchased from AppliChem (VWR International GmbH, Vienna, Austria) or LabM Limited (Dr. Friedrich Bertoni GmbH, Vienna, Austria), respectively. *Pichia* media components were purchased from BD Biosciences (Becton Dickinson Austria GmbH, Vienna, Austria). Zeocin was purchased from InvivoGen (Eubio, Vienna, Austria). α-naphthyl acetate, Fast Blue B, methyl acetate, anti-rabbit IgG (alkaline phosphatase conjugated), tetra base [4,4-methylenebis(N,N-dimethyl-aniline] (98 %) and BCIP/NBT Purple Liquid were ordered from Sigma Aldrich Handels-GmbH (Vienna, Austria). Y-PER was purchased from Fisher Scientific Austria GmbH (Vienna, Austria). Diabur test glucose 5000 was purchased from Roche Diagnostics GmbH (Vienna, Austria). Filter paper discs for alpha-naphthyl acetate assay were ordered from Whatman (VWR International GmbH, Vienna, Austria). Methyl oleate and copper-(II) ethyl-acetoacetate were purchased from ABCR (Karlsruhe, Deutschland). Biodiesel was a kind gift from Prof. Martin Mittelbach (Organic chemistry, Karl Franzens University Graz, Austria) and contained 0.156 vol % MeOH which is therefore neglectable. Mandelonitrile was a kind gift from DSM fine chemicals Austria and contained less than 0.5 % benzaldehyde. *Hevea brasiliensis* HNL primary antibody was a kind gift from Prof. Günther Daum (Institute of Biochemistry, University of Technology Graz, Austria). Phusion polymerase, GeneJet Plasmid Miniprep Kit, dNTPs, DNA ladders, protein mass rulers and restriction enzymes were purchased from Fermentas GmbH, St. Leon-Rot, Germany. WizardSV Gel & PCR Clean up Kit and T4 DNA Ligase were purchased from Promega GmbH (Mannheim, Germany). Vivaspin columns were purchased from Sartorius Mechatronics Austria GmbH (Vienna, Austria). NuPage 12 % Bis/Tris gels and 4 x LDS loading dye were purchased from Invitrogen (Life Tech Austria, Vienna, Austria). Agarose was ordered from

Biozym (Hessisch Oldendorf, Germany). Biodyne A membranes (pore size 0.45 μm) were purchased from PALL Austria GmbH, Vienna. All other chemical reagents used in this study were purchased from Roth/ Lactan (Graz, Austria).

General media and cultivation conditions

E.coli strains were cultivated in Luria-Bertani medium [1 % tryptone, 0.5 % yeast extract, 0.5 % sodium chloride] containing 25 μg/ml zeocin or 40 μg/ml kanamycin. *Pichia* precultures for fermentations were made with YPD [2 % peptone, 1 % yeast extract, 2 % glucose]. Zeocin selection of *P. pastoris* pPpT4_GAP_αS transformants was performed on BYPD Zeocin plates [2 % peptone, 1 % yeast extract, 2 % glucose, 200 mM potassium phosphate buffer pH 7.0, 2 % agar, 100 μg/ml zeocin]. Geneticin selection of pPpKan_S transformants was done on YPD geneticin plates [2 % peptone, 1 % yeast extract, 2 % glucose, 2 % agar, 300 μg/ml geneticin (G418)]. Shake flask cultures of reporter strain control fermentation were grown in ½ BYPD for 24 h and induced continuously for overall 49 h with 100 % methanol to a final methanol concentration of 0.5 % (v/v) during the day (8 h) and 1 % (v/v) during the night (16 h). For alpha-naphthyl acetate assay, *Pichia pastoris* transformants were grown on MD plates [1,34 % yeast nitrogen base w/o amino acids; $4x10^{-5}$ % biotin, 2 % dextrose, 2 % agar].

Generation of autologous esterase/lipase expression plasmids

4 potential annotated esterase or lipase genes were chosen for cloning according to BOGAS and BLAST search results. Genomic DNA of Pichia pastoris strain CBS7435 was prepared by use of the Invitrogen EasyDNA Kit manual. pPpT4_GAP_αS and pPpT4Kan_S vectors were kindly provided by T. Hajek. These vectors do not provide a Kozak sequence, which therefore had to be considered in primer design. Additionally, Kex2p and Ste13p recognition sites for better processing of the secretion signal sequence were added to the primers for pPpT4_GAP_αS constructs (Table 1). PCR reactions for the isolation of esterase/lipase genes were prepared with Finnzymes Phusion polymerase using by default the following conditions: 98 °C 3´//98 °C 10´´/55 °C 30´´/72 °C 30´´(25 cycles)//72 °C 10´/4 °C ∞. For pPpT4_GAP_αS_lipase construct it was necessary to perform overlap extension PCR (oePCR)

to skip the natural signal sequence and to remove internal *Xho*I restriction site. Therefore, 2 primer pairs were created (Table 1). Fragments were generated using the default PCR conditions as described above. Fragments were gel purified, concentration was determined and fragments were used for oePCR. Primers were added after 6 cycles of primerless PCR for an initial annealing and elongation of the fragments. PCR profile was like the following: 98 °C 3´//98 °C 30´´/55 °C 30´´/72 °C 2´(6 cycles)//72 °C 1´/4 °C ∞ followed by profile 2: 98 °C 3´//98 °C 10´´/55 °C 30´´/72 °C 30´´(25 cycles)//72 °C 10´/4 °C ∞. All fragments were gel purified before restriction. For cloning into pPpT4_GAP_αS plasmid, all fragments were restricted with *Xho*I and *Not*I followed by gel purification before ligation and transformation into *E.coli* Top10F´electrocompetent cells. Plasmid DNAs of transformants were isolated, restricted and analyzed by agarose gel electrophoresis. Plasmid DNAs of positive clones were sent for sequencing for confirmation. To analyze the indirect induction effect of the four different esterase/lipase constructs, expression plasmids were planned to be transformed into the reporter strain *P. pastoris* GS115[pHILD 1.17-HNL]. As this strain is already zeocin resistant, the resistance marker of the esterase/lipase constructs had to be changed. For this reason, the expression cassettes were integrated into pPpKan_S vector via *Smi*I and *Not*I restriction sites. Restriction analysis revealed the correct clones which could be right away utilized for *Pichia pastoris* GS115[pHILD 1.17-HNL] transformation.

Screening assays for esterase/lipase activity

For alpha-naphthyl acetate assay, *Pichia pastoris* transformants were grown on MD plates for 2 days at 28 °C. Colonies were lifted with filter paper discs and dried for 5 min. at 28 °C. 1 ml of the screening mix, containing 375 µl alpha-naphthyl acetate [12 mg/ml in acetone], 175 µl Fast Blue B [20 mg/ml in ddH_2O] and 5 ml 0.1 M Tris-HCl buffer pH 7, was dropped onto a glass plate and the dried filter was placed colony-side up onto the solution. Active colonies showed a brownish-violet color after 1-3 min. of incubation. For pH shift assays, *Pichia pastoris* transformants were grown on a biodyne nitrocellulose membrane placed on MD plates for 2 days at 28°C and afterwards dried for 5 min. at 28 °C. Before application of the screening mix, the dried membrane was equilibrated for 30 min. on a filter paper saturated with the same buffer that was used for the screening mix, 10 mM Tris-HCl buffer pH 8. 1 ml of the screening mix, containing 10 g/l phenol red and 10 mM Tris-HCl buffer pH

8, was dropped onto a glass plate and the equilibrated membrane was placed colony-side up onto the solution. After 10-15 minutes of incubation active colonies shifted the color from red to yellow. Rhodamine B assay for the detection of lipases was based on the publication of Kouker&Jaeger (10). Therefore, *Pichia pastoris* transformants were grown on rhodamine B plates consisting of 1.34 % yeast nitrogen base without amino acids, $4x10^{-5}$ % biotin, 0.5 % dextrose, 2 % agar, 1 % methyl oleate, 200 mM potassium phosphate buffer pH 7 and 0.001 % rhodamine B. After 2 days of incubation fluorescence of the transformants containing an active lipase could be detected under UV light. The following control strains were applied in all 3 screening assays: APLE_A1, which is a *Pichia pastoris* strain expressing the alternative pig liver esterase (11) as a positive control for esterase activity, *P. pastoris* CBS7435 wild type strain, *P. pastoris* CBS7435[pPpT4_GAP_αS] and *P. pastoris* GS200 Aox1/Aox2 double knockout strain (IMBT strain collection # 3382, TU Graz, Austria).

SDS PAGE and Native PAGE of the *Pichia pastoris* CBS7435 strains expressing autologous esterases/lipases

350 ml baffled flasks each containing 50 ml YPD medium were inoculated with a single colony of the remaining best active clone of each construct and incubated at 28 °C and 100 rpm. After 24 hours, the main culture containing 125 ml YPD medium with 3 % glucose was inoculated with the pre-culture to an OD_{600} of 0.01. After 36 and 44 hours either 1 % glucose or 0.5 % methyl oleate were added, respectively. After another 12 hours of incubation at 28 °C and 100 rpm fermentation was stopped and samples for PAGE were prepared. For this purpose, fermentation broths were centrifuged and supernatants were concentrated by use of Vivaspin concentrator tubes. For SDS PAGE, 5 µl of the concentrated supernatants were mixed with 5 µl 4 x LDS loading dye and 1 µl 1 M DTT, heated to 95 °C for 10 min. and loaded onto a 12 % NuPAGE gel together with 5 µl of Page Ruler prestained protein ladder. SDS PAGEs were run according to the manufacturer information. Native PAGE was performed according to the manuscript from (12). 24 µl of the concentrated supernatants were mixed with 6 µl 6 x loading dye [150 mM ε-aminocaproic acid, 50 % glycerol, 0.025 % Coomassie G250] and loaded onto the native gel. Running conditions were 200 V, 24 mA for 90 min. at 4 °C. After the run the gel was rinsed with ddH_2O and incubated with the alpha-naphthyl

screening solution (see “Screening assays for esterase/lipase activity”). Active enzymes on ester substrate appeared as brownish violet bands within the gel.

in situ HNL activity assay

This assay is based on the Prussian-Blue reaction described by Feigl and Anger (13). The detection paper was prepared by soaking Whatman filter paper with a chloroform solution, containing 0.5 % (w/v) copper-(II) ethyl-aceto acetate and 0.5 % (w/v) tetra base [4,4-methylenebis(N,N-dimethyl-aniline] (98 %). Dried, almost colourless test-paper was stored in a dark and dry place for two weeks. For preparation of the substrate solution 8 µl racemic mandelonitrile were diluted in 1 ml citrate-phosphate-buffer (pH 3.5, 100 mM). For determination of Hnl activity, *P. pastoris* GS115[pHILD 1.17-HNL] strain transformants carrying the different esterase/lipase constructs were inoculated from glycerol stocks in 250 µl BYPD (1 % glucose) medium in deep well plates. After 24 hours at 28 °C and 320 rpm, cultures were diluted 1:100 with 0.9 % NaCl and stamped onto Biodyne A membranes (pore size 0.45 µm) lying on BMD (1 % glucose) plates, which were incubated for 24 hours at 28 °C. After that, filters were transferred to different induction plates (buffered minimal agar plates [1,34 % yeast nitrogen base w/o amino acids; $4x10^{-5}$ % biotin, 2 % agar] stuffed with different components; for details see Table 2) and incubated for 24 hours. For 48 hours of induction, filters were laid onto fresh induction plates after 24 hours. The membranes with colonies were equilibrated for 30 minutes on a moisture filter paper soaked with a 100 mM citrate-phosphate-buffer pH 5.3. Membranes were then placed on a filter paper soaked with substrate solution colonies upside down. A permeable nylon tissue was placed above it and covered with the detection paper which was fixed with a Petri dish top and was not allowed to move during the developing time, until blue spots emerged on the detection paper (14). The reaction was stopped at two time points, 30 sec. and 2 min. Detection filters were scanned for documentation.

SDS PAGE and Western Blot for indirect induction effect analysis

Based on the *in situ* HNL activity assay data, 2 active clones of each esterase/lipase construct were chosen for baffled shake flask fermentation under methyl oleate or biodiesel induction conditions, respectively. Therefore, precultures, 25 ml BYPD medium each, were inoculated with single colonies of the different strains. After incubation for 24 h at 28 °C and 130 rpm, the main cultures (50 ml ¼ BYPD [0.5 % peptone, 0.25 % yeast extract, 0.5 % glucose, 200 mM potassium phosphate buffer pH 7.0]) were inoculated with the precultures to an OD_{600} of 0.01 and incubated at 28 °C and 130 rpm. OD_{600} and glucose concentration were measured repeatedly. After stationary phase was reached and glucose was depleted, induction phase using methyl oleate, biodiesel or methanol was initiated (see Table 3 for details) and was continued for altogether 49 h. OD_{600} was measured repeatedly during the induction phase and samples were taken at different time points according to the lowest OD_{600} (max. 500 µl). Samples were centrifuged at 10000 rpm for 5 min, supernatants were withdrawn and the pellets were stored at -20 °C until proceeded. For SDS sample preparation, pellets were resuspended in the same volume of 0.1 M phosphate buffer as the sampling volume was. Half of the cell suspension was transferred to weighed tubes, cells were pelleted by centrifugation and the pellets were weighed. 5 ml Yeast Buster reagent and 50 µl of 100 x THP solution were added per g pellet and pellets were resuspended. After incubation for 30 min. at room temperature and 500 rpm and a centrifugation step, 20 µl of the supernatants were mixed with 2 µl 1 M DTT each and incubated for 30 min. at 300 rpm and RT. 3 µl of the samples were mixed with 2 µl of 4 x LDS loading dye and incubated for 10 min. at 70°C before being applied to NuPAGE gel electrophoresis. SDS PAGEs were run according to the manufacturer information. Gels were either stained with Coomassie Brilliant Blue and destained with 10 % acetic acid or used for western blot. Proteins were electro-blotted to a nitrocellulose membrane (0.2 µm) with a transfer buffer containing 0.29 % Tris, 1.44 % glycin and 20 % (v/v) MeOH, Ponceau S stained and rinsed with ddH_2O. Blocking was performed with 1x TBST (0.303 % Tris, 0.876 % NaCl, 0.05 % Tween 20, pH 7.5) with 5 % skimmed milk for 1 h at RT. Primary anti-*Hb*HNL antibody (1:5000 dilution in 1x TBST 5 % skimmed milk) was applied for 2 hours on a shaker. After 5 x 5 min washing steps with 1 x TBST buffer, the membrane was incubated with a 1:30000 dilution in 1 x TBST 5 % skimmed milk of anti-rabbit IgG secondary antibody conjugated with alkaline phosphatase for 1 h at RT. After several washing steps (3 x 5 min with 1 x TBST, 2 x 5 min. with 1 x TBS

without Tween), detection was made with BCIP/NBT substrate. Membranes were scanned for documentation.

Miscellaneous methods

Recombinant DNA methods were performed essentially as described previously by Sambrook *et al*. (15). Plasmid DNA was isolated by use of the GeneJet Plasmid Miniprep Kit from Fermentas GmbH (Thermo Scientific Inc.). Pichia pastoris genomic DNA was prepared using the EasyDNA Kit (Invitrogen Life Tech, Vienna, Austria). Plasmid-DNA digested with restriction enzymes and used for restriction mapping and cloning of fragments, PCR products were separated on Tris-acetate-EDTA agarose gels, DNA fragments were purified from agarose gels using Wizard SV Gel and PCR Clean-up Kit (Promega, Mannheim, Germany). Sequencing was carried out by LGC Genomics GmbH (former Agowa, Berlin, Germany). MsDOS program primer.exe was used for primer design. Gene-specific primers were synthesized by Invitrogen Life tech (Vienna, Austria) or Integrated DNA Technologies Inc. (Munich, Germany). DNA alignments were done with Contig Xpress (VNTI, Invitrogen Life tech, Vienna, Austria), vector maps were designed with VectorNTI 8, Invitrogen Life tech, Vienna, Austria.

RESULTS

Identification of potential esterases and lipases

For the identification of autologous esterases and lipases in *Pichia pastoris*, BOGAS internet platform (16) or NCBI BLAST searches with described fungal esterases or lipases were performed. Four genes were chosen for cloning (Table 4): a methyl carboxyl esterase, a steryl ester hydrolase which is according to BOGAS assumed to be placed on lipid particle membranes and implicated in sterol homeostasis, a serine esterase and a lipase, the two latter containing a natural signal sequence as predicted by SignalP.

Expression vector construction and transformation

The genes of the four chosen enzymes were PCR-amplified from *Pichia pastoris* CBS7435 strain. Purified PCR products were restricted with appropriate restriction enzymes and ligated with restricted and purified plasmid pPpT4_GAP_αS, containing the constitutive glyceraldehyde-3-phosphate-dehydrogenase (GAP) promoter and the *Saccharomyces cerevisiae* α-factor secretion signal for constitutive expression and secretion of the autologous enzymes. Sequenced plasmids and initial plasmid were linearized with *Smi*I, transformed into *P. pastoris* CBS7435 strain and selected on zeocin containing BYPDS agar plates.

Activity screening

For a general esterase activity screening, α-naphthylacetate assays of the Pichia transformants were performed. All tested transformants showed increased activity towards this substrate compared to the negative control (data not shown). Two active colonies of each construct were retested and the preliminary results could be confirmed (Supplemental data; Fig. S1). pH shift assay was the method of choice to test for the substrate specificity of the enzymes or their general ability to cleave methyl esters. Methyl acetate, a methyl ester, was converted and the pH shift was visible as the active colonies turned from red to yellow

(Supplemental data; Fig. S2). As the work focused mainly on the utilization of long chain methyl esters like methyl oleate or biodiesel components, a rhodamine B plate assay was applied. Active colonies, which were able to convert the long chain fatty acids, became fluorescent under UV light (Fig. 1). Table 5 shows a summary of the 3 activity screenings. The four best clones of the preliminary activity screenings were chosen for continuative experiments.

SDS PAGE and Native PAGE analysis:

The 4 hits of the preliminary screenings were fermented in baffled shake flasks with YPD medium or YPD/methyl oleate medium, respectively. After 56 hours, fermentation broths were harvested; supernatants were concentrated with Vivaspin columns and subjected to SDS PAGE and native PAGE analysis. Figure 2 shows the 12 % NuPAGE gel of the *P. pastoris* CBS7435[pPpT4_GAP_αS_MCE_2] transformant, which was the only one to show an additional, distinct band in the approximate size of 47 kDa corresponding to the molecular weight of the over expressed methyl carboxyl esterase. Data could be confirmed by native PAGE analysis. Only *P. pastoris* CBS7435[pPpT4_GAP_αS_MCE_2] concentrated samples, no matter if fermented under glucose or glucose/methyl oleate conditions, gave a clear signal when stained with α-naphthylacetate (Figure 3). Native gels with periplasmic or pellet fractions did not give any signal at all (data not shown).

Vector construction and *Pichia pastoris* reporter strain transformation for indirect induction effect

In order to have an appropriate selection marker system for the co-expression of the different esterase/lipase constructs in the reporter strain *Pichia pastoris* GS115[pHILD 1.17-HNL], a high level intracellular HNL expression strain (9), and in another reporter strain already harboring zeocin resistance (data not shown), the selection marker cassette of the four expression plasmids was changed to kanamycin/geneticin resistance. The four new constructs were linearized and transformed into *Pichia pastoris* GS115[pHILD 1.17-HNL] strain.

HNL plate activity screen for semi-quantitative determination of HNL production

16 clones per constructs were pre-cultured in BYPD media in deep well plates for 24 hours. A 1:100 dilution of the fermentation broth was stamped onto Biodyne A membranes lying on BMD plates. After 24 h, the membranes were transferred to the appropriate induction plate (Table 2) followed by an incubation step of 24 or 48 h, respectively. With these membranes, the in situ HNL assay was performed as described elsewhere (14). The assay was performed in triplicates. Figure 4 shows the results with methyl oleate and Figure 5 the results with biodiesel substrate, respectively. HNL production seemed to take place earlier in the constructs harboring the over-expressed esterase/lipase. In the esterase/lipase co-expressing strains, HNL activity was already detectable after 24 h of induction, whereas HNL activity in the high level reporter strain *P.p.* GS115[pHILD 1.17-HNL] was not seen until 48 h of induction. Furthermore, the signal of the reporter strain appeared much slower compared to the color development in the new co-expression strains. This result implicates an influence of the co-expressed esterase/lipase on the early stage induction of the P_{AOX1}.

SDS PAGE and western blot for quantitative determination of HNL production

2 clones of each construct that gave a positive signal in the in situ HNL assay were chosen for shake flask fermentation under methyl oleate or biodiesel induction conditions over a time period of altogether 49 h. Samples were taken before and after 3, 13, 17, 21, 25, 37, 41 and 49 h of induction. To be able to make a quantitative statement about the HNL expression level, SDS PAGEs and western blots were performed. The reporter strain *P.p.* GS115[pHILD 1.17-HNL] was included as a positive control whereat in this case methanol was chosen as inducing agent; fermentation conditions were the same. Due to intracellular HNL expression, cell suspensions were solubilized with YPER reagent, centrifuged and the supernatant was applied to SDS PAGE and further on western blot detected with specific anti-*Hb*HNL antibody. Figures 6 and 7 show the results for the fermentations of the reporter strain P.p. GS115[pHILD 1.17-HNL] and the best performing strain *P. pastoris* CBS7435[pPpT4_GAP_αS_MCE_1_D1] under biodiesel induction conditions. While the reporter strain is not able to use biodiesel as an inducing agent in a satisfying manner, showing just a basic, weak level of HNL production, clone MCE_D1 shows already a very high

level of HNL expression after 13 h induction that remains stable over the whole period of induction to the same extent reaching at least the expression level of the native reporter strain P.p. GS115[pHILD 1.17-HNL] under methanol induction conditions. These results implicate that an indirect methanol induction using an expression strain constitutively over expressing a methyl carboxyl esterase for biodiesel conversion is possible and is an excellent alternative to methanol induction.

DISCUSSION:

For years, the methanol inducible expression system of the yeast *Pichia pastoris* has been the system of choice in a broad variety of biotechnological and pharmaceutical applications. Unfortunately, a major drawback has to be taken into account: the large-scale use of a highly explosive, toxic component. This study presents an alternative approach, namely the indirect induction of the AOX1 promoter by constitutive overexpression of a methyl esterase to cleave alternative substrates like biodiesel, which is cheap, not as toxic as methanol and made from waste products like old deep fry oil. Expression of autologous, methyl ester cleaving enzymes was performed at a low, constitutive expression level, as the real, native function of the enzymes has not been investigated. A massive over-expression could have a negative effect on cell metabolism or could evoke an alteration in membrane composition, as seen with steryl ester hydrolase constructs, which showed hampered growth (data not shown). Moreover, a slight amount of methanol suffices for AOX1 promoter induction. α-naphthyl acetate assay is a versatile tool for a general screen of potential esterases as the substrate can be cleaved by almost any esterase. It gave just a hint that the autologous enzymes are really constitutively expressed. Employing the pH shift assay, specificity was raised by applying a real short chain methyl ester in the form of methyl acetate. pH shift assay has been developed to analyze esterases expressed in *E.coli* that means the method had not been adapted to *Pichia* before. Due to the acidic pH conditions prevailing in the cytoplasm of a *Pichia* cell, it would have probably been of benefit to test other indicators like e.g. bromocresol purple that changes its color under more acidic conditions. For the success of this study it was crucial to know whether the chosen enzymes are able to react on long chain fatty acid methyl esters like methyl oleate or biodiesel. Rhodamine B assay (10) was developed to test for the ability of lipases to cleave long chain fatty acids e.g. olive oil which is the standard substrate. In case of a positive result the formation of a fluorescent halo around the cell was described. But in our study no halos, but fluorescent colonies became visible under UV light. Probably methyl oleate or biodiesel cannot be taken up by the cell and the expression/secretion levels were rather low. Nevertheless clear results identifying potential candidates for continuative experiments could be obtained. The detection of the constitutively expressed autologous enzymes in *Pichia* on protein level turned out to be problematic because there were no antibodies available and a decision against a protein tag

was made. Therefore the SDS PAGE analysis was made showing solely an additional, distinct band in the size of 47 kDa in case of methyl carboxyl esterase expression. Noticeable were the differences in the protein patterns of the wild type and the strain constitutively expressing the methyl carboxyl esterase, which was probably ascribed to an alteration in the cell metabolism. Repeating the PAGE analysis with a native, non-denaturing gel, an additional band or more a smear was visible after α-naphthyl acetate treatment of the concentrated MCE samples. The smear was probably due to different glycosylation patterns. No other constructs gave a signal on the native gel. The reason could be that the enzymes got stuck in the periplasm and the expression was rather low, maybe below the detection level of the method although concentrated samples were used. Although the prescreens for all other enzyme constructs except the MCE constructs were not that promising, all four expression cassettes were finally transformed into the HNL reporter strain to test for the indirect induction effect on the AOX1 promoter. The problem of the in situ HNL assay is, in general, the reproducibility of the assay as it is very susceptible to environmental factors. Gaseous HCN is detected and so parameters like differences in growth or the airflow under the hood can create different results. To minimize these problems, all HNL assays were done at least in triplicates. The effect of the additionally expressed esterases/lipase was obvious. The signal of the HNL assay meaning the development of the blue dots was much faster than in the reporter strain itself although the overall intensity of the signal was finally the same. SDS PAGE and western blot analysis of 2 clones per construct positively HNL tested showed in case of methyl oleate fermentation just slight differences to the reporter strain. It seems that HNL is to a certain, weak extent capable of methyl oleate cleavage and therefore allocates slight amounts of methanol. The weak expression could on the other hand be caused to leakiness of the AOX1 promoter caused by glucose depletion. Additionally, the supplied methyl oleate was not 100 % pure and could contain free methanol. Altogether, product formation or secretion was not at all comparable to the expression rate reached with the methanol induced reporter strain. Induction of the construct harboring the methyl carboxyl esterase with biodiesel, in contrast, lead to an equal HNL expression level compared to the expression level of the methanol induced reporter strain after an induction time of 49 hours. Remarkably, the expression level had already been reached after 13 hours of induction with biodiesel. A GC analysis of the biodiesel was made to exclude the influence of possible free methanol in the biodiesel fraction resulting in a neglectable free methanol

concentration. Concluding the data of this study, an alternative method to methanol induction was found having a big potential for improvement and optimization that could be reached by e.g. immobilization of an appropriate methyl ester-cleaving enzyme on the surface of the *Pichia* cell or the improvement of fermentation conditions.

Acknowledgements:

Gernot Strohmeier (Institute of Organic Chemistry, University of Technology, Graz) for GC analysis of Biodiesel.

FIGURES AND TABLES

Table 1: Primers used in this study; sequences are given from 5´ to 3´

Enzyme	Primer	Sequence	Additions
Methyl-carboxyl-esterase	G+A642Kozfor	TTCACTAGTGCCACCATGACGGATGGGAATCTAATTTC	*Spe*I, Kozak
	GA+AA642rev	CTAGCGGCCGCTTAATTTTTAACACCCCATTTG	*Not*I
	GA+AA642ste	TAACTCGAGAAAAGAGAGGCTGAAGCTACGGATGGGA ATCTAATTTCTG	*Xho*I, Kex2Ste13
Sterylester-hydrolase	G+A468Kozfor	TTAGGCGCGCCGCCACCATGGATACGCCCGTCAAATC	*Asc*I, Kozak
	GA+AA468rev	CAGGCGGCCGCTTATGTATTTATTATAATG	*Not*I
	38KSF	ACACTCGAGAAAAGAGAGGCTGAAGCTATTACAGCTA ATTTACCAAAG	*Xho*I, Kex2Ste13
	41KSF	ACACTCGAGAAAAGAGAGGCTGAAGCTAATTTACCAAA GTTTGTTCTGC	*Xho*I, *Kex2Ste*13
Serine-esterase	G+A026Kozfor	AGTGGCGCGCCGCCACCATGGCAAACATTCACTTGAT	*Asc*I, Kozak
	GA+AA026rev	CAGGCGGCCGCTCAATTGGGAAACTTGAGTACG	*Not*I
	15KSF	ACACTCGAGAAAAGAGAGGCTGAAGCTAAGGCCGATCA TTTGTCTTATATCC	*Xho*I, Kex2Ste13
Lipase	G+A044Kozfor	ACAACTAGTGCCACCATGAAGTCAAAGTGGGGGATT	*Spe*I, Kozak
	GA+AA044rev	CAGGCGGCCGCTCAAAGACGTAATGCATT	*Not*I
	22KSF	ACACTCGAGAAAAGAGAGGCTGAAGCTCTCCCAACTTA TAATCAAACG	*Xho*I, Kex2Ste13
	044MUTF	ATTGTTGAGTCGTTCCGATTTTC**C**CGAGGTTG	**T-C** exchange
	044MUTR	CAAAGGAAACCCAACCTCG**G**GAAAATCGG	**A-G** exchange
P08220 AOXTTrv		GCACAAACGAAGGTCTCAC	reverse for all plasmids
P08849AOXSyn_end_fw		CAGAAGGAAGCTGCCCTGTC	forward for pPpT4_S and pPpT4_α_S
P08392		GCCGTCGCTGGCAATAATA	forward for pPpT4_GAP_S and pPpT4_GAP_α_S
Seq642for		ACATACCGGGATTCTTTTGC	Carboxyl methyl esterase
Seq642rev		GCAAAAGAATCCCGGTATGT	Carboxyl methyl esterase
SEHfor		GTGCACTGCTGTTGTCAGGT	Sterylester hydrolase
SEHrev		TCTTGGTGTAGTTGCTGGAG	Sterylester hydrolase
Seq468for		GAGGACCACATTGTTCAGACC	Sterylester hydrolase
Seq468rev		GGTCTGAACAATGTGGTCCTC	Sterylester hydrolase
SEfor		CTTGGCTCCTGGAGATGGTA	Serine esterase
SErev		TTCAGCAATCTGTTCGGTG	Serine esterase
Seq026for		AATGGCCAATCCTGCTTCG	Serine esterase
Seq026rev		CGAAGCAGGATTGGCCATTA	Serine esterase
Lipfor		TGCAGGTGGCTTATTGTGAC	Lipase
Liprev		CATCACCGAGTTGAATCCTG	Lipase
Seq044for		TCCGAGGGTCAAGATTTGCC	Lipase
Seq044rev		GGCAAATCTTGACCCTCGGA	Lipase

Table 2: Different agar plates used for induction; BM: buffered minimal

	Agar plates for induction
1	BM Sorbitol 1 %
2	BM MeOH 1 %
3	BM Methyl oleate 1 % Triton 0.1 %
4	BM Sorbitol 1 % Biodiesel 0.2 % Triton 0.1 %
5	BM Biodiesel 1 % Triton 0.1 %
6	BM Sorbitol 1 % Methyl acetate 0.2 %
7	BM Triton 0.1 %
8	BM Glucose 1 %
9	BM Methyl acetate 1 %
10	BM Sorbitol 1 % Methyl oleate 0.2 % Triton 0.1 %
11	BM Sorbitol 1 % MeOH 0.2 %

Table 3: Fermentation conditions for (indirect) methanol induction

Time point	Hours after induction	Methyl oleate/Biodiesel	Methanol
0	0	0.1 %	1%
1	3	0.2 %	
2	13	0.3 %	0.5 %
3	17		
4	21		
5	25	0.4 %	1 %
6	37		
7	41	0.4 %	0.5 %
8	45		
9	49		

Table 4: Identified autologous esterases and lipases from *Pichia pastoris* used in this study; n.d.: not determined

	NCBI accession number	locus	MW [kDa]	Isoelectric point	Signal sequence	Transmembrane domain	family	function
Methyl carboxyl esterase (MCE)	XP_002493642	PAS_chr4_0232	46,87	5,05	no	no	GxSxG superfamily with α/β hydrolase fold	n.d.
Steryl ester hydrolase (SEH)	XP_002492468	PAS_chr3_0252	51,75	6,62	no	yes (aa 1-38 or 1-41)	GxSxG superfamily with α/β hydrolase fold	Sterol homeo-stasis
Serine esterase (SE)	XP_002493026	PAS_chr3_0794	61,32	5,70	yes	no	GxSxG superfamily with α/β hydrolase fold	n.d.
Lipase	CAY67044	C034_0027	57,83	6,09	yes	no	GxSxG superfamily with α/β hydrolase fold	n.d.

Table 5: Summarized activity screening results; ++ very good activity, + good activity, ~ low activity, - no activity; MCE: methyl carboxyl esterase, SHE: steryl ester hydrolase, SE: serine esterase. All esterase/lipase constructs were transformed into *P. pastoris* CBS 7435 wild type strain.

	α-naphthylacetate	pH shift (methyl acetate)	Rhodamine B (methyl oleate)
pPpT4_GAP_αS_MCE_1	+	-	-
pPpT4_GAP_αS_MCE_2	-	+	++
pPpT4_GAP_αS_SEH38_3	+	+	-
pPpT4_GAP_αS_SEH38_4	-	-	~
pPpT4_GAP_αS_SEH41_5	+	+	++
pPpT4_GAP_αS_SEH41_6	+	~	+
pPpT4_GAP_αS_SE_7	+	~	+
pPpT4_GAP_αS_SE_8	-	~	+
pPpT4_GAP_αS_lipase_9	-	-	++
pPpT4_GAP_αS_lipase_10	-	-	-

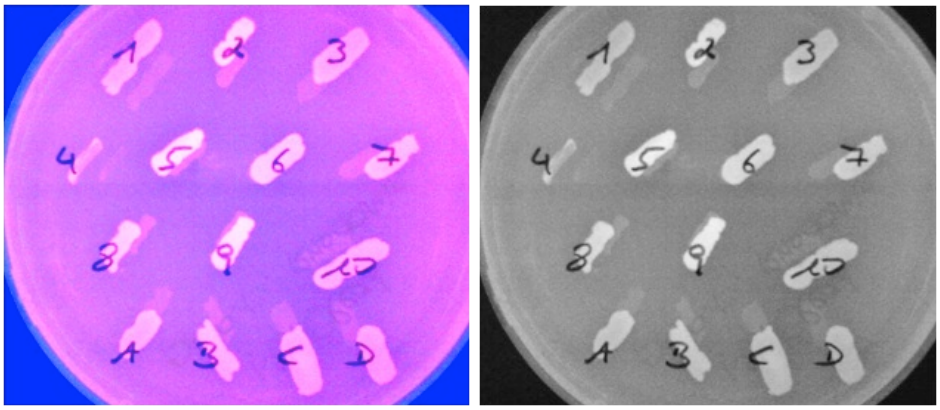

Figure 1: Rhodamine B assay of 2 clones per construct on buffered minimal methyl oleate plate. The left picture shows the streaks on plate in color on UV screen, the right picture shows the same in gray scale to better visualize the contrast. Fluorescent streaks: 2, 5, 6, 8, 9. 1, 2: pPpT4_GAP_αS_MCE; 3, 4: pPpT4_GAP_αS_SEH38; 5, 6: pPpT4_GAP_αS_SEH41; 7, 8: pPpT4_GAP_αS_SE; 9, 10: pPpT4_GAP_αS_Lipase; A: *P. pastoris* APLE_A1; B: *P. pastoris* CBS 7435 wt; C: *P. pastoris* CBS 7435 [pPpT4_GAP_αS], D: *P. pastoris* GS200[*aox1*/*aox2* double knockout]. All esterase/lipase constructs were transformed into *P. pastoris* CBS7435 wild type strain. MCE: methyl carboxyl esterase, SEH: steryl ester hydrolases, SE: serine esterase, APLE: alternative pig liver esterase

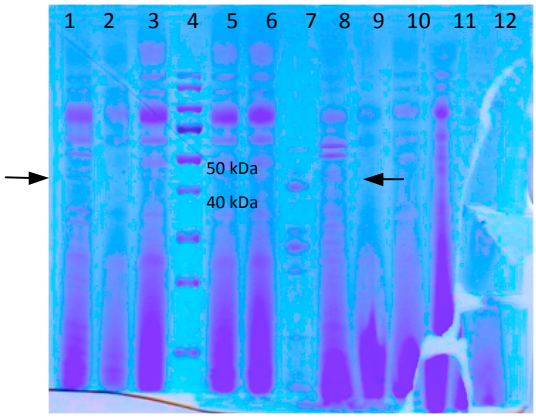

Figure 2: 12 % NuPAGE gel (1 x MOPS) of Vivaspin concentrated supernatants from YPD and YPD-Methyl oleate fermentation. The lanes contain supernatants of transformants and controls: 1: pPpT4_GAP_αS_MCE_2 (YPD), 2: pPpT4_GAP_αS_Lipase_9 (YPD), 3: *P.p.* CBS7435 wild type (YPD), 4: PageRuler Prestained Protein Ladder, 5: pPpT4_GAP_αS (YPD), 6: pGAPZαA-APLE (YPD), 7: Zymolyase [10 mg/ml], 8: pPpT4_GAP_αS_MCE_2 (YPD-Methyl oleate), 9: pPpT4_GAP_αS_Lipase_9 (YPD-Methyl oleate), 10: *P.p.* CBS7435 wild type (YPD-Methyl oleate), 11: pPpT4_GAP_αS (YPD-Methyl oleate), 12: pGAPZαA-APLE (YPD-Methyl oleate); all esterase/lipase constructs were transformed into *P. pastoris* CBS7435 wild type strain. Black arrows indicate an additional protein band of about 47 kDa putatively indicating the carboxyl methyl esterase.

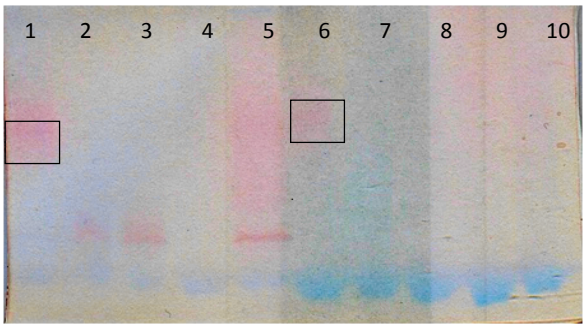

Figure 3: Native PAGE of Vivaspin concentrated supernatants from YPD and YPD-Methyl oleate fermentation stained with α-Naphthylacetate. The lanes contain supernatants of transformants and controls: 1: pPpT4_GAP_αS_MCE_2 (YPD), 2: pPpT4_GAP_αS_Lipase_9 (YPD), 3: *P.p.* CBS7435 wild type (YPD), 4: pPpT4_GAP_αS (YPD), 5: pGAPZαA-APLE (YPD), 6: pPpT4_GAP_αS_MCE_2 (YPD-Methyl oleate), 7: pPpT4_GAP_αS_Lipase (YPD-Methyl oleate), 8: *P.p.* CBS7435 wild type (YPD-Methyl oleate), 9: pPpT4_GAP_αS (YPD-Methyl oleate), 10: pGAPZαA-APLE (YPD-Methyl oleate); all esterase/lipase constructs were transformed into *P. pastoris* CBS7435 wild type strain. Black boxes mark the additional esterase activity.

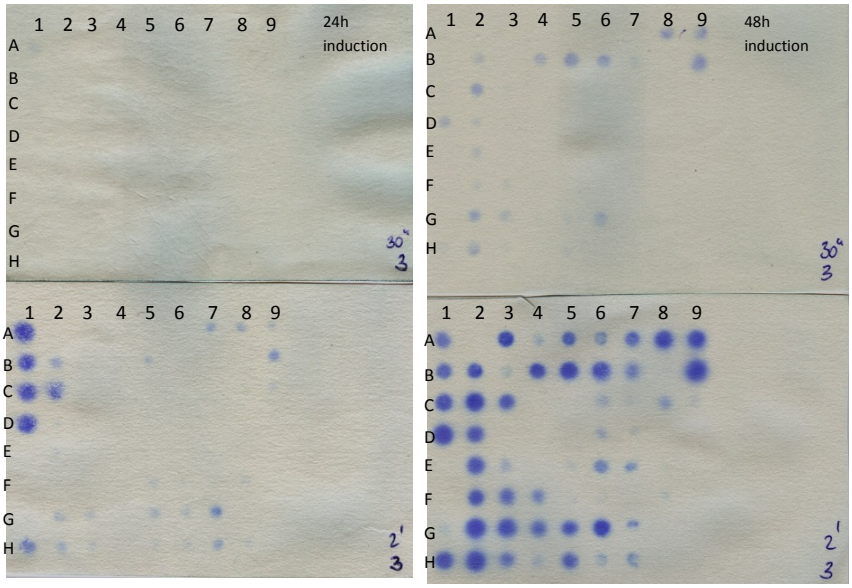

Figure 4: HNL-plate assay result of BM Methyl oleate 1 % + Triton 0.1 % induced esterase/lipase transformants. All constructs were transformed into the reporter strain *Pichia pastoris* GS115[pHILD 1.17-HNL]. The columns contain the following transformants and controls strains: 1+2: pPpT4Kan_GAP_αS_MCE, 3+4: pPpT4Kan_GAP_αS_SEH-41, 5+6: pPpT4Kan_GAP_αS_SE, 7+8: pPpT4Kan_GAP_αS_Lipase, 9: A-C *P.p.* GS115[pHILD 1.17-HNL], 9: D-F *P.p.* CBS7435, 9: G-H sterile control; left pictures 24h and right pictures 48h of induction (upper pictures detected after 30", lower pictures after 2´).

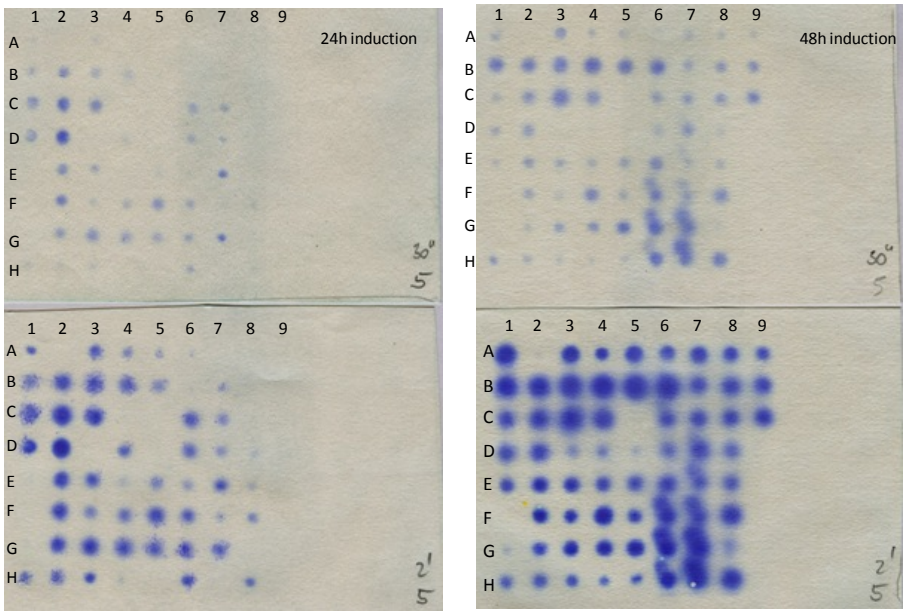

Figure 5: HNL-plate assay result of BM Biodiesel 1 % + Triton 0.1 % induced esterase/lipase transformants. All constructs were transformed into the reporter strain *Pichia pastoris* GS115[pHILD 1.17-HNL]. The columns contain the following transformants and controls strains: 1+2: pPpT4Kan_GAP_αS_MCE, 3+4: pPpT4Kan_GAP_αS_SEH-41, 5+6: pPpT4Kan_GAP_αS_SE, 7+8: pPpT4Kan_GAP_αS_Lipase, 9: A-C *P.p.* GS115[pHILD 1.17-HNL], 9: D-F *P.p.* CBS7435, 9: G-H sterile control; left pictures 24h and right pictures 48h of induction (upper pictures detected after 30", lower pictures after 2´).

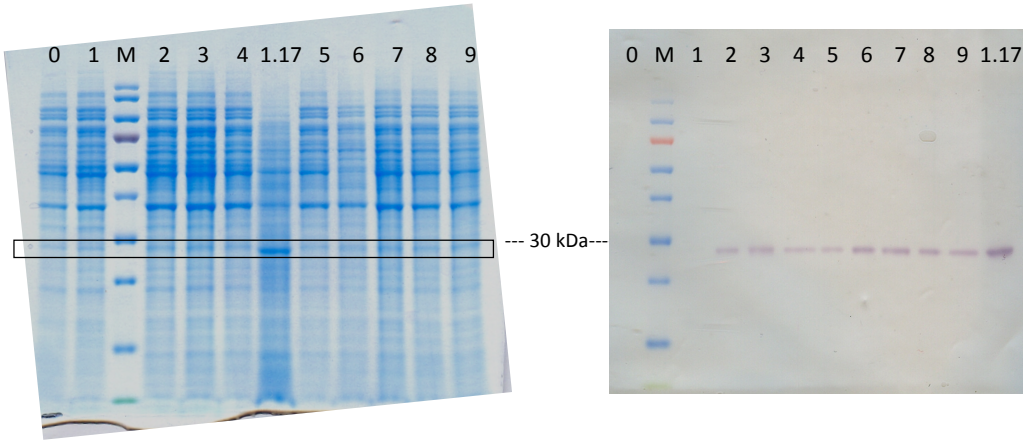

Figure 6: 12 % NuPAGE gel (left) and Western Blot (right) of fermentation broth samples of the reporter strain *P.p.* GS115[pHILD 1.17-HNL] induced with biodiesel. M: PageRuler Prestained Protein Ladder; 0: before induction (+ 0.1 %), 1: 3 h after induction (+ 0.2 %), 2: 13 h after induction (+ 0.3 %), 3: 17 h after induction, 4: 21 h after induction, 5: 25 h after induction (+ 0.4 %), 6: 37 h after induction (+ 0.4 %), 7: 41 h after induction, 8: 45 h after induction, 9: 49 h after induction, 1.17: *P.p.* GS115[pHILD 1.17-HNL] induced with methanol for 49 h (overnight 1 %, day: 0.5 %), the black box highlights the region of Hnl protein (29 kDa).

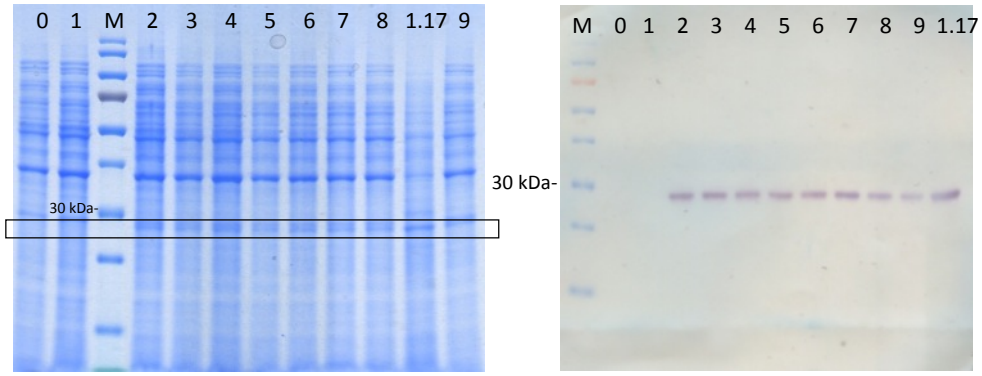

Figure 7: 12 % NuPAGE gel (left) and Western Blot (right) of fermentation broth samples of the strain *P.p.* GS115[pHILD 1.17-HNL pPpT4Kan_GAP_αS_MCE_1_D1] induced with biodiesel. M: PageRuler Prestained Protein Ladder; 0: before induction (+ 0.1 %), 1: 3 h after induction (+ 0.2 %), 2: 13 h after induction (+ 0.3 %), 3: 17 h after induction, 4: 21 h after induction, 5: 25 h after induction (+ 0.4 %), 6: 37 h after induction (+ 0.4 %), 7: 41 h after induction, 8: 45 h after induction, 9: 49 h after induction, 1.17: *P.p.* GS115[pHILD 1.17-HNL] induced with methanol for 49 h (overnight 1 %, day: 0.5 %), the black box highlights the region of Hnl protein (29 kDa).

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SUPPLEMENTAL DATA

Screening assays for esterase/lipase activity

For alpha-naphthyl acetate assay, *Pichia pastoris* transformants were grown on MD plates for 2 days at 28 °C. Colonies were lifted with filter paper discs and dried for 5 min. at 28 °C. 1 ml of the screening mix, containing 375 µl alpha-naphthyl acetate [12 mg/ml in acetone], 175 µl Fast Blue B [20 mg/ml in ddH_2O] and 5 ml 0.1 M Tris-HCl buffer pH 7, was dropped onto a glass plate and the dried filter was placed colony-side up onto the solution. Active colonies showed a brownish-violet color after 1-3 min. of incubation. For pH shift assays, *Pichia pastoris* transformants were grown on a biodyne nitrocellulose membrane placed on MD plates for 2 days at 28°C and afterwards dried for 5 min. at 28 °C. Before application of the screening mix, the dried membrane was equilibrated for 30 min. on a filter paper saturated with the same buffer that was used for the screening mix, 10 mM Tris-HCl buffer pH 8. 1 ml of the screening mix, containing 10 g/l phenol red and 10 mM Tris-HCl buffer pH 8, was dropped onto a glass plate and the equilibrated membrane was placed colony-side up onto the solution. After 10-15 minutes of incubation active colonies shifted the color from red to yellow. Rhodamine B assay for the detection of lipases was based on the publication of Kouker&Jaeger (1). Therefore, *Pichia pastoris* transformants were grown on rhodamine B plates consisting of 1.34 % yeast nitrogen base without amino acids, $4x10^{-5}$ % biotin, 0.5 % dextrose, 2 % agar, 1 % methyl oleate, 200 mM potassium phosphate buffer pH 7 and 0.001 % rhodamine B. After 2 days of incubation fluorescence of the transformants containing an active lipase could be detected under UV light. The following control strains were applied in all 3 screening assays: APLE_A1, which is a *Pichia pastoris* strain expressing the alternative pig liver esterase (2) as a positive control for esterase activity, P. pastoris CBS7435 wild type strain, *P. pastoris* CBS7435[pPpT4_GAP_αS] and *P. pastoris* GS200 Aox1/Aox2 double knockout strain.

Activity screening

For a general esterase activity screening, α-naphthylacetate assays of the Pichia transformants were performed. All tested transformants showed increased activity towards this substrate compaired to the negative control (data not shown). Two active colonies of each construct were retested and the preliminary results could be confirmed (Supplemental data; Fig. 1). pH shift assay was the method of choice to test for the substrate specificity of the enzymes or their general ability to cleave methyl esters. Methyl acetate, a methyl ester, was converted and the pH shift was visible as the active colonies turned from red to yellow (Supplemental data; Fig. 2).

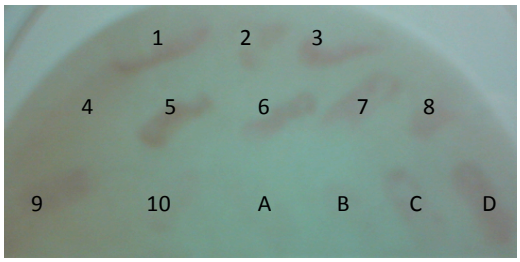

Figure S1: α-Naphthylacetate assay (rescreen) of 2 active clones per construct on MD plate. 1, 2: pPpT4_GAP_αS_MCE; 3, 4: pPpT4_GAP_αS_SEH38; 5, 6: pPpT4_GAP_αS_SEH41; 7, 8: pPpT4_GAP_αS_SE; 9, 10: pPpT4_GAP_αS_Lipase, A: *P. pastoris* APLE_A1; B: *P. pastoris* CBS7435 wt; C: *P. pastoris* CBS7435[pPpT4_GAP_αS], D: *P. pastoris* GS200[*aox1*/*aox2* double knockout]. All esterase/lipase constructs were transformed into *P. pastoris* CBS7435 wild type strain. MCE: methyl carboxyl esterase, SEH: steryl ester hydrolases, SE: serine esterase, APLE: alternative pig liver esterase

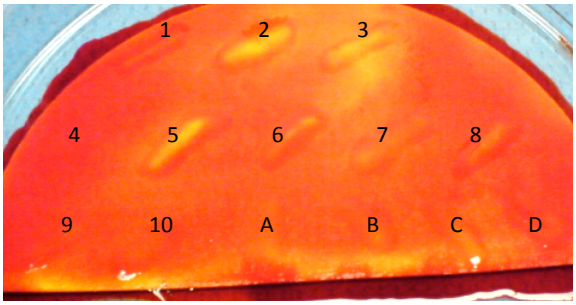

Figure S2: pH shift assay of 2 clones per construct on MD plate. 1, 2: pPpT4_GAP_αS_MCE; 3, 4: pPpT4_GAP_αS_SEH38; 5, 6: pPpT4_GAP_αS_SEH41; 7, 8: pPpT4_GAP_αS_SE; 9, 10: pPpT4_GAP_αS_Lipase; A: *P. pastoris* APLE_A1; B: *P. pastoris* CBS7435 wt; C: *P. pastoris* CBS7435[pPpT4_GAP_αS], D: *P. pastoris* GS200[*aox1*/*aox2* double knockout]. All esterase/lipase constructs were transformed into *P. pastoris* CBS7435 wild type strain. MCE: methyl carboxyl esterase, SEH: steryl ester hydrolases, SE: serine esterase, APLE: alternative pig liver esterase

Chapter 6

Identification and Isolation of Novel Cell Wall Anchor Proteins from *Pichia pastoris* and their Potential for Surface Display of Recombinant Proteins

My contribution to the paper draft is as follows:

- Conceptual formulation (100 %)
- Supervision of project employee T. Nudl (100 %)
- Identification of novel cell wall anchors (100 %)
- Construction of the anchor plasmids (20 %)
- Levanase activity assays (20 %)
- Immunofluorescence microscopy (100 %)
- Writing of the research article (100 %)

Isolation and identification of novel cell wall anchor proteins from *Pichia pastoris* and their potential for surface display of recombinant proteins

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ABSTRACT

A *Pichia pastoris* cell surface display system was developed using new *Pichia pastoris* derived anchors identified based on sequence homologies to GPI anchored or generally cell wall proteins from *Saccharomyces cerevisiae* and *Pichia angusta*. *Bacillus subtilis* levanase (*sacC*) and *Pichia* codon optimized EGFP were used as reporter proteins N-terminally fused to a FLAG tag and to eight different putative anchors. Fusion proteins were expressed under the control of *Pichia pastoris* alcohol oxidase 1 (AOX1) promoter. Surface expression was quantified by levanase activity assay and confirmed by immunofluorescence microscopy. *Pichia pastoris* Sed1p turned out to be a valuable anchor protein for surface display followed by Cwp1p.

INTRODUCTION

Cell surface display of proteins is of big advantage for a variety of biotechnological applications, for instance directed evolution, screening of antibody libraries or use as a whole cell biocatalyst. Smith was the first to report the display of a heterologous protein on a virus surface by fusing it to a filamentous phage protein (1). This technique was refined to finally screen an epitope library for peptide ligands attaching to antibodies or other binding proteins (2). However, to overcome the constriction and fussiness of phage methodology, bacterial surface display systems were developed, the leading one *E. coli* as an example for gram negative bacteria (3)(4)(5) or *Staphylococcus* for gram positive ones (6)(7). Due to severe drawbacks concerning the suitability of bacterial systems for the expression of eukaryotic proteins, the yeast *Saccharomyces cerevisiae* (*Sc*) gained more and more interest as a host for surface expression (8)(9)(10). Over the years, other yeasts were explored as hosts for surface display as well, *Hansenula polymorpha* (11), *Pichia pastoris* (12), and recently *Yarrowia lipolytica* (13).

Until recently, *Pichia pastoris (Pp)* surface display systems were based on well-established *Saccharomyces cerevisiae* anchors, such as α-agglutinin (Aga1p) for N-terminal or Aga2p and FS or FL residues of Flo1p, respectively, for C-terminal fusion proteins. In case of Aga1p, the fusion protein is coupled to the cell wall using a glycosylphosphatidylinositol (GPI) anchor-attachment signal, whereas Aga2p target protein fusion is anchored indirectly to the cell wall as it is conjugated to Aga1p by means of a disulphide bond. FS or FL proteins comprise parts of the flocculation functional domain of Flo1p. These residues are able to mediate noncovalent interaction of the fusion protein with the cell wall by adhesion to mannan chains on the cell surface (14). In 2011, Khasa *et al.* published the development of a *Pichia pastoris* surface display system using the *P. pastoris* homologues of the *Sc*Pir1p as anchors (15). Not only the choice of the anchor itself, but also other considerations are important for a successful display of foreign proteins on the *Pichia* cell surface. A good signal sequence for an efficient transport to the cell membrane as well as secretion of the fusion protein is indispensable. In case of *Pichia pastoris*, the *S. cerevisiae* alpha factor secretion signal might be the signal peptide of choice, as well as other available signal peptides for protein secretion including native signal sequences of recombinant proteins to be immobilized on

the cell surface. The influence of the positioning and the length of the anchor with respect to the sustainment of stability and activity of the target protein must be kept in mind, thus strongly contingent upon the application. The length and the amino acid content of the spacer between the anchor and the target protein should be optimized for best functionality and substrate accessibility of the displayed protein (14 and references therein).

In this work we identified and isolated several novel cell wall anchors originating from *Pichia pastoris* and investigated their suitability for the display of recombinant proteins on the surface of a *Pichia pastoris* cell. To demonstrate their potential we used the *Bacillus subtilis* levanase (*sacC*) to investigate and quantify the efficiency of the novel surface display expression system (16). Immunofluorescence microscopy was performed for confirmation of immobilization of the target protein on the cell surface. At least one efficient *Pichia pastoris* anchor for the expression of *B. subtilis* levanase could be identified, whereas at least one more anchor seemed to have good potential for surface display.

MATERIALS AND METHODS:

Strains and materials

E.coli Top 10F´[lacI^{q} Tn10(tet^{R})] mcrAΔ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 deoRnupG recA1 araD139 Δ(ara-leu)7697 galUgalKrpsL(Str^{R}) endA1 λ^{-}] was used as host for recombinant DNA manipulations. *P. pastoris* CBS7435 wildtype strain was used as the host for surface display. Plasmid DNA was isolated using Fermentas GeneJet Plasmid Miniprep Kit, Phusion polymerase (Finnzymes), dNTPs and restriction enzymes were also purchased from Fermentas GmbH, St. Leon-Rot, Germany. Mouse IgG monoclonal anti-FLAG M2 antibody, Poly-L-Lysine and sucrose were purchased from Sigma Aldrich Handels-GmbH (Vienna, Austria), goat anti-mouse polyclonal IgG DyLight 488 conjugated antibody was ordered from Fisher Scientific Austria GmbH (Vienna, Austria). *E.coli* media components were purchased from AppliChem (VWR International GmbH, Vienna, Austria) or LabM Limited (Dr. Friedrich Bertoni GmbH, Vienna, Austria), respectively. *Pichia* media components were purchased from BD Biosciences (Becton Dickinson Austria GmbH, Vienna, Austria). Glucose-Hexokinase UV reagent was purchased from Dipromed (Weigelsdorf, Austria). T4 DNA Ligase and Wizard Gel and PCR Clean-up system were purchased from Promega GmbH (Mannheim, Germany). Vectashield was ordered from Vectorlabs (Szabo-Scandic, Vienna, Austria). All other chemical reagents used in this study were purchased from Lactan (Graz, Austria).

Media and cultivation conditions

E.coli was cultivated in Luria-Bertani medium [1% tryptone, 0.5 % yeast extract, 0.5 % NaCl] containing 100 µg/ml ampicillin. His^{+} selection of pAaHSwa transformants was done on MD plates [1,34 % yeast nitrogen base w/o amino acids; 4x10^{-5} % biotin, 2 % dextrose, 2 % agar]. *P. pastoris* was grown in ½ BYPD [1 % peptone, 0.5 % yeast extract, 1 % dextrose, 200 mM potassium phosphate buffer pH 7.0]. Heterologous protein expression was induced in deep well plates with BMM [1,34 % yeast nitrogen base w/o amino acids; 4x10^{-5} % biotin; 0,5 % or 1 % methanol, 200 mM potassium phosphate buffer pH 7.0], in shake flasks with 0.5 % or 1 % methanol. Mut^{+}/Mut^{S} selection was done on colony level on MD [1,34 % yeast nitrogen

base w/o amino acids; $4x10^{-5}$ % biotin, 2 % dextrose, 2 % agar] and 1 % MM [1,34 % yeast nitrogen base w/o amino acids; $4x10^{-5}$ % biotin, 1 % methanol, 2 % agar] plates. Prescreens of *Pichia pastoris* Mut^S transformants were performed in deep well plates. Therefore cells were grown in 250 µl ½ BYPD for 24 h, then 250 µl of 2 % (v/v) BMM was added for induction. Cultures were continuously induced for overall 48 h with BMM twice daily. Shake flask cultures were grown in ½ BYPD for 24 h and induced continuously for overall 48 h with 100 % methanol to a final methanol concentration of 0.5 % (v/v) during the day (8 h) and 1 % (v/v) during the night (16 h).

Plasmid construction

P. pastoris CBS7435 genome was searched for homologous GPI anchored or cell wall proteins in general based on *Saccharomyces cerevisae* sequences and *Hansenula polymorpha* (*Pichia angusta*) (17). Eight genes thereof were chosen for subsequent cloning into pPpT4alphaS or pAaHSwa vector, respectively (Fig. 1, Table 1). Anchor ORFs were PCR amplified from *P. pastoris* CBS 7435 genomic DNA using primers given in Table 2. Anchor primers included FLAG tag sequence and *Spe*I and *Not*I restriction sites (Table 2). *Pichia pastoris* codon optimized EGFP was amplified from pGAPZ_A_EGFP plasmid (IMBT culture collection number 5208, University of Technology, Graz, Austria) with the primers EGFP XhoKexStefor and EGFP SpeNotrev (Table 2). pGAPZPhusion Polymerase (Finnzymes) was used for PCR reactions according to the manufacturer´s manual using by default the following conditions: 98°C 3´//98°C 30´´/55°C 30´´/72°C 1´(25 cycles)//72°C 10´/4°C ∞. *Xho*I/*Not*I opened pPpT4alphaS vector was ligated with *Xho*I/*Not*I digested EGFP fragment, transformed in *E.coli* Top10F´ and sequenced. After restriction with *Spe*I and *Not*I the FLAG anchor fragments were ligated. Constructs were transformed into E.coli Top10F´cells. For levanase constructs *sacC* gene was amplified from Levanase plasmid (IMBT culture collection number 856, University of Technology, Graz, Austria) using the primers Lev2EA_XhoIF and SpeI_Lev_Rev. pPpT4_alphaS_EGFP-FLAG-anchor plasmids were cut with *Xho*I and *Spe*I to remove EGFP fragment and were ligated with *Xho*I/*Spe*I digested *sacC* PCR fragment. After transformation in *E.coli* Top10F´ pPpT4_alphaS_sacC-FLAG-anchor were sequenced for confirmation and later on digested with *Xho*I and *Not*I to isolate the *sacC*FLAG-anchor

fragments for the ligation with *Xho*I/*Not*I opened pAaHSwa vector. All constructs were sent to LGC Genomics (Berlin, Germany) for sequencing (Table 1).

Plasmid transformation and expression

pAaHSwaLevFLAGanchor plasmids were digested with *Swa*I and transformed into the host strain *Pp* CBS7435*Δhis* (kind gift from T. Hajek, Institute of Molecular Biotechnology, Graz University of Technology) . His+ transformants were tested for Mut^S/Mut^+ phenotype and solely Mut^S transformants were cultivated in 96 deep well plates in ½ BYPD and BMM medium for levanase activity prescreens. Hits of the prescreens were subjected to shake flask fermentations in 300 ml baffled flasks containing 50 ml of ½ BYPD medium at 28 °C and 120 rpm. Initial induction took place after 24 h with 250 µl 100 % methanol. Cells were further induced after 8, 24 and 32 hours with 250 µl or 500 µl 100 % methanol, respectively. Cells were harvested after 48 h and objected to levanase activity assay. *Pichia pastoris* strain CBS7435*Δhis*[pAaHBglLev]_SC5/13 were used as secretory levanase expression control, *Pichia pastoris* CBS7435*Δhis*[pAHBglLev] as intracellular levanase expression control and *P.p.* CBS7435 wild type strain was used as negative control.

Immunofluorescence microscopy

OD_{600} of the induced cultures of EGFP and levanase constructs was measured and cultures were diluted to an OD_{600} of 1 with 100 mM potassium phosphate buffer pH 7.5. 100 µl of 37 % formaldehyde was added to each 1 ml of OD 1 cultures and incubated for 30 min. at 30 °C. After centrifugation at 2500 rpm for 5 min., cell pellets were washed once with 100 mM KP_i buffer, pH 7.5, 100 mM KPi buffer/4% formaldehyde, 100 mM KP_i buffer/1.2 M Sorbitol and 1.2 M Sorbitol. Slides were coated with Poly-L-Lysine [0.1 % in ddH_2O]; fixed cells were pipetted into the wells and incubated for 30 min. at room temperature. Residual cell suspensions were removed, slided were washed 5 times with ddH_2O and slides were treated with blocking buffer [40 mM K_2HPO_4, 40 mM KH_2PO_4, 150 mM NaCl, 0.1 % NaN_3, 0.1 % Tween 20, 2% skimmed milk] for 30 min. Blocking solution was removed and cells were first incubated with IgG monoclonal anti-FLAG M2 antibody, diluted 1:500 in PBS [100 mM KP_i

buffer pH 7.5, 2 % bovine serum albumin (BSA)] for 30 min at room temperature and then washed 8 times with 100 mM KP_i buffer pH 7.5/ 2 % BSA. The resulting cells were reacted with secondary goat anti-mouse polyclonal IgG DyLight 488 conjugated antibody (diluted 1:500 in PBS) for 1h at room temperature in the dark in a humid chamber. After washing 5 times with 100 mM KP_i buffer pH 7.5/ 2 %BSA, 3 µl of Vectashield per well were applied, slides were covered with the cover slip and sealed with nail polish. Preparations were examined using a Leica fluorescence microscope. Filter I3 (excitation BP450-490/emission LP515) was used for DyLight 488 detection.

Levanase activity assay

The most promising transformants were inoculated in 300ml shake flasks. Therefore precultures were made. 20 ml of BYPD medium was inoculated with a single colony from freshly prepared streaks in a 100 ml flask. After 24 h the OD_{600} of the precultures were measured and 50 ml of ½ BYPD medium in 350 ml baffled flasks was inoculated to a starting OD_{600} of 0.2 and incubated at 28 °C and 150 rpm. After 24 h the glucose concentration of the main culture was determined with Diabur 5000 glucose test kit (Roche). The whole glucose should be metabolized. Initial induction took place after 24 h with 250 µl 100 % methanol. Cells were further induced after 8, 24, 32 hours and so forth with 250 µl or 500 µl 100 % methanol, respectively. 1 ml samples were taken after 0, 8, 24, 32, 48, 56 and 72 hours of induction. 100 µl of the samples were used for OD_{600} measurements. The remaining 900 µl of culture were centrifuged at 4 °C and 4000 rpm for 20 min, washed twice with ddH_2O and finally resuspended in 900 µl of ddH_2O. 50 µl of the supernatant or the resuspended washed cells were mixed with 50 µl 50 mg/ml sucrose and incubated for 10 min. at 37 °C. Subsequently, supernatant samples were directly heat inactivated by heating them up to 95 °C for 5 min. in a PCR machine. Cell suspensions were centrifuged for 1 min. at 4 °C followed by the heat inactivation step. All of the samples, except the 0 h and 8 h time points, were diluted 1:10 with ddH_2O for the measurements. Finally, 10 µl of the (diluted) samples were mixed with 190 µl of the Dipromed UV hexokinase substrate, incubated for 10 min. at 37 °C followed by absorption measurement at 340 nm in a plate reader. For the standard curve, 10 µl of different glucose concentrations (2.5, 1.6667, 1.25, 0.625, 0.3125, 0.15625, 0.078125 mg/ml) were mixed with 190 µl Dipromed UV hexokinase substrate incubated for 10 min. at

37 °C followed by absorption measurement at 340 nm in a plate reader. One unit of activity was defined as the amount of enzyme required to liberate 1 mmol of glucose per min. (18).

RESULTS AND DISCUSSION

Identification of GPI-anchored protein coding genes

To identify putative new anchor proteins for surface display in *Pichia pastoris* BLAST searches of the recently published *P.pastoris* CBS7435 *wt* strain (GenDB) (19) were performed based on GPI anchored or cell wall proteins from *Saccharomyces cerevisiae* and on patented sequences of GPI anchored proteins used for surface display in *Pichia angusta* (17).The chosen genes were annotated as *sed1*, *gas1*, *gas2*, *cwp1*, *tir3* and *wsc2*, which are known to comprise surface anchoring motifs (20)(10)(11).

Sed1 is described to be the predominant structural cell wall protein in *S. cerevisiae* in stationary-phase cells (21) and is suggested to play an important role in imparting resistance to biotic and abiotic stresses (22). Protein BLAST sequence alignment of the 135 amino acids of the amplified PCR product (*Pp*Sed1) showed 54 % identity with *Pichia angusta* Sed1 and 39 % identity with *Sc*Sed1. Additionally, PpSed1 was much shorter than ScSed1, lacking the repeating domains found therein. SignalP (http://www.cbs.dtu.dk/services/SignalP/) analysis of *Pp*Sed1 predicted a putative secretion signal sequence of 17 amino acids and PredGPI (http://gpcr.biocomp.unibo.it/predgpi/) a putative GPI anchoring sequence with a ω-site at position 112. MotifScan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) results showed two potential glycosylation sites and a threonine-rich region, covering almost the whole protein.

During anaerobiosis, a couple of *S. cerevisiae* cell wall mannoproteins, the DAN/TIR proteins, are expressed within hours whereas expression of CWP 1&2, the major cell wall mannoproteins, is correspondingly shut down. It could be shown, that the Cwp proteins are generally regulated by oxygen and temperature (23). Smits *et al.* were able to localize Cwp1p at the birth scars of *Saccharomyces cerevisiae* but its function there still remains unclear (24). 2 annotated PpCwp1 proteins were detected (GenDB ID 199427 and 153002) by BLAST search using the *Pichia angusta* (*Pa*) and the *Saccharomyces cerevisiae* Cwp1 sequence. PpCwp1 (199427), a protein of 289 amino acids, showed no identity with *Pichia angusta* Cwp1, but 46 % identity with the *S. cerevisiae* counterpart. SignalP predicted a putative secretion signal sequence of 18 amino acids, but PredGPI showed no GPI anchor. MotifScan analysis points out an aspartic acid rich region and a putative cell wall binding repeat profile.

Pp*cwp1* (153002) annotated gene encodes a protein with 399 amino acids, which has predicted N-terminal signal peptide of 22 amino acids, but no GPI anchor. It shows 34 % identity with *Sc*Cwp1, but none with *Pa*Cwp1.

*Sc*Wsc2 belongs to a family of five proteins (Wsc1-3, Mid2, Mtl1), the so-called membrane-spanning cell wall integrity sensors, which most likely transfer the signal to the GDP/GTP exchange factor Rom2 so that the small GTPase Rho1 is activated by GTP binding. Thus, protein kinase C is converted into its active state. The signal is transmitted to the transcription factor Rlm1 by the MAPK (mitogen-activated protein kinase) cascade and further on to the heterodimeric SBF complex (formed by Swi4 and Swi6), whereby transcriptional response is initiated ((25)(26) and references therein). Wsc2 was also found in a screen for suppressors of mutants defective in glycerol synthesis (27) and in the heat-shock response (hsf1;(28)).*Pp* annotated *wsc2* gene encodes a protein of 372 amino acids with a PSORT (http://psort.hgc.jp/) predicted N-terminal signal peptide of 16 amino and no predicted GPI anchor. It shows no identity with the *Hansenula polymorpha* Wsc2, but 36 % identity with the *Saccharomyces cerevisiae* Wsc2. Protein analysis tools revealed an aspartic acid and a serine rich region together with a wsc domain profile.

*Pp*Gas1p has been described for the first time in 2006 by Marx *et al.* and belongs, like its 4 paralogues, to the GH-72 family of glycosyl hydrolases catalyzing a trans-glycosylation with β-1,3-glucan as a substrate (30). It comprises a protein of 538 amino acids and has an N-terminal secretory signal sequence of 16 amino acids, several putative N-glycosylation sites, a C-terminal serine-rich region and a GPI anchor. Gas1 deletion led to a 2-fold improvement of *Rhizopus oryzae* lipase (Rol) secretion and Rol overexpression in a *Pichia pastoris* HAC1/Δgas1 double mutant, constitutively over expressing *Sc*HAC1, even resulted in a 7-fold increase, whereat triggering of the unfolded protein response positively affected Rol productivities of the process (29)(31). Gas1 homologues have already been described in *Schizosaccharomyces pombe* (*Sp*), where *Sp*Gas1 is needed to sustain cell integrity during vegetative growth (32), and in *Candida albicans* (PHR1 and PHR2)(33). In contrast, *Sc*Gas2 expression is triggered in cells undergoing sporulation whereas the time of highest expression level is coincident with the stage of spore wall formation. There is no expression at the vegetative state (34). *Pp*GAS2 is a protein of 596 amino acids with a cleavable N-

terminal signal sequence of 19 amino acids, a putative GPI anchor motif with a ω-site at position 571 and a predicted X8 domain. *Pp*GAS2 shows 52 % identity with *Sc*GAS2.

*Sc*Tip1 was primarily described in 1991 as a new type of stress-inducible gene the expression of which was induced by cold as well as heat shock and was therefore referred to as "**t**emperature shock-**i**nducible **p**rotein gene" (35). Together with *Sc*Tir3 it belongs to a group of homologous genes encoding mannoproteins implicated in anaerobic adaptation. In addition to being oxygen regulated, *Sc*Tip1 was induced during cold shock, while CWP1 was correspondingly down-regulated and TIR3 remained uninduced (22 and references therein). *P. pastoris* annotated *tir3* (Tip1 related protein) gene encoded a 194 amino acid protein, which showed 30 % identity with *Sc*TIP1 and 31 % identity with *Sc*TIR3 while no identities with *Hansenula polymorpha* TIP1 could be detected. Protein analysis tools revealed a glutamic acid-rich region and a seripauperin and TIP1 family motif. A PSORT N-terminal predicted signal peptide of 18 amino acids and a GPI anchoring motif were found as well.

Expression vector construction and transformation for surface display

2 different proteins were chosen for surface display. Plasmids for EGFP surface display were constructed as shown in Fig. 1. Primer extension PCR was performed to add *Xho*I restriction site (N-terminal fusion) and *Spe*I/*Not*I restriction site (C-terminal fusion) to *Pichia pastoris* codon optimized EGFP. PCR product was ligated with the *Xho*I/*Not*I opened pPpT4alphaS plasmid carrying *S. cerevisiae* alpha mating factor secretion signal. Eight putative GPI anchor proteins could be identified by GenDB BLAST homology search based on *H. polymorpha* and/or *S. cerevisiae* sequences. A FLAG tag was fused N-terminally to the anchors via PCR together with *Spe*I restriction site, whereas *Not*I site was fused at the C-terminus. Anchor-FLAG PCR products were fused C-terminally to EGFP so that the FLAG tag is located between EGFP and the anchor. For levanase expression, *sacC* gene was amplified from Levanase plasmid adding *Xho*I restriction site (N-terminal fusion) and *Spe*I/*Not*I restriction site (C-terminal fusion). pPpT4_alphaS_EGFP-FLAG-anchor plasmids were cut with *Xho*I and *Spe*I to remove EGFP fragment and were ligated with *Xho*I/*Spe*I digested *sacC* PCR fragment. After transformation in pPpT4_alphaS_sacC-FLAG-anchor were sequenced for confirmation and

later on digested with *Xho*I and *Not*I to isolate the *sacC*-FLAG-anchor fragments for the ligation with *Xho*I/*Not*I opened pAaHSwa vector.

Enzyme activity

For the determination of enzyme activity, prescreens of the eight different anchor levanase constructs were performed. Therefore 80 solely Mut^S transformants per anchor construct were fermented in 96 deep well plates. The 8 best performing clones of each construct were chosen for further deep well fermentations. One 96 well master plate was made harboring the best 8 clones of each construct. Fermentations were made in triplicates; samples were taken at 24h and 48 hours of induction. Finally 2 best clones of each construct were chosen and analyzed in 4 replicates each in deep well fermentations (Fig. 2). The most interesting anchors in combination with *Bacillus subtilis* levanase turned out to be anchor E (Sed1), followed by anchor C (Cwp1) and to a much weaker extent also anchor I (Tir3). Results were confirmed by final shake flask experiments of the 2 most promising clones, both with Sed1p anchor protein (anchor E). Measurements were done in 24 replicates each. Clone E4 showed a continuous increase in levanase activity by time in the washed pellet fraction and in the supernatant, but activity in the supernatant was comparatively low (Fig. 3). As clone E 20 showed comparable values to E4, it is not shown in Fig. 3. Overall, clearly higher activity levels than the best levanase-secreting clone D1 (secretory control) could be reached with the two best clones with levanase immobilized on the *Pichia pastoris* surface. Practically no activity was detectable with the pellet fraction of the secretory control and the intracellular levanase expression control, confirming the reliability of the levanase activity measurement protocol.

Immunofluorescence microscopy

The FLAG tag between levanase and the different anchors was detected by immunofluorescence microscopy. Therefore, cells were induced in BMM medium containing 1% (v/v) methanol for 64 hours. Cells expressing levanase on the surface showed an equal distribution of fluorescent dye on the cell surface (Fig. 4). On control cells, no fluorescence could be observed.

CONCLUSION

In this study we investigated the potential of eight newly isolated cell wall proteins of *Pichia pastoris* for the surface display of heterologous proteins. The use of *Pichia´*s own cell wall anchor can be advantageous for the correct processing and folding of the fusion protein followed by efficient and stable surface expression. Surface display of *Bc*SacC showed a very high level of levanase activity on the cell surface, but almost none in the supernatant, when anchored with *Pichia pastoris* Sed1p although employment of anchor C also mediated good immobilization on the cell wall, but much less levanase activity (Fig. 2). It could be shown for the sacC-Sed1 strain E4 that the surface expression of levanase seems to be stable as the activity is still increasing until the final time point at 72 hours. EGFP surface display explicitly worked for anchor A (GenDB annotated gas1) and anchor C (GenDB annotated cwp1) (supplemental data), whereat immunofluorescence detection with anti-FLAG antibody was also successful with anchor I (GenDB annotated tir3) (data not shown). At least in case of anchor C certain suitability for the surface display of both reporter proteins could be shown. If an anchor works excellently with one protein one cannot conclude that it would be the perfect choice for all other proteins as well. Unfortunately sufficient surface display has to fulfill various parameters and in the end it is in many cases a matter of trial and error to find the appropriate system for ones needs. Finally one can conclude from this study, that *Pp*Sed1p has a high potential to be utilized as anchor for efficient immobilization and expression of heterologous proteins on the *Pichia* cell wall, but at least one more protein could be identified (*Pp*Cwp1p) that showed a good suitability which needs further investigations.

FIGURES AND TABLES

Table 1: Recombinant plasmids used in this study. *Pichia pastoris* GPI anchor proteins were identified based on homology searches of GPI anchored or cell wall proteins in general based on *Saccharomyces cerevisiae* and *Hansenula polymorpha* (*Pichia angusta*) sequences.

Plasmid	Purpose	Anchor (GenDB annotation)	GenDB ID
pPpT4_αS_EGFP/A	EGFP surface display	Full length PpGas1p	148978
pPpT4_αS_EGFP/B	EGFP surface display	Full length PpGas1p	148979
pPpT4_αS_EGFP/C	EGFP surface display	Full length PpCwp1p	153002
pPpT4_αS_EGFP/D	EGFP surface display	Full length PpWsc2p	191035
pPpT4_αS_EGFP/E	EGFP surface display	Full length PpSed1p	192092
pPpT4_αS_EGFP/F	EGFP surface display	Full length PpCwp1p	199427
pPpT4_αS_EGFP/G	EGFP surface display	Full length PpGas2p	199999
pPpT4_αS_EGFP/I	EGFP surface display	Full length PpTir3p	208766
pAaHSwaLev/A	SacC surface display	Full length PpGas1p	148978
pAaHSwaLev/B	SacC surface display	Full length PpGas1p	148979
pAaHSwaLev/C	SacC surface display	Full length PpCwp1p	153002
pAaHSwaLev/D	SacC surface display	Full length PpWsc2p	191035
pAaHSwaLev/E	SacC surface display	Full length PpSed1p	192092
pAaHSwaLev/F	SacC surface display	Full length PpCwp1p	199427
pAaHSwaLev/G	SacC surface display	Full length PpGas2p	199999
pAaHSwaLev/I	SacC surface display	Full length PpTir3p	208766

Table 2: Primers used in this study

Primer	Sequence (5´ to 3´)
EGFP XhoKexStefor	TGACTCGAGAAGAGAGAGGCCGAAGCTGCTAGCAAAGGAGAAGAACTTTTC
EGFP SpeNotrev	AGTGCGGCCGCTTTTTTACTAGTCTTGTACAATTCATCCATGC
Lev2EA_XhoIF	CACTCGAGAAAAGAGAGGCTGAAGCTGCCGATTCAAGCTACTATG
SpeI_Lev_Rev	TCACTAGTAGACTCCTTCGTTACATTC
148978SpeFLAGfor	AATACTAGTGATTACAAGGACGATGACGATAAGATGTTTAAATCTCTGTGCATG
148978Notrev	AGT-GCGGCCGCTTAGAATGACATAATCATTCC
148979SpeFLAGfor	AATACTAGTGATTACAAGGACGATGACGATAAGATGTTGTCCATTTTAAGTGC
148978Notrev	TGAGCGGCCGCCTATAAAAGAGTGTAAGTGAATCC
153002SpeFLAGfor	AATACTAGTGATTACAAGGACGATGACGATAAGATGTTGTTGAAGTTGATTTGG
153002Notrev	AGTGCGGCCGCTTAGGCATTATTTCCTGGGGTC
191035SpeFLAGfor	AATACTAGTGATTACAAGGACGATGACGATAAGATGACCAAGTTTATATTGATATTGG
191035Notrev	TGAGCGGCCGCCTAAACTTCATCATCTGTGG
192092SpeFLAGfor	AATACTAGTGATTACAAGGACGATGACGATAAGATGCAATTCTCTATCGTCG
192092Notrev	AGTGCGGCCGCTTACAAGAAGTAAGCAGC
199427SpeFLAGfor	AATACTAGTGATTACAAGGACGATGACGATAAGATGTTCAACCTGAAAACTATTCTC
199427Notrev	AGTGCGGCCGCTTATTTCTCCCATACTTTAAGG
199999SpeFLAGfor	AATACTAGTGATTACAAGGACGATGACGATAAGATGCTGGGTTTCAAGGACTTC
199999Notrev	AGTGCGGCCGCCTAAGTAAATAATATTACTAAG
201375SpeFLAGfor	AATACTAGTGATTACAAGGACGATGACGATAAGATGAGATTTTCTAACGTCGTTTTAAC
201375Notrev	AGTGCGGCCGCTTACAAGGCAAAGACTCCGAAAG
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208766Notrev	AGTGCGGCCGCTTACAAGGCAAAGACTCCGAAAG

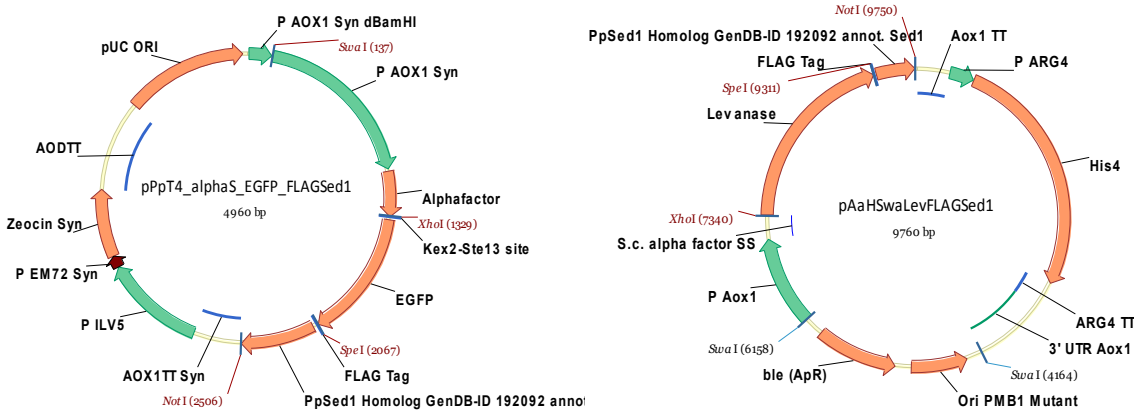

Fig.1: Schematic diagram of the plasmids encoding the EGFP-Sed1 or levanase-Sed1 fusion protein, respectively. Left plasmid map: The sequence encoding *EGFP* was fused at the C-terminus to a FLAG tag and to the sequence encoding *P. pastoris* Sed1p (anchor E). The FLAG-anchor fragment was inserted into pPpT4_alphaS_EGFP vector after restriction with *Spe*I and *Not*I. The fusion protein was expressed under the control of AOX1 promoter. P AOX1 Syn dBamHI: part of *P. pastoris* P_{AOX1} (synthetic); P AOX1 Syn: synthetic *Pichia pastoris* AOX1 promoter; Kex2-Ste13 site: cleavage site for Kex2/Ste13 for processing of the alpha factor secretion signal; AOX1TT Syn: AOX1 transcription termination (synthetic); P ILV5: ILV5 promoter amplified from *Pichia pastoris* CBS 7435 strain; P EM72 Syn: Synthetic consensus sequence of an E.coli promoter; Zeocin Syn: synthetic, mixed codon optimized for the expression in *E.coli* and *P. pastoris*; AODTT: AOD transcription termination amplified from *Pichia pastoris* CBS 7435 strain; pUC ori: pUC replication origin for E.coli from pBR322 plasmid. Right plasmid map: The sequence encoding *sacC* was fused at the C-terminus to a FLAG tag and to the sequence encoding *P. pastoris* Sed1 (anchor E). The sacC-FLAG-anchor fragment from pPpT4_alphaS_LevFLAGSed1 was inserted into pAaHSwa vector after restriction with *Xho*I and *Not*I. The fusion protein was expressed under the control of AOX1 promoter. P Aox1: AOX1 promoter; S.c. alpha factor SS. S. cerevisiae alpha factor secretion signal; AOX1 TT: AOX1 transcription termination; P ARG4: ARG4 promoter; His4: HIS4 coding region for selection in *Pichia pastoris*; ARG4 TT: ARG4 transcription termination; 3´ UTR Aox1: 3´ untranslated region of AOX1 for homologous recombination into AOX1 locus; Ori PMB1 Mutant: replication origin for *E.coli*; ble (ApR): ampicilline resistance cassette for selection in *E.coli*; all parts of the plasmid except the *E.coli* part, *S.c.* alpha factor and levanase were amplified from *P. pastoris* CBS 7435 wild type strain.

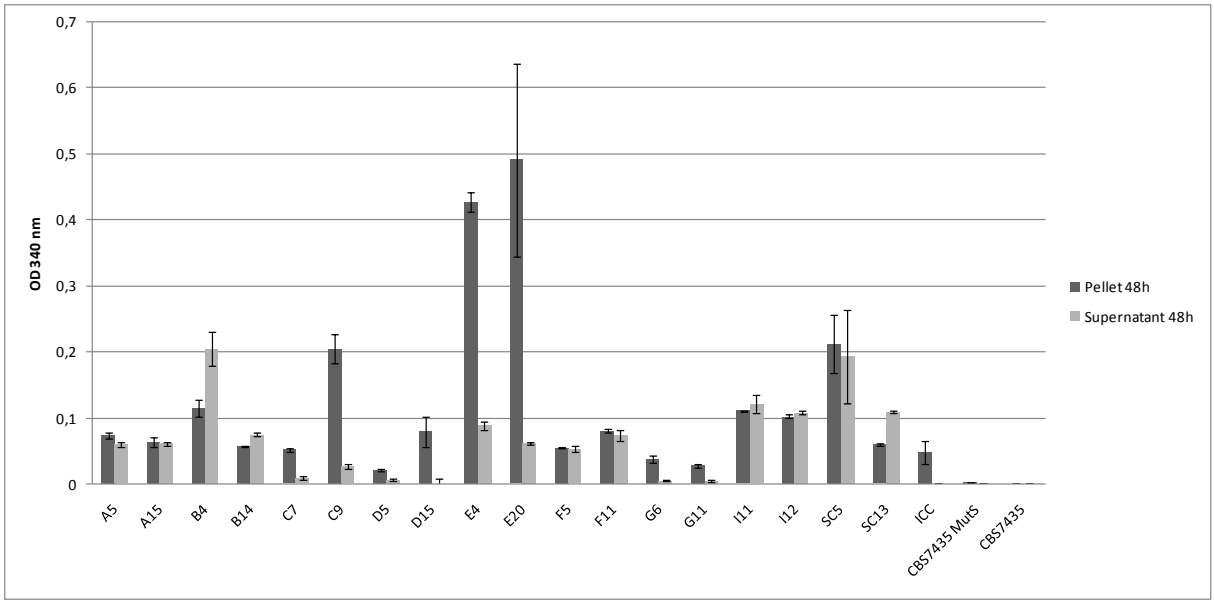

Fig. 2: Plate reader data (OD_{340}) from the 48 h measurement of levanase activity in the supernatant / washed pellet of the best 2 selected clones per anchor construct. Measurements were done in 4 replicates each. Letters A-I signify the different anchors (see Table 1), numbers signify the clone number. SC: secretory control *P. pastoris* CBS7435*Δhis*[pAaHBglLev]_SC5/13, ICC: intracellular control *P. pastoris* CBS7435*Δhis*[pAHBglLev].

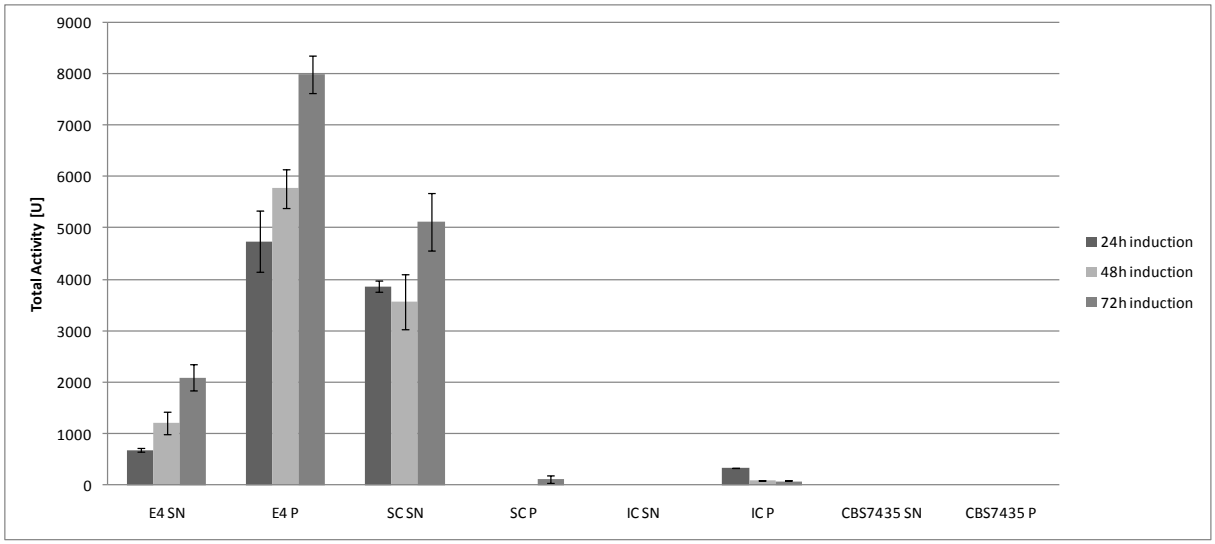

Fig. 3: Diagram of the levanase activity assay performed with the supernatant / washed pellet of *P. pastoris* CBS 7435[pAaHSwaLev/E4]. E4 was the best performing clone in the shake flask experiments. For simplicity, only values for the 24 h, 48 h and 72 h of induction were included into the diagram. Values are given in mean +/- standard deviation. As the overall OD_{600} values were almost the same, values were not normalized to OD_{600}. Measurements were performed in 24 replicates each. SN: supernatant; P: pellet; SC: secretory control *P. pastoris* CBS7435*Δhis*[pAaHBglLev]_SC13; IC: intracellular control *P. pastoris* CBS7435*Δhis*[pAHBglLev], CBS7435 wild type strain.

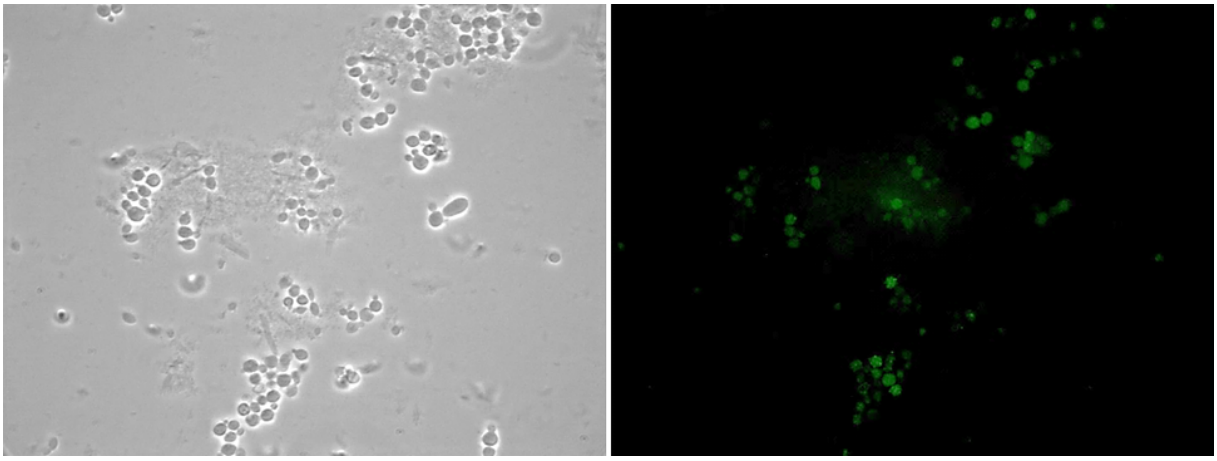

Fig. 4: Micrographs of *Bacillus subtilis* levanase surface displaying *Pichia pastoris* strain CBS 7435[pAaHSwaLev/E4] using anchor E (GenDB annotated *sed1*). Left picture: transmitted light micrograph; right picture: fluorescence micrograph; cells were labeled with anti-FLAG antibody and DyLight 488 anti-mouse IgG

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SUPPLEMENTAL DATA

MATERIALS AND METHODS

Strains and materials:

E.coli Top 10F´[lacI^{q} Tn10(tet^{R})] mcrAΔ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 deoRnupG recA1 araD139 Δ(ara-leu)7697 galUgalKrpsL(Str^{R}) endA1 λ^{-}] was used as host for recombinant DNA manipulations. *P. pastoris* CBS7435 wildtype strain was used as the host for surface display. Plasmid DNA was isolated using Fermentas GeneJet Plasmid Miniprep Kit, dNTPs, Phusion polymerase (Finnzymes) and restriction enzymes were also purchased from Fermentas GmbH, St. Leon-Rot, Germany. Mouse IgG monoclonal anti-FLAG M2 antibody, Poly-L-Lysine and sucrose were purchased from Sigma Aldrich Handels-GmbH (Vienna, Austria), goat anti-mouse polyclonal IgG DyLight 594 conjugated antibody was ordered from Fisher Scientific Austria GmbH (Vienna, Austria). *E.coli* media components were purchased from AppliChem (VWR International GmbH, Vienna, Austria) or LabM Limited (Dr. Friedrich Bertoni GmbH, Vienna, Austria), respectively. *Pichia* media components were purchased from BD Biosciences (Becton Dickinson Austria GmbH, Vienna, Austria). Zeocin was purchased from InvivoGen (Eubio, Vienna, Austria). Glucose-Hexokinase UV reagent was purchased from Dipromed (Weigelsdorf, Austria). T4 DNA Ligase and Wizard Gel and PCR Clean-up system were purchased from Promega GmbH (Mannheim, Germany). Vectashield was ordered from Vectorlabs (Szabo-Scandic, Vienna, Austria). All other chemical reagents used in this study were purchased from Lactan (Graz, Austria).

Media and cultivation conditions:

E.coli was cultivated in Luria-Bertani medium [1 % tryptone, 0.5 % yeast extract, 0.5 %NaCl] containing 100 µg/ml ampicillin. Zeocin selection of *P.pastoris* pPpT4_alphaS transformants was performed on BYPD Zeocin plates (2 % peptone, 1 % yeast extract, 2 % glucose, 200 mMpotassium phosphate buffer pH 7.0, 2% agar, 100 µg/ml zeocin]. *P. pastoris* was grown in ½ BYPD [1% peptone, 0.5 % yeast extract, 1 % dextrose, 200 mM potassium phosphate

buffer pH 7.0]. Heterologous protein expression was induced in deep well plates with BMM [1,34 % yeast nitrogen base w/o amino acids; $4x10^{-5}$ % biotin; 0,5 % or 1 % methanol, 200 mM potassium phosphate buffer pH 7.0], in shake flasks with 0.5 % or 1 % methanol. Transformants were grown in 250 µl ½ BYPD for 24 h, then 250 µl of 2 % (v/v) BMM was added for induction. Cultures were continuously induced for overall 48 h with BMM twice daily to a final methanol concentration of 0.5 % (v/v) during the day (8 h) and 1 % (v/v) during the night (16 h).

Plasmid construction

P. pastoris CBS7435 genome was searched for homologous GPI anchored or cell wall proteins in general based on *Saccharomyces cerevisae* sequences and *Hansenula polymorpha* (*Pichia angusta*)(1). Eight genes thereof were chosen for subsequent cloning into pPpT4_alphaS (Fig. 1). EGFP CDS was PCR amplified from pGAPZA_EGFP plasmid DNA (IMBT culture collection #5208) using the primers EGFPXhoKexStefor and EGFPSpeNotrev. Anchor ORFs were PCR amplified from *P. pastoris* CBS7435 genomic DNA. Phusion Polymerase (Finnzymes) was used for PCR reactions according to the manufacturer´s manual using by default the following conditions: 98°C 3´//98°C 10´´/55°C 30´´/72°C 30´´(25 cycles)//72°C 10´/4°C ∞. Anchor primers included FLAG tag sequence and *Spe*I and *Not*I restriction sites (Table 1). *Xho*I/*Not*I opened pPpT4_alphaS vector was ligated with EGFP fragment, transformed in *E.coli* Top10F´ and sequenced. After restriction with *Spe*I and *Not*I the FLAG anchor fragments were ligated. All constructs were sent to LGC Genomics (Berlin, Germany) for sequencing.

Plasmid transformation and expression

pPpT4alphaS_EGFP_FLAGanchor plasmids were linearized with *Swa*I and transformed into the host strain Pp CBS7435 by Gene Pulser XCell Electroporation System (Bio-Rad) according to the condensed transformation protocol (2). Zeocin resistant transformants were selected on BYPD Zeocin plates. EGFP transformants were pre-cultivated in ½ BYPD medium in 96 deep well plates at 28 °C and 320 rpm for 24 h. Cells were diluted 1:100 with 0.9 % sodium

chloride and stamped onto 1 % BMM plates. After 48 h fluorescent colonies were detected on a UV screen. Positive EGFP transformants were cultivated in 96 deep well plates in ½ BYPD and BMM medium for immunofluorescence microscopy.

Immunofluorescence microscopy

OD_{600} of the induced cultures of EGFP constructs was measured and cultures were diluted to an OD_{600} of 1 with 100 mM potassium phosphate buffer pH 7.5. 100 µl of 37 % formaldehyde was added to each 1 ml of OD1 cultures and incubated for 30 min. at 30 °C. After centrifugation at 2500 rpm for 5 min., cell pellets were washed once with 100 mM KP_i buffer, pH 7.5, 100 mM KP_i buffer/4 % formaldehyde, 100 mM KP_i buffer/1.2 M Sorbitol and 1.2 M Sorbitol. Slides were coated with Poly-L-Lysine [0.1 % in ddH_2O]; fixed cells were pipetted into the wells and incubated for 30 min. at room temperature. Residual cell suspensions were removed, slided were washed 5 times with ddH_2O and slides were treated with blocking buffer [40 mM K_2HPO_4, 40 mM KH_2PO_4, 150 mM NaCl, 0.1 % NaN_3, 0.1 % Tween 20, 2% skimmed milk] for 30 min. Blocking solution was removed and cells were first incubated with IgG monoclonal anti-FLAG M2 antibody, diluted 1:500 in PBS [100 mM KP_i buffer pH 7.5, 2 % bovine serum albumin (BSA)] for 30 min at room temperature and then washed 8 times with 100 mM KP_i buffer pH 7.5/ 2 % BSA. The resulting cells were reacted with secondary goat anti-mouse polyclonal IgG DyLight 594 conjugated antibody (diluted 1:500 in PBS) for 1h at room temperature in the dark in a humid chamber. After washing 5 times with 100 mM KP_i buffer pH 7.5/ 2 % BSA, 3 µl of Vectashield per well were applied, slides were covered with the cover slip and sealed with nail polish. Filter I3 (excitation BP450-490/emission LP515) was used for EGFP detection, filter N2.1 (excitation BP515-560/emission LP 590) was used for DyLight 594 detection. For the surface display of EGFP, a merge of green (native EGFP fluorescence) and red (DyLight 594 FLAG tag detection) fluorescence was visualized with Image J software (http://rsbweb.nih.gov/ij/).

RESULTS

Expression vector construction and transformation for surface display

Plasmids for EGFP surface display were constructed as shown in Fig. S1. Primer extension PCR was performed to add *Xho*I restriction site (N-terminal fusion) and *Spe*I/*Not*I restriction site (C-terminal fusion) to *Pichia pastoris* codon optimized EGFP. PCR product was ligated with the *Xho*I/*Not*I opened pPpT4alphaS plasmid carrying *S. cerevisiae* alpha mating factor secretion signal. Eight putative GPI anchor proteins could be identified by GenDB BLAST homology search based on *H. polymorpha* and/or *S. cerevisiae* sequences. A FLAG tag was fused N-terminally to the anchors via PCR together with *Spe*I restriction site, whereas *Not*I site was fused at the C-terminus. Anchor-FLAG PCR products were fused C-terminally to EGFP so that the FLAG tag is located between EGFP and the anchor. The FLAG tag was used for immunofluorescent detection. All EGFP constructs were transformed into *Pichia pastoris* CBS7435 wild type strain and selected on Zeocin plates. Table S1 shows a list of the recombinant plasmids and Table S2 shows a list of the primers used in the supplemental data.

Immunofluorescence microscopy

The FLAG tag between EGFP and the different anchors was detected by immunofluorescence microscopy. Therefore, cells were induced in BMM medium containing 1% (v/v) methanol for 64 hours. Surface displaying EGFP cells using anchor A (GenDB annotated *gas1*) showed fluorescent spots on the surface most probably at budding sites, whereas on CBS7435[pPpT4alphaS-EGFP-FLAG-Anchor C] cells a fluorescent ring around the edge of the cells could be visualized (Fig. S2). Cells expressing levanase on the surface showed an equal distribution of fluorescent dye on the cell surface (Fig. S2). On control cells, no fluorescence could be observed.

FIGURES AND TABLES:

Table S1: Recombinant plasmids used in this study. *Pichia pastoris* GPI anchor proteins were identified based on homology searches of GPI anchored or cell wall proteins in general based on *Saccharomyces cerevisiae* and *Hansenula polymorpha* (*Pichia angusta*) sequences.

Plasmid	Purpose	Anchor (GenDB annotation)	GenDB ID
pPpT4_αS_EGFP/A	EGFP surface display	Full length PpGas1p	148978
pPpT4_αS_EGFP/B	EGFP surface display	Full length PpGas1p	148979
pPpT4_αS_EGFP/C	EGFP surface display	Full length PpCwp1p	153002
pPpT4_αS_EGFP/D	EGFP surface display	Full length PpWsc2p	191035
pPpT4_αS_EGFP/E	EGFP surface display	Full length PpSed1p	192092
pPpT4_αS_EGFP/F	EGFP surface display	Full length PpCwp1p	199427
pPpT4_αS_EGFP/G	EGFP surface display	Full length PpGas2p	199999
pPpT4_αS_EGFP/I	EGFP surface display	Full length PpTir3p	208766

Table S2: Primers used in this study

Primer	Sequence (5´to 3´)
EGFPXhoKexStefor	5'-TGACTCGAGAAGAGAGAGGCCGAAGCTGCTAGCAAAGGAGAAGAACTTTTC-3'
EGFPSpeNotrev	5'-AGTGCGGCCGCTTTTTTTACTAGTCTTGTACAATTCATCCATGC-3'
148978SpeFLAGfor	5'-AATACTAGTGATTACAAGGACGATGACGATAAGATGTTTAAATCTCTGTGCATG-3'
148978Notrev	5'-AGTGCGGCCGCTTAGAATGACATAATCATTCC-3'
148979SpeFLAGfor	5'-AATACTAGTGATTACAAGGACGATGACGATAAGATGTTGTCCATTTTAAGTGC-3'
148978Notrev	5'-TGAGCGGCCGCCTATAAAAGAGTGTAAGTGAATCC-3'
153002SpeFLAGfor	5'-AATACTAGTGATTACAAGGACGATGACGATAAGATGTTGTTGAAGTTGATTTGG-3'
153002Notrev	5'-AGTGCGGCCGCTTAGGCATTATTTCCTGGGGTC-3'
191035SpeFLAGfor	5´-AATACTAGTGATTACAAGGACGATGACGATAAGATGACCAAGTTTATATTGATATTGG-3'
191035Notrev	5'-TGAGCGGCCGCCTAAACTTCATCATCTGTGG-3'
192092SpeFLAGfor	5'-AATACTAGTGATTACAAGGACGATGACGATAAGATGCAATTCTCTATCGTCG-3'
192092Notrev	5'-AGTGCGGCCGCTTACAAGAAGTAAGCAGC-3'
199427SpeFLAGfor	5'-AATACTAGTGATTACAAGGACGATGACGATAAGATGTTCAACCTGAAAACTATTCTC-3'
199427Notrev	5'-AGTGCGGCCGCTTATTTCTCCCATACTTTAAGG-3'
199999SpeFLAGfor	5'-AATACTAGTGATTACAAGGACGATGACGATAAGATGCTGGGTTTCAAGGACTTC-3'
199999Notrev	5'-AGTGCGGCCGCCTAAGTAAATAATATTACTAAG-3'
201375SpeFLAGfor	5'-AATACTAGTGATTACAAGGACGATGACGATAAGATGAGATTTTCTAACGTCGTTTTAAC-3'
201375Notrev	5'-AGTGCGGCCGCTTACAAGGCAAAGACTCCGAAAG-3'
208766SpeFLAGfor	5'-AATACTAGTGATTACAAGGACGATGACGATAAGATGAGATTTTCTAACGTCG-3'
208766Notrev	5'-AGTGCGGCCGCTTACAAGGCAAAGACTCCGAAAG-3'

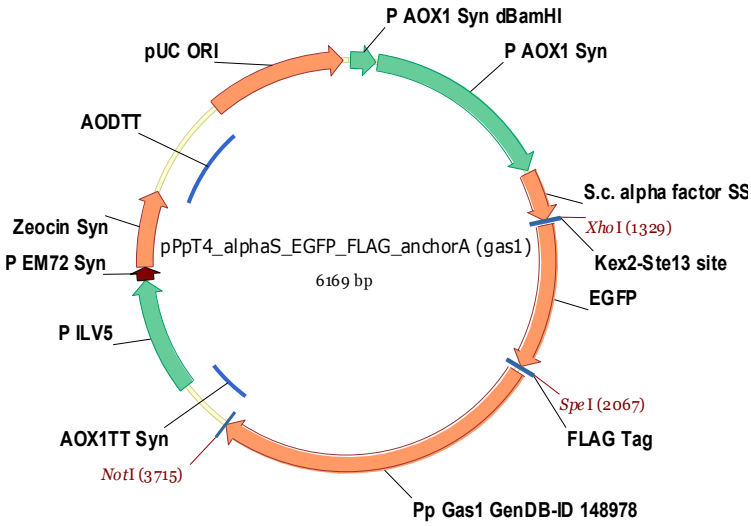

Fig. S1: Schematic diagram of the plasmid encoding the EGFP-Gas1 fusion protein. The sequence encoding *EGFP* was fused at the C-terminus to a FLAG tag and to the sequence encoding *P. pastoris* Gas1p (anchor A). The FLAG-anchor fragment was inserted into pPpT4_alphaS_EGFP vector after restriction with *Spe*I and *Not*I. The fusion protein was expressed under the control of AOX1 promoter. P AOX1 Syn dBamHI: part of *P. pastoris* P_{AOX1} (synthetic); P AOX1 Syn: synthetic *Pichia pastoris* AOX1 promoter; Kex2-Ste13 site: cleavage site for Kex2/Ste13 for processing of the alpha factor secretion signal; AOX1TT Syn: AOX1 transcription termination (synthetic); P ILV5: ILV5 promoter amplified from *Pichia pastoris* CBS 7435 strain; P EM72 Syn: Synthetic consensus sequence of an E.coli promoter; Zeocin Syn: synthetic, mixed codon optimized for the expression in *E.coli* and *P. pastoris*; AODTT: AOD transcription termination amplified from *Pichia pastoris* CBS 7435 strain; pUC ori: pUC replication origin for E.coli from pBR322 plasmid.

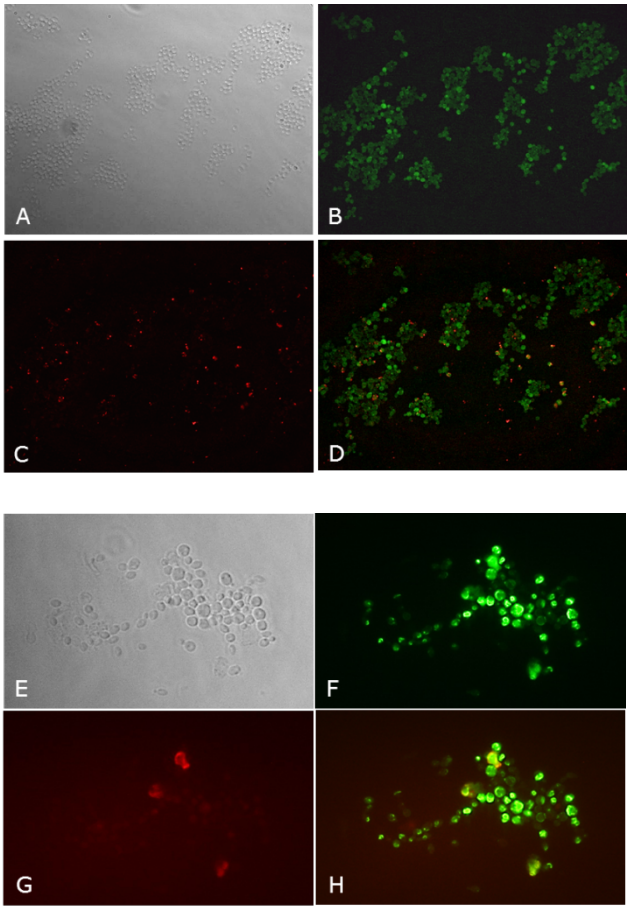

Fig. S2: Micrographs of EGFP surface displaying *Pichia pastoris* cells. A-D: anchor A (GenDB annotated *gas1*); E-H: anchor C (GenDB annotated *cwp1*). A, E: transmitted light microscopy; B, F: EGFP fluorescence micrographs using filter I3 (excitation BP450-490/emission LP515); C, G: fluorescence micrographs; cells were labeled with anti-FLAG antibody and DyLight 594 anti-mouse IgG and detected with filter N2.1 (excitation BP515-560/emission LP 590); D, H: Merge (ImageJ)

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Chapter 7

Pichia pastoris Surface Expression of the Hexameric *E. coli* Branched-chain Amino Acid Aminotransferase IlvE

My contribution to the paper draft is as follows:

- Conceptual formulation (100 %)
- Supervision of S. Moser (100 %)
- Construction of the IlvE plasmids (20 %)
- Deepwell fermentation (80 %)
- Shake flask fermentation (70 %)
- Immunofluorescence microscopy (80 %)
- Writing of the research article (100 %)

Pichia pastoris surface expression of the hexameric *E. coli* branched-chain amino acid aminotransferase IlvE

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ABSTRACT

Branched-chain amino acid aminotransferase (BCAT) coded by *ilvE* gene is one of 42 identified aminotransferases in *Escherichia coli* and is highly specific for the hydrophobic amino acids isoleucine, leucine and valine. So far it has been intracellularly over-expressed in *Escherichia coli* and *Pichia pastoris*. In this study we present the efficient surface expression of IlvE hexamer by immobilization of the protein on the *Pichia* cell surface using the well established *S. cerevisiae* alpha-agglutinin (*Sc*Aga1p) anchor. IlvE activity was determined and surface localization was confirmed by immunofluorescence microscopy and western blot. To our knowledge, this study describes for the first time the successful surface display of a functional hexamer and is therefore an impressive example for the use of *Pichia pastoris* as whole cell biocatalyst.

INTRODUCTION

Aminotransferases or transaminases are ubiquitously expressed enzymes in nature and fundamental for the composition and decomposition of amino acids. They reversibly catalyze transamination of various amino acids to 2-oxoglutarate requiring pyridoxal-5´-phosphate as a cofactor. Several examples for industrial applications can be found in literature mostly dealing with the production of optically pure amines, but also with the synthesis of unnatural amino acids or of alternative intermediates for the production of pharmaceutical components ((1)(2)(3)(4)). Branched-chain amino acid aminotransferase (BCAT) is one of 42 identified aminotransferases in *Escherichia coli* and is highly specific for the hydrophobic amino acids isoleucine, leucine and valine. In 1953, it has been described for the first time in literature (5) and in 1979 Lee-Peng *et al.* were able to solve the quaternary structure of the so-called Transaminase B. They proposed the enzyme to exist in a hexamer conformation with evidently identical subunits collocated as double-trimers (6). They estimated the molecular weight of the multimeric protein with 185 kDa and 31.5 kDa for the monomer and determined BCAT to be a part of the ilvGEDA operon. A few years later the nucleotide and the amino acid sequence of IlvE was solved by Kuramitsu *et al.* (7). Inoue *et al.* over-expressed BCAT in *E.coli*, purified it, determined the chemical and physical properties and supported the results of Lee-Peng *et al.* by electron microscopy (8). Finally, in 1997, Okada et al. solved the three-dimensional structure of BCAT and re-confirmed on the one hand the hexamer conformation but on the other hand they found out that three dimer units are positioned around a three-fold axis giving it the form of a triangular prism. The monomeric subunit is separated into a small and a large domain (9). Recently, Weinhandl *et al.* described the development of a new transaminase activity assay and the intracellular expression of IlvE in *Pichia pastoris* (10). Industrial applications of branched-chain amino acid aminotransferases can be mostly found in the field of nutrition (aroma compounds) (11). BCAT inhibitors are applied as pharmaceuticals for the treatment of neurodegenerative diseases (12).

Surface display in *Pichia pastoris* combines the advantages of the yeast *Pichia pastoris* as an expression host for recombinant proteins with the immobilization of the enzyme on the surface to create a whole cell biocatalyst for versatile applications. *Pichia pastoris* expression

system is well established and characterized; it is a perfect option for the high-level production of recombinant proteins. *Pichia pastoris* is easy to handle, grows on cheap media to high cell densities, has GRAS status, is able to secrete high product amounts into the fermentation broth, is relatively easy to manipulate as the genome sequence is available and even offers the opportunity to create e.g. pharmaceutical compounds with human glycosylation patterns. Until recently, *Pichia pastoris* surface display systems were based on well established *Saccharomyces cerevisiae* anchors, such as α-agglutinin (Aga1p) for N-terminal or Aga2p and FS or FL residues of Flo1p, respectively, for C-terminal fusion proteins. In case of Aga1p, the fusion protein is coupled to the cell wall using a glycosylphosphatidylinositol (GPI) anchor-attachment signal, whereas Aga2p target protein fusion is anchored indirectly to the cell wall as it is conjugated to Aga1p by means of a disulphide bond. FS or FL proteins comprise parts of the flocculation functional domain of Flo1p. These residues are able to mediate noncovalent interaction of the fusion protein with the cell wall by adhesion to mannan chains on the cell surface (13). In 2011, Khasa *et al.* published the development of a *Pichia pastoris* surface display system using the *P. pastoris* homologues of the *Sc*Pir1p as anchors (14). Not only the choice of the anchor itself, but also other considerations are important for a successful display of foreign proteins on the *Pichia* cell surface. A good signal sequence for an efficient transport to the cell membrane as well as secretion of the fusion protein is indispensable. In case of *Pichia pastoris*, the *S.c.* alpha factor secretion signal might be the signal peptide of choice, as well as other available signal peptides for protein secretion including native signal sequences of recombinant proteins to be immobilized on the cell surface. The influence of the positioning and the length of the anchor with respect to the sustainment of stability and activity of the target protein must be kept in mind, thus strongly contingent upon the application. The length and the amino acid content of the spacer between the anchor and the target protein should be optimized for best functionality and substrate accessibility of the displayed protein (12 and references therein).

Surface display of multimeric proteins is mostly limited to bacterial, phage or spore display systems. But even there it seems to be restricted to hetero-/homodimers (15)(16)(17) . Recently, Hwang *et al.* reported the expression of a *Bacillus subtilis* ω-transaminase which is functional a as dimer by use of a bacterial spore display system (18). Dozens of examples can be found in literature regarding the display of antibody fragments or libraries in phages

although in the last years display of antibody libraries and manipulations thereof or the display of immunological proteins like MHC became more and more abundant in yeast (reviewed by 18)(20). But to our knowledge, no example of the surface expression of a trimer, tetramer or hexamer has been reported yet.

In this study we describe for the first time the surface display of the functional *E.coli* BCAT hexamer on *Pichia pastoris* cells. *ilvE* gene was anchored with the commonly used *S. cerevisiae* alpha-agglutinin. A FLAG tag was introduced between the *ilvE* coding sequence and the membrane anchor for detection. BCAT activity was measured using a recently developed high throughout assay (10). Immunofluorescence microscopy, enterokinase treatment and finally western blotting was used to confirm the surface expression of the transaminase and hence the appropriateness of *Pichia pastoris* as whole cell biocatalyst.

MATERIALS AND METHODS

Strains and materials:

E.coli Top 10F´[lacI^{q} Tn10(tet^{R})] mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str^{R}) endA1 λ^{-}] was used as host for recombinant DNA manipulations. *P. pastoris* CBS 7435 wild type and CBS 7435 Δ*his* strains were used as the host for surface display. Plasmid for *ilvE* amplification (pPpT4GAPalphaS:ilvE#16#74 confirmed) was a kind gift from Andrea Camattari (Institute of Molecular Biotechnology, University of Technology, Graz). Plasmid DNA was isolated using Fermentas GeneJet Plasmid Miniprep Kit. Wizard Gel and PCR clean-up kit and T4 DNA Ligase were purchased from Promega GmbH (Mannheim, Germany). Enterokinase from porcine intestine, mouse IgG monoclonal anti-FLAG M2 antibody (#F3165), glutamate oxidase, L-Leucine, L-tert-leucine (TBG), 2-oxo-glutarate, pyridoxal-5-phosphate (PLP), 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) and horseradish peroxidase (HRP) were purchased from Sigma Aldrich Handels-GmbH (Vienna, Austria), goat anti-mouse polyclonal IgG DyLight 488 conjugated antibody was ordered from Fisher Scientific Austria GmbH (Vienna, Austria). Goat anti-mouse IgG antibody conjugated with alkaline phosphatase was purchased from Santa Cruz Biotechnology Inc. (Szabo-Scandic, Vienna, Austria). *E.coli* media components were purchased from AppliChem (VWR International GmbH, Vienna, Austria) or LabM Limited (Dr. Friedrich Bertoni GmbH, Vienna, Austria), respectively. *Pichia* media components were purchased from BD Biosciences (Becton Dickinson Austria GmbH, Vienna, Austria). Zeocin was purchased from InvivoGen (Eubio, Vienna, Austria). Phusion polymerase, restriction enzymes and prestained protein ladder mix were purchased from Fermentas GmbH (St. Leon-Rot, Germany). 12 % NuPAGE Bis/Tris gels, 4 x LDS sample buffer, 10 x MOPS buffer and BCIP/NBT kit were purchased from Invitrogen (LifeTech Austria, Vienna, Austria). All other chemical reagents used in this study were purchased from Lactan (Graz, Austria).

Media and cultivation conditions:

E.coli was cultivated in Luria-Bertani medium [1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl] containing 100 µg/ml ampicillin. Zeocin selection of *P. pastoris* pPpT4_alphaS transformants was performed on BYPD Zeocin plates (2 % peptone, 1% yeast extract, 2% glucose, 200 mM potassium phosphate buffer pH 7.0, 2 % agar, 100 µg/ml zeocin]. His^+ selection of pAaHSwa transformants was done on MD plates [1,34 % yeast nitrogen base w/o amino acids; 4 x 10^{-5} % biotin, 2 % dextrose, 2 % agar]. *P. pastoris* was grown in ½ BYPD [1 % peptone, 0.5 % yeast extract, 1 % dextrose, 200 mM potassium phosphate buffer pH 7.0]. Prescreens of *Pichia pastoris* transformants were performed in deep well plates. Therefore cells were grown in 250 µl ½ BYPD for 24 h, then 250 µl of 2 % (v/v) BMM was added for induction. Cultures were continuously induced for overall 48 h with BMM to a final methanol concentration of 0.5 % (v/v) during the day (8 h) and 1 % (v/v) during the night (16 h). Shake flask cultures were grown in ½ BYPD for 24 h and induced continuously for overall 48 h with 100 % methanol to a final methanol concentration of 0.5 % (v/v) during the day (8 h) and 1 % (v/v) during the night (16 h) in case of Mut^S clones and 1 % (v/v) during the day (8 h) and 2 % (v/v) during the night (16 h) in case of Mut^+ strains. Mut^+/Mut^S selection was performed on colony-level on MD and 1 % MM [1,34 % yeast nitrogen base w/o amino acids; 4 x 10^{-5} % biotin, 1 % methanol, 2 % agar] plates, respectively. Therefore plates were incubated for 48 to 72 hours.

Plasmid construction

ilvE CDS was originally PCR amplified from pPpT4GAPalphaS:ilvE#16#74 plasmid DNA. Alpha-Agglutinin was PCR amplified from *Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA using the primers agglutininEcoAscFLAGfornew110713 (5´-TTAGAATTCAAAAAAGGCGCGCC**AGCA**GATTACAAGGACGATGACGATAAGGGTCGGAACCTCGGTACAGC- 3´) and agglutininNotrev (5´-TGTGCGGCCGCTTAGAATAGCAGGTACGACAAAAGCAG-3´). PCR program was as follows: 98° C 3´//98° C 30´´/60° C 30´´/72° C 1´// 72° C 10´/ 4° C ∞. pPpT4_alphaS plasmid and gel purified PCR fragments were digested with *Eco*RI / *Not*I followed by PCR clean up, ligation and *E.coli* transformation. *ilvE* CDS was PCR amplified from pAaHSph_ilvE-ATG plasmid DNAs (kind gift from M. Ahmad) using the primers

ScalphaF_EcoRIfor (5'-ACGAATTCTTCGAAACG-ATGAGATTCCCATCTATTTTCACC-3') and ilvEAscrev (5'-ATTGGCGCGCC-CTGGTTGACCTGGTCCAAC-3') or PpalphaF_EcoRIfor and ilvEAscrev, respectively. pPpT4_alphaS_alpha-agglutinin plasmid and PCR fragments were *Eco*RI / *Asc*I digested followed by PCR clean up, ligation and *E.coli* transformation. Resulting pPpT4 plasmids were sequenced for confirmation. pPpT4 plasmids containing the *S.c.* or *P.p.* alpha factor, respectively, *ilvE* CDS, FLAG tag and alpha-agglutinin were restricted with *Eco*RI and *Not*I to release the secretion signal-ilvE-FLAG-anchor expression cassette as well as pAaHSph vector was cut with the same restriction enzymes. After gel purification, ligation and transformation in *E.coli*, resulting plasmids were sequenced for confirmation.

Plasmid transformation and expression

pPpT4_Sc alpha factor/Pp alpha factor_ilvE-ATG_α-agg plasmids were linearized with *Swa*I and transformed into the host strain *P.p.* CBS 7435 by Gene Pulser XCell Electroporation System (Bio-Rad) according to the condensed transformation protocol (21). Zeocin resistant transformants were selected on BYPD Zeocin plates. pAa(*Sc*)/(*Pp*)HSph_ilvE-ATG_FLAG_α-agg plasmids were digested with *Sph*I and transformed into the host strain Pp CBS 7435 *Δhis*. Transformants were cultivated in 96 deep well plates in ½ BYPD and BMM medium for IlvE activity prescreens. Hits of the prescreens were subjected to Mut^S/Mut^+ selection on MD and 1 % MM plates followed by shake flask fermentations in 300 ml baffled flasks containing 50 ml of ½ BYPD medium at 28° C and 120 rpm. Initial induction took place after 24 h with 250 µl 100 % methanol. Cells were further induced after 8, 24 and 32 hours with 250 µl or 500 µl of 100 % methanol, respectively. Cells were harvested after 48 h and objected to IlvE activity assay, immunofluorescence preparation and enterokinase treatment/western blotting. Copy number detection of the selected clones was done as described elsewhere (22). *Pichia pastoris* strain *P.p*. CBS 7435[pAaHSphilvE-ATG_C6] (Mut^+) was used as secretory *ilvE* expression control, *P.p.* CBS 7435[pAHSphilvE-ATG_A6] (Mut^+) as intracellular ilvE expression control and *P.p.* CBS 7435 wild type strain was used as negative control. qPCR of the two best performing surface display BCAT strains was performed as described elsewhere (22).

Porcine enterokinase treatment

1 ml of OD_{600} 5 or OD_{600} 20 induced fermentation broth harvested after 48 hours were centrifuged at 4000 g for 5 minutes at 4° C. Supernatant was withdrawn and pellet was washed twice with 10 mM Tris/HCl buffer pH 7.8 containing 2 mM $CaCl_2$ and 50 mM NaCl. Finally, pellet was resuspended in 250 µl of the upper washing buffer containing 2 units (OD 5 samples) or 5 units (OD 20 samples) porcine enterokinase and incubated at 4° C for 4 hours. After a final centrifugation step at 4000 g for 10 minutes at 4° C supernatant was carefully withdrawn; remaining cells were washed twice with PBS buffer and stored at 4° C for immunofluorescence. Supernatant was precipitated with 15 % TCA over night at 4° C. After two washing steps with each 500 µl distilled water, pellet was solubilized with 14 µl of ddH_2O and subjected to SDS PAGE.

BCAT activity assay

Fermentations were done as described in „plasmid transformation and expression". Prescreens were performed on deep well level, hits of the prescreens were subjected to shake flask fermentations. IlvE activity assays were performed in 96 well microtiter plates in replicates. Therefore 110 µl of each well of the deep well cultures were withdrawn, 10 µl were used for OD_{600} measurement. The remaining cultures were pipetted into 96 well v-bottom microplates, centrifuged for 10 min. at 3000 rpm. 1 ml samples from shake flask fermentations were taken and centrifuged for 5 minutes at 5000 rpm. Supernatants were transferred to a new 96 well microplate or to a new Eppendorf tube and stored at 4° C until measurement; pellets were washed twice with ddH_2O to remove any supernatant remnants. The method was described in (10). Shake flask experiments were repeated for five times and BCAT activity assays were always performed in 4 to 8 replicates per assay. Two substrates were used: L-Leucine which is taken up by the cell and L-tert-Leucine (TBG) which cannot be uptaken. General assay procedure was described in (10). This assay is optimized for crude lysate preparations of intracellularly IlvE expressing strains and had therefore to be adapted for pellet / supernatant measurements. Pellets were resuspended in 500 µl substrate solution containing 25 mM L-Leucine / TBG, 25 mM 2-oxo-glutarate, 0.5 mM pyridoxal-5-phosphate (PLP), 100 mM sodium-phosphate buffer pH 7.5. 200 µl of the supernatants were

mixed with 200 µl of the same substrate solution. Samples were incubated for 30 minutes at room temperature and centrifuged for 10 minutes at 4000 rpm. 50 µl of this supernatants were mixed with 50 µl enzyme mix containing 5 mM 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS), 20 mU/ml L-glutamate oxidase and 20 µg/ml horseradish peroxidase. Kinetics measurements were performed at 30° C and 419 nm in the Synergy-Mx Biotek plate reader (Biotek GmbH, Germany).

Immunofluorescence microscopy

OD_{600} of the induced cultures of *ilvE* constructs was measured and cultures were diluted to an OD_{600} of 1 with 100 mM potassium phosphate buffer pH 7.5. 100 µl of 37 % formaldehyde was added to each 1 ml of OD 1 cultures and incubated for 30 min. at 30° C. After centrifugation at 2500 rpm for 5 min., cell pellets were washed once with 100 mM KP_i buffer pH 7.5, 100 mM KP_i buffer / 4 % formaldehyde, 100 mM KP_i buffer / 1.2 M Sorbitol and 1.2 M Sorbitol. Cells were first incubated with IgG monoclonal anti-FLAG M2 antibody (diluted 1:500) for 30 minutes at room temperature and then washed 8 times with 100 mM KP_i buffer pH 7.5 / 2 % BSA. The resulting cells were reacted with secondary goat anti-mouse polyclonal IgG DyLight 488 conjugated antibody (diluted 1:500) for 1 h at room temperature in the dark in a humid chamber. After washing 5 times with 100 mM KP_i buffer pH 7.5 / 2 % BSA, cells were examined using a Leica fluorescence microscope. Filter I3 (excitation BP450-490/emission LP515) was used for DyLight 488 detection.

Western blotting

1 ml fermentation broth samples harvested after 48 hours were centrifuged at 10000 rpm for 5 minutes, supernatants were withdrawn and the pellets were stored at -20° C until proceeded. For SDS sample preparation, pellets were resuspended in the same volume of 10 mM phosphate buffer pH 7 as the sampling volume was. 12 µl of the resuspended pellet were mixed with 3 µl of 0.1 N NaOH, 2 µl 1M DTT and 6 µl 4 x LDS sample buffer. 14 µl of the supernatant were mixed with 1 µl 1 M DTT and 5 µl 4 x LDS loading dye. After heating for 10 min. at 95° C, samples were stored on ice until proceeded. Samples were loaded onto a 12 %

Bis/Tris NuPAGE gel and SDS PAGE was run in a NuPAGE chamber according to the manufacturer information. Gels were either stained with Coomassie Brilliant Blue and destained with 10 % acetic acid or used for western blot. Proteins were electroblotted to a nitrocellulose membrane (0.2 µm) with a transfer buffer containing 0.29 % Tris, 1.44 % glycin and 20 % (v/v) methanol, Ponceau S stained and rinsed with ddH_2O. Blocking was performed with 1x TBST (0.303 % Tris, 0.876 % NaCl, 0.05 % Tween 20, pH 7.5) with 5 % skimmed milk for 1 h at RT. Primary mouse anti-FLAG antibody (1:1000 dilution in 1x TBST 5 % skimmed milk) was applied for 2 hours on a shaker. After 5 x 5 min washing steps with 1x TBST buffer, the membrane was incubated with a 1:5000 dilution in 1x TBST 5% skimmed milk of goat anti-mouse IgG secondary antibody conjugated with alkaline phosphatase for 1 h at RT. After several washing steps (3 x 5 min. with 1x TBST, 2 x 5 min. with 1 x TBS without Tween), detection was made with BCIP/NBT substrate. Membrane was scanned for documentation.

RESULTS

Expression vector construction, transformation and *ilvE* surface expression

Two different vector systems (pPpT4alphaS and pAaHSph) and two different alpha factor secretion signals (from *S.c.* and from *P.p*.) were chosen for surface display. *Pichia pastoris* alpha factor secretion signal was identified from members of this working group and was published in 2011 (23). *ilvE* CDS was cloned without ATG. Plasmids for *ilvE* surface display were constructed as shown in Fig.1. FLAG-alpha-agglutinin *Eco*RI / *Not*I digested PCR product was ligated with the *Eco*RI/*Not*I opened pPpT4alphaS plasmid carrying *S. cerevisiae* alpha mating factor secretion signal*. S. cerevisiae* or *P. pastoris* alpha factor secretion signal, respectively, and *ilvE* CDS without ATG were PCR amplified from ilvE secretory plasmids pAa(*Sc*)/(*Pp*)HSphilvE-ATG (kind gift from M. Ahmad), *Eco*RI / *Asc*I digested and ligated with the *Eco*RI / *Asc*I opened pPpT4_alpha-agglutinin plasmid. The two resulting pPpT4 plasmids carrying the different alpha factor signal sequences, ilvE-ATG, a FLAG tag and the alpha-agglutinin anchor were sequenced for confirmation. For pAa(*Sc*)/(*Pp*)SphilvE-ATG cloning the two pPpT4 plasmids were cut with *Eco*RI and *Not*I and were ligated with the *Eco*RI / *Not*I opened pAaHSwa plasmid. The two resulting pAa(*S.c.*)/(*P.p.*)SphilvE-ATG-FLAG-alpha-agglutinin plasmids were again sequenced for confirmation. The FLAG tag was used for immunofluorescent detection, enterokinase treatment and western blotting. pPpT4 constructs were linearized with *Swa*I, transformed into *Pichia pastoris* CBS 7435 wildtype strain and selected on Zeocin plates, whereas pAaHSph constructs were digested with *Sph*I, transformed into *Pichia pastoris* CBS 7435 *Δhis* strain and selected on MD plates.

BCAT activity assay

Prescreens in deep well plates were made where 80 transformants per construct were inoculated in a 96 well microplate together with the secretory and intracellular control and the *P.p.* CBS 7435 wild type strain. Constructs carrying the *Pichia pastoris* alpha factor signal sequence were much weaker in expression compared to the *Saccharomyces cerevisiae* alpha factor constructs (data not shown) and were therefore considered being neglectable. The

three best, three middle and three weakly performing clones of the *S.c.* alpha factor constructs were chosen for further deep well experiments and were inoculated in 8 replicates each. Mut^+/Mut^S selection was performed from these clones. A clear difference between the pPpT4 and the pAaHSph vectors became apparent. There was just one pPpT4_*Sc*alphaS clone that showed approximately the same expression or activity level as the best three pAaHSph clones. Interestingly, all pPpT4 transformants turned out to be Mut^+, whereas pAaHSph transformants were both Mut^+ and Mut^S. As the best results were obtained with pAaHSph transformants, continuous experiments focused on these clones. Shake flask experiments were performed with the two best clones, named *P.p.* CBS 7435[pAaHSph_ilvE(-ATG)-FLAG-Aga1p] G1 (Mut^S) and *P.p.* CBS 7435[pAaHSph_ilvE(-ATG)-FLAG-Aga1p] G2 (Mut^+), and one middle expressing clone *P.p.* CBS 7435[pAaHSph_ilvE(-ATG)-FLAG-Aga1p] G5 (Mut^S). Control strains used were *P.p*. CBS 7435[pAaHSphilvE-ATG] C6 (Mut^+) for secretory control and *P.p.* CBS 7435[pAHSphilvE-ATG] A6 (Mut^+) for intracellular control of *ilvE* expression. P.p. CBS 7435 wild type strain was used as negative control. Fig. 2 shows a diagram with the activity assay data. Quantitative real time PCR of the two best performing surface display BCAT strains determined the following copy numbers: 4 copies in the two Mut^S strains G1 and more than 20 copies in the Mut^+ strain G2. Activity levels in the supernatants of G1 and G2 were almost zero with L-Leucine and TBG substrate, the pellets showed really high activity comparable or to activity levels reached with the secretory control (M. Ahmad, personal communication). Interestingly, in this study, just weak activity was detectable with the supernatant of the secretory control. The intracellular IlvE expression control pellet fraction gave relatively high signal with L-Leucine, the substrate that enters the cell and no signal with TBG (L-tert-Leucine), the substrate that cannot be taken up by the cell, confirming the reliability of the ilvE activity measurement protocol and the maintenance of cell stability despite the 2 washing steps.

Immunofluorescence microscopy

The FLAG tag between ilvE and alpha-agglutinin anchor was detected by immunofluorescence microscopy. Therefore, cells were fermented as described above and harvested after 48h. Surface displaying IlvE cells showed either fluorescent regions on the surface most probably at budding sites, or a fluorescent ring with dots around the edge of

the cells could be visualized (Fig. 3). G2 strain showed a really high number of fluorescent cells, whereas in G1 cells *ilvE* expression on the surface seemed much weaker, although the measured activity levels were almost the same (data not shown). After porcine enterokinase treatment, fluorescence disappeared (data not shown). On control cells, no fluorescence could be observed.

Western Blot

The two best performing clones G1 and G2 and the weakly performing clone G5 were chosen for western blotting (Fig. 4). Therefore, the washed fermentation pellets and the enterokinase supernatants were subjected to SDS PAGE followed by western blotting. In the pellets fractions, three (four?) bands were clearly visible, a double band at around 30-40 kDa probably corresponding to the glycosylated/unglycosylated monomer, a weaker band at about 60 kDa which could correspond to the dimer, a band at around 80 kDa that should theoretically correspond to the trimer and a huge band above 140 kDa corresponding to the hexamer (6). The same bands were visible in the TCA precipited enterokinase supernatants, whereas the upper two bands seemed to be more prominent that the lower ones. That makes sense as the enzyme should be surface displayed in the functional multimerized, hexamer form being demonstrated by BCAT activity.

DISCUSSION

To date, functional surface expression of multimeric, mostly dimeric proteins is virtually confined to phage, bacterial and spore display systems. Expression of antibody fragments and MHC class II molecules are the only reports of attempted multimeric protein production on the yeast surface (20)(19). In this study we report for the first time to our knowledge the functional expression of a hexameric protein, the *E.coli* branched chain amino acid aminotransferase (BCAT) IlvE, on the surface of *Pichia pastoris*. Two different vector systems, both developed in-house, were used in parallel for a direct comparison. On the one hand the pPpT4 vector system that uses a synthetic P_{AOX1} based on the original P_{AOX1} from *Pichia pastoris*, a synthetic AOX1 termination site and a *P.p.* codon optimized synthetic zeocin selection marker cassette and on the other hand the pAaHSwa vector system using the original *Pichia pastoris* P_{AOX1} mimicking at the best the original situation in the *P.p.* AOX1 expression cassette avoiding junk DNA in the immediate vicinity of the Kozak sequence/ATG and His4 auxotrophy as selection marker. Recently, the *Pichia pastoris* alpha mating factor secretion signal was identified in our group (23). Hence, *S.c.* and *P.p.* alpha factor secretion signals were used for attempted *ilvE* surface display. The two secretion signals structurally differ from each other. One has to consider that we included the hexa-peptide KREAEA implying the Kex2p and Ste13p processing site connecting the pro-sequence with the mature alpha factor peptide into the pPpT4 expression vectors marking the transition point of the secretion signal to the coding sequence of the protein to be secreted/surface displayed. This hexa-peptide is virtually ideal for the *S.c.* alpha mating factor secretion signal but in recent experiments based on the published *Pichia pastoris* alpha factor sequence it became apparent that the *Pichia pastoris* alpha mating factor secretion signal works better the more EA peptides are included (M. Ahmad, unpublished results), that´s why we included 5EA repeats into the constructs with the *P.p.* alpha factor secretion signal. Already the first prescreens showed a distinct tendency and confirmed our surmise – the *Pichia pastoris* alpha factor, at least in this constellation, was not the secretion signal of choice in this case. Finally, the pAaHSwa vector turned out to be the winner. The transformants constantly showed the best performance throughout the first prescreens which was later on also confirmed by deep well fermentations of selected clones done in replicates. In IlvE secretion

experiments in our group an interesting phenomenon occurred. Only Mut^+ clones were able to secrete functional, active IlvE (M. Ahmad, unpublished results). This lead to discussions about the ideal integration locus and the existence of transcriptional hot spots. When we made a Mut^+/Mut^S selection of the selected clones it turned out that two of the three best performing clones were Mut^+ and one was interestingly Mut^S. *ilvE* activity assay based on the conversion of L-Leucine or L-tert-Leucine to L-Glutamate and further metabolism of the L-Glutamate to H_2O_2 detected with ABTS (10). This assay is very sensitive and had to be adapted for surface display as the washed pellets were the basis of our measurements. Assays were performed with the substrates L-leucine that is able to enter the cell and with the synthetic substrate L-tert leucine that cannot be uptaken by the *Pichia* cell. The disadvantage of the synthetic substrate is that it is worse metabolized than L-leucine meaning it gives a much weaker signal/activity in the assay. Nevertheless, the data were reliable and coherent. However, a problem with the secretory expression control became apparent. Although the performance was initially, in deep well fermentations, as it should be, almost no activity in the shake flask fermentation supernatants could be detected. More detailed and repeated shake flask experiments with secretory clones revealed a relation between the media composition and the level of secretion. Obviously minimal media instead of complex media as used in this study increased the productivity of the secretory strains a lot (M. Ahmad, personal communication). As seen for the intracellular expression control, the pellet washing steps with ddH_2O did not cause any cell breakage that could have biased the results. For any reason, immunofluorescence analysis didn´t work out in the beginning. We speculated that the complexity or size of the hexamer complex (ca. 180 kDa) could possibly cover the short peptide sequence bound by the anti-FLAG antibody but in the end we succeeded. Enterokinase cleavage of the FLAG tag between the *ilvE* coding sequence and the alpha-agglutinin anchor to release the enzyme complex into the media as a final evidence for surface anchoring proved to be rather difficult and we needed quite a lot of cells to see something in the western blot. Two possible problems came into our mind: again the accessibility of the cleavage site for the enterokinase enzyme and the preference of the enterokinase as it was not clear from the manufacturer´s informations whether the enzyme is capable of cleaving between two proteins. But higher OD for the enterokinase treatment and the increase of the enzyme amount followed by trichloroacetic acid precipitation of enterokinase cleavage supernatant was able to solve the problem. On the

western blot four (five) distinct bands could be detected: a double band between 30 and 40 kDa corresponding to the glycosylated/unglycosylated monomer. These bands were also prominent in the supernatant of the ilvE secretory expression clones and the upper band disappeared after treatment with endoglycosidase H (M. Ahmad, unpublished data). The upper bands seemed to correspond to the dimer, trimer and finally hexamer (ca. 180 kDa). Concluding, surface display of the multimeric branched-chain amino acid aminotransferase IlvE is a versatile tool and an impressive example for the use of *Pichia pastoris* as whole cell biocatalyst.

FIGURES

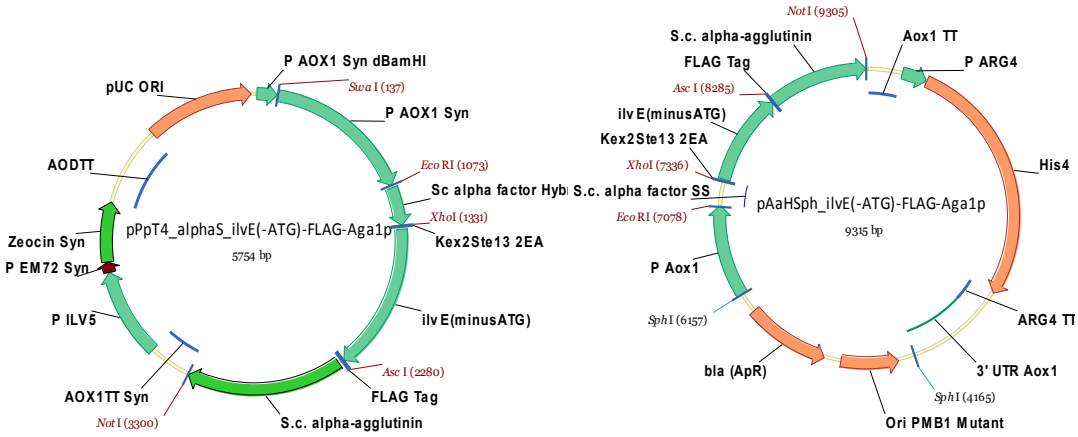

Fig. 1: Schematic diagram of the plasmids encoding the ilvE-FLAG-Aga1p fusion protein. Left plasmid map: The sequence encoding *ilvE(minusATG)* was fused at the C-terminus to a FLAG tag and to the sequence encoding *S. cerevisiae* alpha-agglutinin (Aga1p). The *ilvE* fragment was inserted into pPpT4_alphaS_FLAG-Aga1p vector after restriction with *Xho*I and *Asc*I. The fusion protein was expressed under the control of AOX1 promoter. P AOX1 Syn dBamHI: part of *P. pastoris* P_{AOX1} (synthetic); P AOX1 Syn: synthetic *Pichia pastoris* AOX1 promoter; Kex2-Ste13 site: cleavage site for Kex2/Ste13 for processing of the alpha factor secretion signal; AOX1TT Syn: AOX1 transcription termination (synthetic); P ILV5: ILV5 promoter amplified from *Pichia pastoris* CBS 7435 strain; P EM72 Syn: Synthetic consensus sequence of an *E.coli* promoter; Zeocin Syn: synthetic, mixed codon optimized for the expression in *E.coli* and *P. pastoris*; AODTT: AOD transcription termination amplified from *Pichia pastoris* CBS 7435 strain; pUC ori: pUC replication origin for E.coli from pBR322 plasmid. Right plasmid map: The sequence encoding *ilvE(minusATG)* was fused at the C-terminus to a FLAG tag and to the sequence encoding *S. cerevisiae* alpha-agglutinin (Aga1p). The ilvE-FLAG-Aga1p fragment from pPpT4_alphaS_ilvE(minusATG)-FLAG-Aga1p was inserted into pAaHSph vector after restriction with *Xho*I and *Not*I. The fusion protein was expressed under the control of AOX1 promoter. P Aox1: AOX1 promoter; S.c. alpha factor SS. S. cerevisiae alpha factor secretion signal; AOX1 TT: AOX1 transcription termination; P ARG4: ARG4 promoter; His4: HIS4 coding region for selection in *Pichia pastoris*; ARG4 TT: ARG4 transcription termination; 3´ UTR Aox1: 3´ untranslated region of AOX1 for homologous recombination into AOX1 locus; Ori PMB1 Mutant: replication origin for *E.coli*; ble (ApR): ampicilline resistance cassette for selection in *E.coli*; all parts of the plasmid except the *E.coli* part, *S.c.* alpha factor and levanase were amplified from *P. pastoris* CBS 7435 wild type strain.

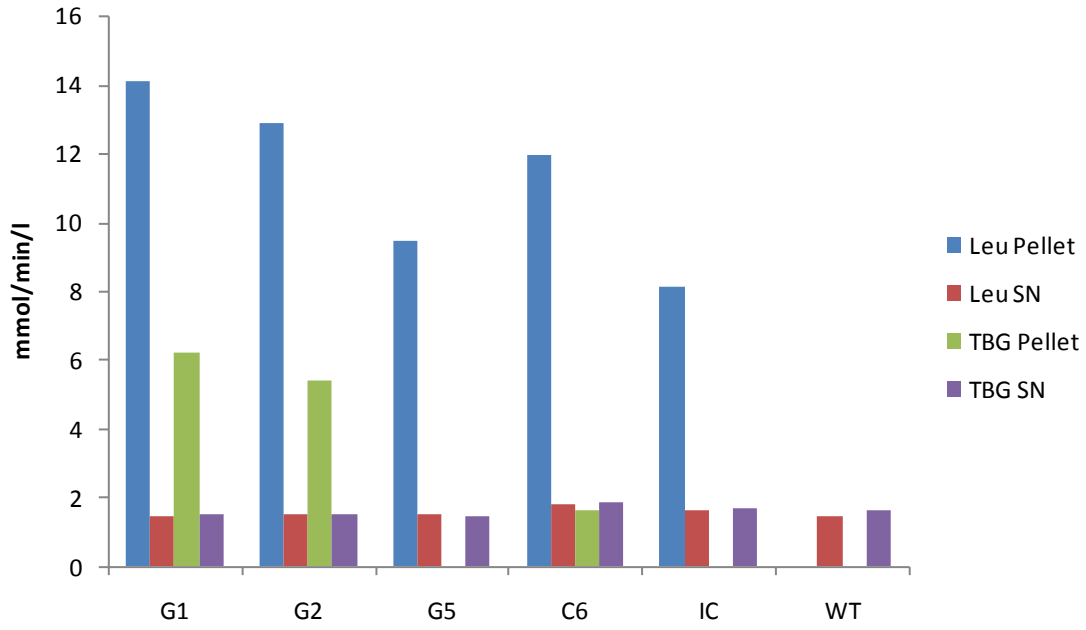

Fig. 2: Diagram of the BCAT activity assay performed with the supernatants / washed pellets of *P. pastoris* CBS 7435[pAaHSph_ilvE(minusATG)-FLAG-Aga1p] clones. Measurements were performed after 48 hours of induction. Values are given in mean +/- standard deviation. As the overall OD_{600} values were almost the same, values were not normalized to OD_{600}. Measurements were performed in 8 replicates each. Leu: L-Leucine; TBG: L-tert-Leucine; SN: supernatant; G1: *P. pastoris* CBS 7435[pAaHSph_ilvE(minusATG)-FLAG-Aga1p] clone G1 (mutS); G2: *P. pastoris* CBS 7435[pAaHSph_ilvE(minusATG)-FLAG-Aga1p] clone G2 (mut+); G5 *P. pastoris* CBS 7435[pAaHSph_ilvE(minusATG)-FLAG-Aga1p] clone G5 (mutS); C6: *P. pastoris* CBS 7435[pAaHSph_ilvE(minusATG)]_C6 secretory control; IC: *P. pastoris* CBS 7435[pAHSph_ilvE(minusATG)]_A6 intracellular control; WT: P. pastoris CBS 7435 wild type strain.

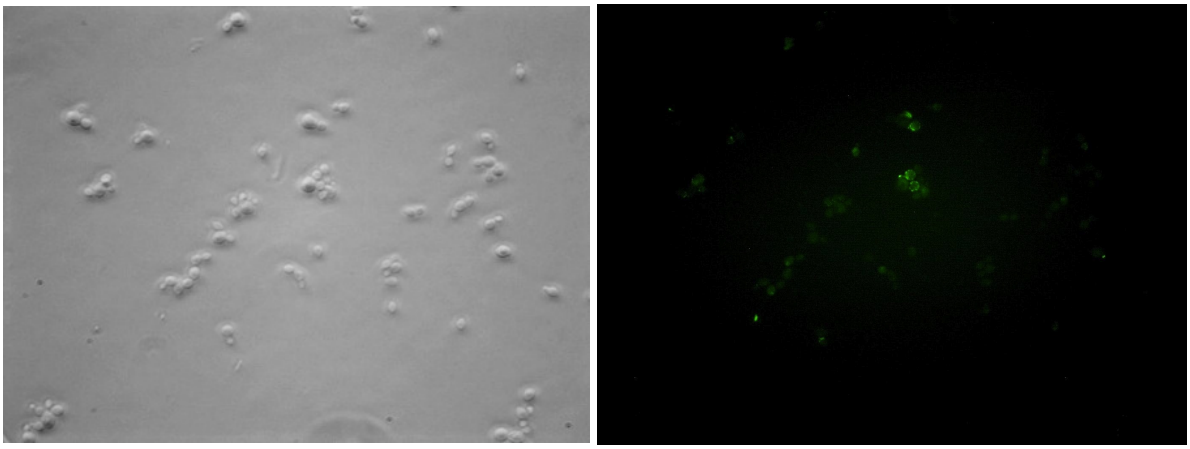

Fig. 3: Micrographs of *Escherichia coli* BCAT *(ilvE)* surface displaying *Pichia pastoris* strain CBS 7435[pAaHSph_ilvE(minusATG)-FLAG-Aga1p] clone G2. Left picture: transmitted light micrograph; right picture: fluorescence micrograph; cells were labeled with anti-FLAG antibody and DyLight 488 anti-mouse IgG; Exposure Time 540 ms; Enhancement 7,2x; Pseudocolors 529 nm

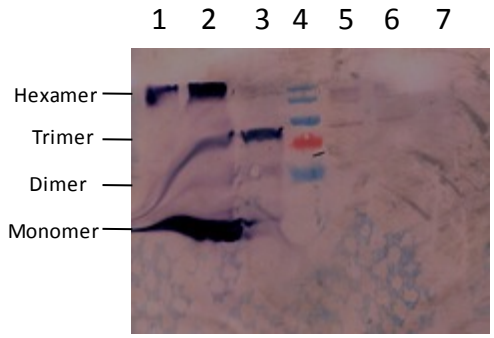

Fig. 4: Western Blot of cell pellets and enterokinase supernatants (TCA precipited) of the three IlvE surface displaying *Pichia pastoris* strains CBS 7435[pAaHSph_ilvE(minusATG)-FLAG-Aga1p] G1, G2 and G5. Shake flask fermentation was finished and samples were taken after 48 hours of methanol induction. 1: resuspended pellet of clone G1; 2: resuspended pellet of clone G2; 3: resuspended pellet of clone G5; 4: PageRuler Prestained Protein Ladder; 5: TCA precipited enterokinase supernatant of clone G1; 6: TCA precipited enterokinase supernatant of clone G2; 7: TCA precipited enterokinase supernatant of clone G5. The red band of the prestained protein ladder is the reference band with the size of 65 kDa.

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FINAL DISCUSSION AND OUTLOOK

In the last years a lot of pioneering has been done with respect to the clarification of the genomic background of host strains used for recombinant gene expression. In 2009, draft genome sequences of Pichia pastoris GS115 and CBS704 have been published (1)(2) thereby providing the tools for targeted (metabolic) engineering of host strains and enhanced recombinant protein production. Finally, in 2011, we published the high quality genome sequence of the parental strain NRRL Y-11430, which has been deposited as CBS7435 in the CBS (Centraalbureau voor Schimmelcultures) and has been classified as *Komagataella phaffii* (3). 2 sequencing approaches (454 sequencing and illumine method) were combined with Sanger sequencing of PCR amplicons for gap closure (which was mainly my part). As one result, the first complete mitochondrial genome of a methylotrophic yeast was reported. A resequencing of the MUT pathway genes was also performed; thereby the incorrect assembly of DAS1 and DAS2 in the *P. pastoris* GS115 sequence was corrected. A previous attempt to create a das1/das2 knockout, which would be attractive, as growth should be abolished on methanol as single carbon source, failed due to the erroneous sequence. A direct comparison of next generation sequencing results and Sanger reads on PCR amplified MUT pathway genes showed a perfect match. Only a slight distinction to the published GS115 AOX2 gene became apparent in form of a single nucleotide polymorphism at position 785. Moreover, the *Pichia pastoris* alpha mating factor was identified and isolated (which was also my part) representing a good alternative for the commonly used *Saccharomyces cerevisiae* alpha mating factor secretion signal. In first experiments, the secretory expression of *Bacillus subtilis* comparing both secretion signals reached comparable expression rates (M. Ahmad, personal communication). Indeed, the architecture of *P. p.* alpha mating factor protein turned out to be far more complex.

In a second approach, a special head-to-head promoter situation was unraveled probably being responsible for the high-yield intracellular expression of *Hevea brasiliensis* hydroxynitrile lyase. This has been done in the doctoral thesis of B. Krammer and the master thesis of S. Nussbaumer. My part was now to analyse the head-to-head promoter sequence and to build up an expression system based on this novel AOX1 fusion promoter. Analysis of the head-to-head promoter fragment by Matinspector software showed no

formation of novel transcription factor binding sites, but the loss or multiplication of possible positive or negative acting factor binding sites, e.g. Adr1 or some of the claimed Mxr1 response elements (4). It could also be the case that the integration of the expression cassette next to another strong promoter might enhance the transcription of the integrated one, which makes sense considering chromosome packaging and chromatin remodelling and the effect on the accessibility of the DNA for regulatory elements. First pinpointed by Schadt *et al.*, expression quantitative trait loci (eQTL) "hot spots", i.e., transcription activity hot spots, defined as chromosome regions that contain more eQTL, that would have been detected by chance, have been points of research interest in almost all studies that search for genetic regulators for gene expression (5). Hot spots of gene regulation are most prominent in yeast (6; 7), where 8 have been detected, which were functionally subsumed under e.g. mating or mitochondrial. Nevertheless, the construction of new AOX1 head-to-head promoter expression vectors carrying HNL W128F mutant as model protein and some *Pichia pastoris* transformants thereof were able to reach expression and activity levels comparable to the *Hb* HNL expression level described by Hasslacher *et al.* in 1997. As a final step, it would be interesting to clarify the genome sequence of the original high level expression strain as the integration locus of the additional head-to-head Aox1 promoter expression cassette is still not known and the promoter rearrangement is probably not the only thing that happened during the spheroplast transformation.

In a third line, the work of M. Tscherner, which was done in frame of his diploma thesis, was continued. At the point I took over his work, initial results including the set up of a screening system based on *Bacillus subtilis* levanase, the screening of a *Pichia pastoris* library for regulatory elements of the AOX1 promoter and the identification of transformants containing fragments encoding functional AOX1 that show methanol free induction of the AOX1 promoter. The construction of active site AOX1 mutants followed by transformation into the AOX1/AOX2 double knockout strain and western blot analysis revealed the expression of a non-functional AOX1 protein. Growth assays of these mutants and of an AOX1 stop mutant confirmed the preliminary results and the hypothesis that arose from M. Tscherners work. AOX1 seems to have an auto-regulatory function on the AOX1 promoter. Furthermore, the two mutagenized amino acids are obviously essential for the active site of AOX1 protein. Interestingly, differences between growth on sucrose or inulin became obvious. Whereas strains with a functional AOX1 protein were able to grow on agar plates

containing sucrose, they didn´t grow on inulin. This might as well be another hint or proof for an auto-induction effect of AOX1. Another possible explanation could be that levanase, although theoretically secreted via *S. cerevisiae* alpha factor, is expressed but gets stuck in the periplasm. Sucrose, much less complex than inulin, diffuses to periplasm, where it was cleaved by levanase. Additionally, an acidification of the media surrounding the emerging colonies caused by cell growth could have a supportive effect. We also noticed that inulin is a very sensitive substrate to work with. Another explanation could be that *Pichia pastoris* might have an intrinsic source of tiny amounts of methanol that induced levanase expression.

It´s not proven so far that methanol is the inducer of the P_{AOX1}. The inducer might be a secondary metabolite like formaldehyde or for example dihydroxyacetone (DHA) formed by dihydroxyacetone synthase. The growth assays that were performed on plates grown under formaldehyde atmosphere led to the assumption that formaldehyde could be a possible inducer as an enhancing growth effect on sucrose and inulin plates was reached, even for the wild type strain *P.p.* CBS 7435 that should not be able to grow on sucrose or inulin. These result supported the fact that growth under formaldehyde atmosphere probably has a toxic effect on the cells. Liquid levanase assays were performed under methanol and formaldehyde inducing conditions whereas formaldehyde has definitely no inductive effect. A comparison of the four different AOX1 mutants in contrast showed that the N616A strain possesses an interesting feature. The strain cannot use methanol but under methanol inducing conditions levanase expression respectively activity reaches the same or even a higher level compared to the original gene library transformant strain having a functional AOX cassette (Fig. 6). The other mutants, no matter if carrying the H567 or the stop mutations, shows almost the same level of levanase activity as well as the double knock-out strain. This is not astonishing as all these strains do not have a functional AOX1 but P_{AOX1} is still induced by methanol and leads to levanase expression. Growth plate assays showed that the N616A strain doesn´t grow on methanol, but grows weakly on sucrose and strongly on methanol sucrose plates, whereas the retransformant strain grows strongly on methanol and methanol sucrose, but slightly on sucrose plates. These final results are contradictory to the former results as we postulated a functional AOX1 to be essential for the auto-regulatory function. The exact reasons for this effect remain to be elucidated but it is of great benefit

for a lot of biotechnological applications to have a strain in hand that needs just tiny amounts of methanol for high effective high yield production.

Chapter 4 describes another possibility to avoid direct methanol induction. Wet work of the study was mostly conducted by T. Nudl in frame of her diploma thesis. Conceptual formulation, supervision and correction of the thesis were my part. The idea of the study was to create a *Pichia* host strain for recombinant protein expression in which the induction of the strong AOX1 promoter takes place without the addition of methanol. Therefore different autologous esterases and one lipase were identified via bioinformatic tools. These enzymes were constitutively expressed in *Pichia pastoris* and tested for functionality applying three different esterase/lipase assays, which primarily had to be adapted for *Pichia pastoris*. We decided for constitutive expression as the native function of the enzymes is not known and too high expression levels could have a negative effect on cell metabolism. The three different assays were chosen to get as much information as possible about a moderate functional expression of the enzyme in Pichia. First, the alpha-naphthyl acetate assay that gave us just an idea that the chosen enzymes have something like an esterase activity followed by pH shift assay which increased specificity as real methyl esters were applied although we had to fight some setbacks as the acidic pH prevailing in the cytosol of *Pichia* cells caused problems. It would have probably been advantageous to test other pH indicators whose color changing takes place in a more acidic range. The third assay using Rhodamine B was chosen because the ability of the cloned enzymes on long chain fatty acid methyl esters should be evaluated. In case of a conversion the formation of a fluorescent halo around the cell was described (8). But no halos could be detected whereas whole colonies became fluorescent under UV light. Probably methyl oleate or biodiesel cannot be taken up by the cell and the expression/secretion levels were rather low. The analysis of constitutively expressed esterases/lipase on protein level ran into difficulties as no tag was added and no antibodies were available. Therefore the SDS PAGE analysis was made showing solely an additional, distinct band in the size of 47 kDa in case of methyl carboxyl esterase expression. The differences in the protein patterns of the wild type and the strain constitutively expressing the methyl carboxyl esterase were remarkable probably caused by an alteration in the cell metabolism. PAGE analysis with a native, non-denaturing gel, revealed an additional band or more a smear, which was probably caused by different glycosylation patterns, becoming visible after α-naphthyl acetate treatment of the

concentrated MCE samples. No other constructs gave a signal on the native gel. The reason could be that the enzymes got stuck in the periplasm and the expression was rather low, maybe below the detection level of the method although concentrated samples were used. All four expression cassettes were finally transformed into the HNL reporter strain and assayed for the indirect induction effect on the AOX1 promoter. *in situ* HNL assay is problematic as the reproducibility of the assay is very sensitive to environmental factors. Gaseous HCN is detected and so parameters like differences in growth or the airflow under the hood can create different results. To minimize these problems, all HNL assays were done at least in triplicates. An effect of the additionally expressed esterases/lipase could clearly be seen. The signal of the HNL assay meaning the velocity of blue color development of the transformants was much higher than in the reporter strain itself although the overall intensity of the signal was finally the same. SDS PAGE and western blot analysis of 2 clones per construct positively HNL tested showed in case of methyl oleate fermentation just slight differences to the reporter strain. It seems that HNL is to a certain, weak extent capable of methyl oleate cleavage and therefore allocates slight amounts of methanol. Another possibility could be that leakiness of the AOX1 promoter caused by glucose depletion provokes a weak expression. Additionally, the supplied methyl oleate was not 100 % pure and could contain free methanol. Altogether, product formation or secretion was not at all comparable to the expression rate reached with the methanol induced reporter strain. Induction of the construct harboring the methyl carboxyl esterase with biodiesel, in contrast, lead to an equal HNL expression level compared to the expression level of the methanol induced reporter strain after an induction time of 49 hours. Remarkably, the expression level could already be obtained after 13 hours of biodiesel induction. A GC analysis of the biodiesel was made to exclude the influence of possible free methanol in the biodiesel fraction resulting in a neglectable free methanol concentration. Altogether, methanol induction could be successfully circumvented by the development of the methyl carboxyl esterase co-expressing strain. It provides a promising tool and bears a big potential for improvement and optimization. One option would be to immobilize an appropriate methyl carboxyl esterase on the *Pichia* cell surface to optimize methanol availability. In case of the already available strain, improvement of fermentation conditions could also be of benefit. The development of a novel *Pichia pastoris* surface display system using newly identified and isolated *Pichia* cell wall proteins was strived to overcome secretion bottleneck. 8 putative

anchors were chosen to be analyzed for immobilization capacity of 2 reporter genes (*Bacillus subtilis* levanase and *Pichia* codon optimized EGFP) on *Pichia* surface. The use of *Pichia´*s own cell wall anchors can be advantageous for the correct processing and folding of the fusion protein followed by efficient and stable surface expression. An important point that has to be considered is the influence of the positioning and the length of the anchor with respect to the sustainment of stability and activity of the target protein (9). Surface display of *Bc*SacC showed a very high level of levanase activity on the cell surface, but almost none in the supernatant, when anchored with *Pichia pastoris* Sed1p. Application of anchor C (Cwp1p) also mediated good immobilization on the cell wall, but much less levanase activity. It could be shown for the *sacC*-Sed1 strain E4 that the surface expression of levanase seems to be stable as the activity is still increasing until the final time point at 72 hours. EGFP surface display explicitly worked for anchor A (GenDB annotated gas1) and anchor C (GenDB annotated cwp1), whereat immunofluorescence detection with anti-FLAG antibody was also successful with anchor I (GenDB annotated tir3) (data not shown). In case of anchor C suitability for the surface display of both reporter proteins could be shown to a certain extent. If an anchor works excellently with one protein one cannot conclude that it would be the perfect choice for all other proteins as well. Unfortunately sufficient surface display has to fulfill various parameters and in the end it is in many cases a matter of trial and error to find the appropriate system for ones needs. Nevertheless, *Pp*Sed1p can be considered as interesting candidate for efficient *Pichia* surface display of other recombinant proteins, but at least one more protein could be identified (*Pp*Cwp1p) that showed a good suitability, which needs further investigations.

The last part of my thesis describes the attempt to show the potential of *Pichia pastoris* as a whole cell biocatalyst for the expression of complex, multimeric enzymes. Therefore, *Escherichia coli* branched chain amino acid aminotransferase (BCAT, *ilvE*) was anchored on the Pichia cell surface by use of the well-established Aga1p (alpha-agglutinin)-system of Saccharomyces cerevisiae. Two different in-house developed vector systems were applied in parallel for a direct comparison. On the one hand the pPpT4 vector system that uses a synthetic P_{AOX1} based on the original P_{AOX1} from *Pichia pastoris*, a synthetic AOX1 termination site and a *P.p.* codon optimized synthetic zeocin selection marker cassette and on the other hand the pAaHSwa vector system using the original *Pichia pastoris* P_{AOX1} mimicking at the best the original situation in the *P.p.* AOX1 expression cassette avoiding junk DNA in the

immediate vicinity of the Kozak sequence/ATG and His4 auxotrophy as selection marker. *Saccharomyces cerevisiae* and *Pichia pastoris* alpha factor secretion signals were used for attempted *ilvE* surface display. The two secretion signals structurally differ from each other. One has to consider that we included the hexa-peptide KREAEA implying the Kex2p and Ste13p processing site connecting the pro-sequence with the mature alpha factor peptide into the pPpT4 expression vectors marking the transition point of the secretion signal to the coding sequence of the protein to be secreted/surface displayed. This hexa-peptide is virtually ideal for the *S.c.* alpha mating factor secretion signal but in recent experiments based on the published *Pichia pastoris* alpha factor sequence it became apparent that the *Pichia pastoris* alpha mating factor secretion signal works better the more EA peptides are included (M. Ahmad, unpublished results), that´s why we included 5EA repeats into the constructs with the *P.p.* alpha factor secretion signal. Already the first prescreens showed a distinct tendency and confirmed our surmise – the *Pichia pastoris* alpha factor, at least in this constellation, was not the secretion signal of choice in this case. Finally, the pAaHSwa vector turned out to be the winner. The transformants constantly showed the best performance throughout the first prescreens which was later on also confirmed by deep well fermentations of selected clones done in replicates. *ilvE* activity assay is based on the conversion of L-Leucine or L-tert-Leucine to L-Glutamate and further metabolism of the L-Glutamate to H_2O_2 detected with ABTS (10). This assay is very sensitive and had to be adapted for surface display as the washed pellets were the basis of our measurements. Assays were performed with the substrates L-leucine that is able to enter the cell and with the synthetic substrate L-tert leucine that cannot be uptaken by the *Pichia* cell. The disadvantage of the synthetic substrate is that it is worse metabolized than L-leucine meaning it gives a much weaker signal/activity in the assay. Nevertheless, the data were reliable and coherent. However, a problem with the secretory expression control became apparent. Although the performance was initially, in deep well fermentations, as it should be, almost no activity in the shake flask fermentation supernatants could be detected. More detailed and repeated shake flask experiments with secretory clones revealed a relation between the media composition and the level of secretion. Obviously minimal media instead of complex media as used in this study increased the productivity of the secretory strains a lot (M. Ahmad, personal communication). As seen for the intracellular expression control, the pellet washing steps with ddH_2O did not cause any cell breakage that could have biased

the results. For any reason, immunofluorescence analysis didn´t work out in the beginning. We speculated that the complexity or size of the hexamer complex (ca. 180 kDa) could possibly cover the short peptide sequence bound by the anti-FLAG antibody but in the end we succeeded. Enterokinase cleavage of the FLAG tag between the *ilvE* coding sequence and the alpha-agglutinin anchor to release the enzyme complex into the media as a final evidence for surface anchoring proved to be rather difficult and we needed quite a lot of cells to see something in the western blot. Two possible problems came into our mind: again the accessibility of the cleavage site for the enterokinase enzyme and the preference of the enterokinase as it was not clear from the manufacturer´s informations whether the enzyme is capable of cleaving between two proteins. But higher OD for the enterokinase treatment and the increase of the enzyme amount followed by trichloroacetic acid precipitation of enterokinase cleavage supernatant was able to solve the problem. On the western blot four (five) distinct bands could be detected: a double band between 30 and 40 kDa corresponding to the glycosylated/unglycosylated monomer. These bands were also prominent in the supernatant of the ilvE secretory expression clones and the upper band disappeared after treatment with endoglycosidase H (M. Ahmad, unpublished data). The upper bands seemed to correspond to the dimer, trimer and finally hexamer (ca. 180 kDa). Concluding, surface display of the multimeric branched-chain amino acid aminotransferase IlvE is a versatile tool and an impressive example for the use of *Pichia pastoris* as whole cell biocatalyst.

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ABBREVIATIONS

µg	micro gram
µl	micro liter
AGE	Agarosegel electrophoresis
bp	base pairs
c	concentration
DNA	Desoxyribonucleic acid
dNTPs	Desoxyribonucleotides
DTT	1,4-Dithiothreitol
DWP	Deep-Well-Plate
E.coli	*Escherichia coli*
e.g.	example given
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidiumbromide
EtOH	Ethanol
g	gram
h	hour(s)
Hnl	hydroxynitrile lyase
ddH_2O	distilled water
kDa	kilo Dalton
l	liter
LB	Luria-broth
MCS	Multiple Cloning Site
MeOH	Methanol
mg	milligram
min	minute(s)
ml	milliliter
MOPS	3-(N-morpholino) propanesulfonic acid
(B)MD	(Buffered) Minimal Dextrose Media
(B)MM	(Buffered) Minimal Methanol Media
ng	nanogramm

nr.	number
OD_{600}	optical density λ = 600 nm
o.n.	over night
ONC	over night culture
oePCR	overlap extension PCR
PCR	Polymerase chain reaction
P.pastoris	*Pichia pastoris*
rpm	rotations per minute
RT	room temperature
S.cerevisiae	*Saccharomyces cerevisiae*
SDS-PAGE	Sodiumdodecylsulphate- Polyacrylamide gel electrophoresis
Std	standard
TCA	trichloroacetic acid
Tris	Tris(hydroxymethyl)-aminomethan
UV	ultra violet
YNB	Yeast Nitrogen Base
(B)YPD(S)	(Buffered) Yeast Extract Peptone Dextrose (Sorbitol) Media

MATERIALS AND METHODS COLLECTION
Strains and growth conditions

Bacterial strain

The bacterial strain used for general cloning strategies during this thesis is described in Table 2. *E.coli* was generally grown at 37°C.

Table 2: Bacterial strain

Strain	Genotype	Reference
E.coli K12 Top10F	F'[lacI^q Tn10(tet^R)] mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZ ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str^R) endA1 λ$^-$	www.stratagene.com

Media and solutions

LB Medium per 1 liter:

1 % tryptone

0,5 % yeast extract

0,5 % NaCl

(2 % agar)

20g LB-Media (Lennox) were dissolved in H_2O_{dest} in a final volume of 1L and autoclaved for 20 min at 120°C. For the preparation of plates 20g Agar were added before. The appropriate antibiotics were added when the autoclaved media has cooled down to ~55°C.

The used antibiotics (Table 3) were provided from Roche and all stock solutions were filter-sterilized (d = 0,2 µm) and stored at -20°C. The filter sterilization was performed with a Filtropur S 0,2 PAT US4900449 syringe filter from SARSTEDT.

Table 3: Antibiotic stocks used for *E.coli*

antibiotic	c_{stock}[mg/mL]	c_{final} [mg/L]
Ampicillin	100	100
Kanamycin	40	40
Zeocin	100	25

Yeast strain

Yeast strains which were used during this thesis are listed in Table 4. *Pichia pastoris* strains were generally grown at 28°C.

Table 4: Yeast strains used

Strain	Genotype	Phenotype	Reference
P.pastoris CBS7435		Mut^+	http://www.cbs.knaw.nl
P.pastoris GS115	*his*4	Mut^+ His^-	www.invitrogen.com
P.pastoris G115 1.17	His4, *aox*1Δ::*HbHNL*	Mut^S	M. Hasslacher, IBMT strain collection # 2386
P. pastoris GS115 1.17pGAPaox1_C3	His4, *aox*1Δ::*HbHNL*, P_{GAP}-*AOX*1	Mut^-, Zeo^R	IMBT, PhD I. Anderl

Media and Solutions

(B)YPD(S)-Medium per 1 liter:

1 % yeast extract

2 % peptone

2 % dextrose

(2% agar)

(1M sorbitol)

(200mM potassium phosphate buffer pH 7)

For YPD 10g Bacto™ Yeast Extract and 20g Bacto™ Peptone were dissolved in a final volume of 900 ml H_2O_{dest} and autoclaved for 20 min at 120°C. After the media has cooled down to ~55°C 100 ml of sterile 10x D were added.

For YPDS medium 186g sorbitol were added before autoclaving. To prepare BYPD medium the Bacto™ Yeast Extract and Bacto™ Peptone were dissolved in a final volume of 700ml H_2O_{dest} and after the autoclaved medium had cooled down 200ml of 1M potassium phosphate buffer pH 7 were added additionally to 10x D. For the production of BYPDS 186g sorbitol were used.

For the preparation of plates 20g agar were added before autoclaving. The appropriate antibiotics were added when the autoclaved media had cooled down to ~55°C.

The used antibiotics (Table 5) were provided from Roche and all stock solutions were filter-sterilized (d = 0,2 µm) and stored at -20°C. The filter sterilization was performed with a Filtropur S 0,2 PAT US4900449 syringe filter from SARSTEDT.

Table 5: Antibiotic stocks for *P. pastoris*

antibiotic	c_{stock}[mg/mL]	c_{final} [mg/L]
Geneticin (G418)	100	300
Zeocin	100	100

(B)MM per 1 liter:

1,34% YNB

$4*10^{-5}$% biotin

1% MeOH

(2% agar)

(200mM potassium phosphate buffer pH 7)

888ml H_2O_{dest} were autoclaved for preparing MM medium and 688ml H_2O_{dest} when producing BMM medium. For the preparation of plates 20g agar were added before autoclaving. After the medium had cooled down 100ml 10x YNB, 2ml 500x biotin and 10ml MeOH were added. For BMM medium 200ml 1M potassium phosphate buffer pH 7 were added.

(B)MD per 1 liter:

1,34% YNB

$4*10^{-5}$% biotin

2% dextrose

(2% agar)

(200mM potassium phosphate buffer pH 7)

798ml H_2O_{dest} were autoclaved for making MM medium and 598 H_2O_{dest} when making BMM medium. For the preparation of plates 20g agar were added before autoclaving. After the medium had cooled down 100ml 10x YNB, 2ml 500x biotin and 100ml 10x dextrose were added. For BMM medium 200ml 1M potassium phosphate buffer pH 7 were additionally added.

10x dextrose per 1L:

20% dextrose

200g dextrose were dissolved in 1L H_2O_{dest} and autoclaved for 20min at 120°C.

500x biotin:

0,02% biotin

20mg biotin were dissolved in 100ml H_2O_{dest} and filter-sterilized with a Filtropur S 0,2 PAT US4900449 syringe filter from SARSTEDT.

10x YNB:

13,4% Yeast Nitrogen Base with Ammonium Sulfate without amino acids

134 g Difco Yeast Nitrogen Base with Ammonium Sulfate and without amino acids were dissolved in 1 L H_2O_{dest} and autoclaved for 20 min at 120°C.

Potassium phosphate buffer:

A: 1M K_2HPO_4: 174.18g K_2HPO_4 were dissolved in 1 L H_2O_{dest}

B: 1M KH_2PO_4:136.09g were dissolved in 1 L H_2O_{dest}

Depending on the desired pH value x ml of A was mixed with x ml of B. To adjust the exact pH value phosphoric acid or KOH was used. It was autoclaved for 20 min at 120°C.

Transformation protocols

***E.coli* K12 Top10F´**

Preparation of electro competent cells

All centrifugation steps were done at 4°C and all intermediate steps were performed on ice. The first centrifugation steps were performed in an Avanti™ J-20 XP Centrifuge from Beckman Coulter with a JA-10 rotor from Beckman Coulter. The last two centrifugation steps were performed in an Eppendorf Centrifuge 5810 R with an Eppendorf A-4-62 rotor. OD_{600} was measured with a Beckman Coulter DU® 800 spectrophotometer.

Material:

- LB-Media
- sterile 10% Glycerol (ice-cold)
- liquid nitrogen
- Procedure: 150ml LB-Media were inoculated with a single colony of *E.coli* K12 Top10F` the day before. The cells were incubated over night at 37°C and 110rpm in a shaking incubator AG20 from Infors HT. The next day 6 main cultures – each 250ml LB-Media in a 2L flask – were inoculated with the ONC to an OD_{600} of 0,1 and incubated at 37°C and 110rpm till they reached an OD_{600} of 0,8. Cultures were kept on ice for 1 hour. Cells were pelleted by centrifugation for 7 min at 4500rpm. The supernatant was discarded and each pellet was gently resuspended in 400ml ice-cold 10% Glycerol. The cells were again centrifuged for 7min at 3500rpm. The resulting pellets were each gently resuspended in 100ml ice-cold 10% Glycerol. Cells were harvested again at the same parameters. The resulting pellets were all gently resuspended in 50ml ice-cold 10% Glycerol and cells were harvested again. The resulting pellet was gently resuspended in 5ml ice-cold 10% Glycerol. All resuspension steps were performed on ice. Aliquots of 70µl of were filled into Eppendorf vials, immediately frozen with liquid nitrogen and stored at -70°C.

<u>10% Glycerol</u>

100 ml Glycerol were mixed with 900 ml H_2O_{dest} and autoclaved for 20 min at 120°C.

Transformation in E.coli K12 Top10F´

The electroporation was performed with a BIO-RAD Micropulser™ and electroporationcuvettes (2µm; article number 748020) from Biozyme.

Material:

- electro competent *E.coli* Top10F`cells
- Plasmid-DNA or ligation sample
- LB-Media
- LB-Agarplates (with appropriate antibiotic)

Per transformation 1µl of Plasmid-DNA or 10µl of a desalted ligation and 70µl of electro competent *E.coli* Top10F` cells in a pre-cooled electroporationcuvette and incubated on ice for 10min. The transformation was performed with the program "bacteria: *Ec*2". Immediately after the electroporation 1ml LB-Media was added into the cuvette, mixed by inversion and decanted into a sterile Eppendorf-vial. Cells were regenerated for 1h at 37°C and 600rpm in a thermocycler from Eppendorf. Per reaction 50µl, 100µl and 200µl were plated on LB-Agarplates with the appropriate antibiotic and incubated over night at 37°C in an incubator from Binder.

Pichia pastoris

Preparation of electro competent P.pastoris cells

Preparation of electro competent cells and the transformation in *P.pastoris* were performed according to the Condensed protocol from Lin-Cereghino et al [**Fehler! Verweisquelle konnte nicht gefunden werden.**]. The centrifugation steps were performed in an Eppendorf centrifuge 5810 R with an Eppendorf rotor A-4-62 at RT. The electroporation was done with a BIO-RAD Micropulser™ and electroporationcuvettes (2µm article number 748020) from Biozyme. OD_{600} was measured with a Beckman Coulter DU® 800 spectrophotometer.

Material:

- *P.pastoris*
- YPD-Medium
- BEDS
- 1M DTT

Procedure: 5ml YPD-Media were inoculated with a single colony of *P.pastoris* the day before. Cells were incubated over night at 28°C and 110rpm in a shaking incubator AG20 from Infors HT. The next day the main culture – 50ml YPD-Media – was inoculated with the ONC to an OD_{600} of 0,2 and incubated at 28°C and 110rpm till an OD_{600} of 0,8 was reached. The cells were harvested by centrifugation for 5min at 1600rpm. The supernatant was discarded and the pellet gently resuspended in 9ml ice-cold BEDS and 1ml 1M DTT. The cell suspension was shaken slightly by hand for 5min. Then the cells were again pelleted via centrifugation for 5min at 1600rpm. The resulting pellet was gently resuspended in 1ml ice-cold BEDS and kept on ice.

<u>BEDS</u>

1M sorbitol

10mM bicine

3% ethylenglycol

5% DMSO

0,4080g bicine and 45,5g sorbitol were dissolved in 230ml $H_2O_{dest.}$ The pH was adjusted to 8,3 with 2M NaOH. 7,5ml ethylenglycol and 12,5ml DMSO were added and mixed. The solution was filter-sterilized (d= 2µm) and stored at 4°C.

<u>1M DTT</u>

1,54 g DTT were dissolved in 10 ml H_2O_{dest} and filter sterilized. 1 ml aliquots were stored at -20°C.

Transformation into Pichia pastoris

Material:

- electro competent *Pichia pastoris* cells
- 1M Sorbitol
- linearized Plasmid-DNA
- BYPDS-agarplates (with appropriate antibiotic)

2-4µg linearized Plasmid-DNA were mixed with 100µl of competent *P.pastoris* cells in pre-cooled electroporationcuvettes and incubated for 2min on ice. The reaction mixture was electroporated with the program "yeast – *Pic*". Immediately after the electroporation 0,5ml 1M Sorbitol were added into the cuvette, mixed by inversion and decanted into a sterile Falcon Tube. Cells were regenerated at 28°C and 110rpm. After 1h 0,5ml YPD were added to the cells and they were incubated for another hour at 28°C and 110rpm. Per reaction 50µl, 100µl and 200µl were plated on BYPDS-Agarplates and incubated for 2-3 days at 28°C in an incubator from Binder. The antibiotics depended on the plasmid used.

1M sorbitol

182 g Sorbitol were dissolved in 1 L H_2O_{dest} and autoclaved for 20 min at 120°C.

Glycerolstocks

E.coli

Material:

- 30% Glycerol
- LB-agarplate (with appropriate antibiotic)

Procedure:

A single colony of the desired *E.coli* clone was plated out on a LB-Agarplate the day before and incubated over night at 37°C. The next day 2ml of 30% Glycerol were added on the plate and the cells were elutriated with a spatula. This suspension was filled into sterile vials (Cryo.s^{TM}, PP, with screw cap) from Greiner Bio-One and stored at -70°C.

P.pastoris
Material:

- 30% Glycerol
- BYPD
- DeepWell Plate
- Microplate

A 96well DeepWell Plate was filled with 250µl BYPD per well and inoculated with thr transformants. They were incubated at 28°C and 320rpm in a shaking incubator AG20 from Infors HT. 250µl 30% Glycerol were added to each well after 24h of incubation. 200µl of this mixture was filled into a 96well PP-microplate with flat bottom from Greiner Bio-One and stored at -70°C.

30% Glycerol:

300 ml Glycerol were mixed with 700 ml H_2O_{dest} and autoclaved for 20 min at 120°C.

DNA methods

Plasmid DNA Isolation
Plasmid DNA from *E.coli* was isolated according to the GeneJeT™ Plasmid Miniprep Kit Manual from Fermentas. All centrifugation steps were performed with an Eppendorf centrifuge 5415 R and a F45-24-11 rotor at 11000rpm.

Material:

- GeneJET™ Plasmid Miniprep Kit (Fermentas)
- LB-Agarplate (with appropriate antibiotic)

A single colony which carries the plasmid was streaked on a LB-Agarplate (with antibiotic) and incubated at 37°C over night in an incubator from Binder. The cells were harvested with a sterile toothpick and resuspended in a sterile Eppendorf-vial with 250µl of Resuspension Solution. After the resuspension 250µl of Lysis Solution was added and gently mixed through inverting the vial 6-times till the solution becomes viscous and clear. Afterwards 350µl of Neutralization Solution was added and gently mixed through inverting the vial 6-times. To get rid of cell debris and chromosomal DNA the bacterial lysate was spinned for 5min. The supernatant was transferred into a GeneJet™ spin column and centrifuged for 1min. After two washing steps with 500µl Wash Solution and 1min centrifugation steps the empty GeneJet™ spin column was centrifuged to remove EtOH residues. The column was transferred into a sterile Eppendorf-vial and 50µl H_2O_{dest} (Fresenius) were added to the spin column membrane to elute the plasmid. After about 5min incubation plasmid DNA was eluted by centrifugation for 2min.

Agarose gel electrophoresis (AGE)
Material:

- 6x MassRuler™ DNA Loading Dye (Fermentas)
- SUB-CELL® GT (Bio-Rad)
- PowerPac-Basic (Bio-Rad)
- 1x TAE-buffer
- GeneRuler™ DNA Ladder Mix (Fermentas SM0331)
- GeneRuler™ 1kb DNA Ladder (Fermentas SM0311)
- Agarose (Biozyme LE)

Procedure:

1% agarose in 1x TAE buffer was used to determine the concentration of DNA or to check the size of certain fragments. 0,8% agarose in 1x TAE buffer was used for DNA isolation. The agar solution was heated in a microwave till the agarose was completely melted. After cooling down the solution to about 60°C 1µl Ethidiumbromide [10mg/ml] was added. The solution was filled into a casting tray to allow solidification. 2 combs were put into it before to provide the slots for the samples. The chamber was filled with 1x TAE-buffer and the solidified gel was put into it.

Additionally to the samples, 5µl of a standard was loaded into one of the slots. The samples had to be complemented with the right amount of 6x MassRuler™ DNA Loading Dye. The gel run with 120V for 60min and after the electrophoresis the gel was photographed with the GelDoc-It™ Imaging System from UVP. The sizes of the fragments and the concentration of the DNA could be estimated by comparison with the standard.

50x TAE buffer:

484g Tris, 29,2g EDTA and 114,2ml acetic acid were filled up with H_2O_{dest} to a final volume of 2L.

For preparing 1x TAE buffer the 50x TAE buffer was diluted 1:50 with H_2O_{dest} .

DNA isolation and PCR purification from AGE

For the isolation of a certain DNA fragment from an agarose gel the Wizard® SV Gel and PCR Clean-Up System from Promega was used. All centrifugation steps were performed with an Eppendorf centrifuge 5415 R at 13000rpm and the rotor type was F45-24-11.

Material:

- Wizard® SV Gel and PCR Clean-Up System (Promega)
- DNA sample

The restricted DNA or the PCR reaction was mixed with the corresponding quantity of 6x MassRuler™ DNA Loading Dye and loaded onto an 0,8% agarose gel for electrophoresis. The gel was run at 90V for 90min. The right DNA fragment was cut out with a scalpel under UV light and the gel slice was transferred to a sterile Eppendorf-vial which was weighed before. Per 10mg gel slice 10µl of the Membrane Binding Solution from the Kit were added and the sample was heated in a thermoshaker from Eppendorf at 60°C and 600rpm for about 10min till it was completely dissolved. A SV Minicolumn was put into a Collection tube and the solution was filled into it and centrifuged for 1min. After this the membrane was washed with 700µl Wash Solution and centrifuged for 5min. The flow through was discarded and the membrane was washed again with 500µl of the Wash solution and centrifuged for 1min. The flow through was discarded and the empty column was spinned for 1min to remove the residual EtOH. The SV Minicolumn was transferred into a new sterile Eppendorf-vial and 50µl H_2O_{dest} was added onto the membrane and incubated for 2min. During the last centrifugation step for 1min the DNA was eluted from the membrane.

Restriction of DNA

Material:

- Restriction enzymes (Fermentas)
- 10x buffer (Fermentas)
- Template-DNA

Procedure:

1U of restriction enzyme is defined as the amount of enzyme which is able to cut 1µg of λ-DNA in 1h. In a reaction mix, the amount of enzyme should not exceed 10% of the reaction volume and this volume should not exceed 100µl. The right buffer condition for a double digestion can be looked up at the Homepage of Fermentas with the tool "DoubleDigest" [**Fehler! Verweisquelle konnte nicht gefunden werden.**].

The samples were incubated at the right temperature (mostly 37°C) for at least 2h or when FastDigest enzymes were used for 30min.

The restriction samples were purified through AGE followed by gel isolation.

Setup of standard restriction digests for restriction control:

3µl DNA

2 µl 10x restriction buffer

0,5µl restriction enzyme (Fermentas)

H_2O_{dest} to a final volume of 20µl

Setup of standard preparative restriction digests:

30µl DNA

5µl 10x restriction buffer

2µl restriction enzyme (Fermentas)

H_2O_{dest} to a final volume of 50µl

Enzymes used are provided in the appendix (**Fehler! Verweisquelle konnte nicht gefunden werden.**).

Ligation
Material:

- T4 DNA Ligase 3U/µl (Promega)
- 10x T4 Ligase buffer (Promega)
- restricted DNA (vector and insert)

Per ligation reaction 50-100ng vector DNA were used. The right amount of Insert-DNA was calculated with the following formula:

ng Insert = 3/1 * [(ng vector*bp insert)/bp vector]

Standard ligation mix setup:

xµl vector DNA

xµl insert DNA

1µl T4 Ligase buffer

0,5µl T4 DNA Ligase

H_2O_{dest} to a final volume of 10µl

Additionally a ligation mix without the insert DNA was performed as a control. The ligation mixes were incubated at 16°C over night. Before the transformation into electro competent *E.coli* Top10F`cells the reaction had to be desalted. Therefore the whole reaction was pipetted onto a nitrocellulose membrane from Millipore lying on the surface of distilled water. After 30min the whole reaction was removed from the membrane and completely transformed into electro competent *E.coli* Top10F`cells.

Polymerase Chain Reaction (PCR)

To isolate the genes of interest PCR reactions were performed.

Material:

- genomic DNA from *Pichia pastoris* CBS7435 or plasmid DNA with Primer (forward and reverse)
- Phusion polymerase [2U/µl] (Finnzymes, F-530L))
- 5x Phusion HF buffer (Finnzymes, F-518)
- dNTP mix (Fermentas)
- GeneAmp® PCR System 2700 (AB Applied Biosystems)

Table 6: PCR reaction mix

	Volume [µl]
genomic DNA from *P.pastoris* CBS7435 or plasmid DNA	1
Forward Primer [10pmol/µl]	2,5
Reverse Primer [10pmol/µl]	2,5
Phusion Polymerase	0,5

Phusion HF buffer	10
dNTP Mix [2,5mM each]	5
H_2O	28,5
Total volume	**50µl**

Table 7: Reaction conditions

Phase	**Temperature [°C]**	**Time**	**Cycle**
Denaturation	98	3 min	1
Denaturation	98	10 sec	25
Annealing	55	30 sec	
Extension	72	30 sec	
Final Extension	72	10 min	1
Cooling	4	∞	

oePCR (Overlap Extension PCR)
An oePCR consists of 2 steps: Amplification of the two overlapping mutated fragments by a normal PCR reaction and the following oePCR where the fragments are joined together and amplified.

Table 8: PCR reaction mix for fragment generation

	Volume [µl]
genomic DNA from *P.pastoris* CBS7435	1
Forward Primer [5pmol/µl]	5
Reverse Primer [5pmol/µl]	5
Phusion Polymerase	0,5
Phusion HF buffer	10
dNTP Mix [2,5mM each]	5
H_2O	23,5
Total volume	**50µl**

Table 9: PCR conditions for fragment generation

Phase	Temperature [°C]	Time	Cycle
Denaturation	98	3 min	1
Denaturation	98	10 sec	25
Annealing	55	30 sec	
Extension	72	30 sec	
Final Extension	72	10 min	1
Cooling	4	∞	

Table 10: oePCR reaction mix

	Volume [µl]
Fragment 1	1
Fragment 2	0,5
Phusion Polymerase	0,5
Phusion HF buffer	10
dNTP Mix [2,5mM each]	5
H_2O	23
Total volume	**40µl**

Table 11: oePCR reaction profile 1 (without primer)

Phase	Temperature [°C]	Time	Cycle
Denaturation	98	3 min	1
Denaturation	98	30 sec	6
Annealing	55	30 sec	

Extension	72	2 min	
Final Extension	72	1 min	1
Cooling	4	∞	

Table 12: oePCR reaction profile 2 (with primer)

Phase	**Temperature [°C]**	**Time**	**Cycle**
Denaturation	98	3 min	1
Denaturation	98	10 sec	18
Annealing	55	30 sec	
Extension	72	30 sec	
Final Extension	72	10 min	1
Cooling	4	∞	

The PCR and oePCR reactions were loaded onto a preparative Agarosegel. The right fragments were cut out and isolated from the gel.

Restriction, ligation and transformation into *E.coli* K12 Top10F´

The isolated PCR fragments and the vectors were restricted with the appropriate restriction enzymes purchased from Fermentas. The restricted samples were purified through AGE and after that the concentration of the cut PCR products and vectors were determined through AGE. The ligation was performed like described in point 0. Before the transformation into electro competent *E.coli* K12 Top10F` cells the reaction had to be desalted. For the desalination the whole reaction was pipetted on to a nitrocellulose filter from Millipore which was placed on water. After 30min the whole reaction was transformed into electro competent *E.coli* Top10F`cells.

Control of the plasmids

Transformants of each construct were randomly chosen and the plasmids were isolated with the GeneJeT™ Plasmid Miniprep Kit from Fermentas. At first a restriction control was performed. The isolated plasmid DNA was restricted with the appropriate enzymes to cut the insert out and restriction samples were analysed through AGE.

Samples of each construct which showed right fragment sizes were sent to AGOWA genomics [**Fehler! Verweisquelle konnte nicht gefunden werden.**] for sequencing to check if mutations had occurred during the PCR reaction. Therefore 10µl of the sample had to be complemented with 4µl of a sequencing primer [5pmol/µl].

Screening Assays

α-Naphthylacetate filter assay

Material:

- α-Naphthylacetate [12mg/ml in acetone] from Sigma
- FastBlueB [20mg/ml in H_2O] from Sigma
- Tris-HCl 0,1M pH 7
- Whatman filterpaper

Principle:

When the enzyme (e.g. esterase) is active, the α-Naphthylacetate is cleaved into α-Naphthol. The α-Naphthol binds to the FastBlueB salt and they build a Diazo coloured complex (

Figure 1). Active colonies change their color from white to brownish violet. The substrate α-Naphthylacetate is accepted by most of the esterases. Therefore this is not a very specific assay.

Reaction scheme:

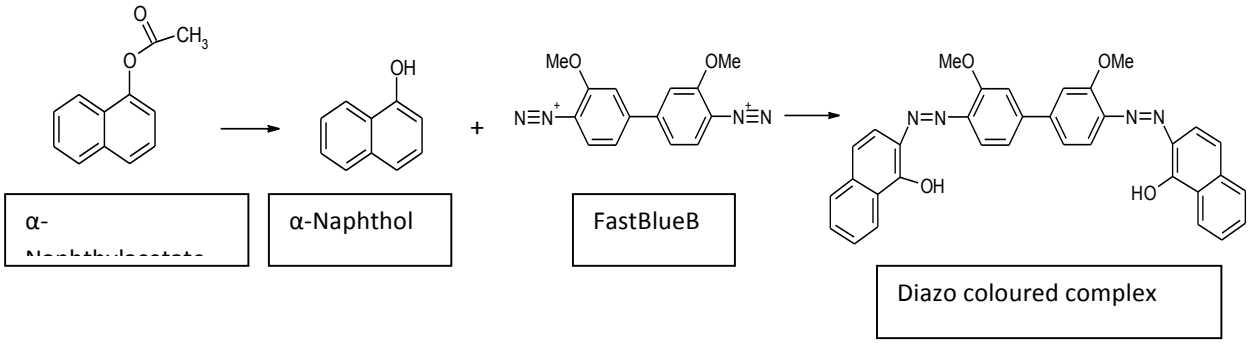


Figure 1: Reaction of α-Naphthylacetate with FastBlueB

Prodcedure:

The *P.pastoris* transformants were grown on MD or MM agarplates without antibiotic at 28°C for 2 days. For the screening they were lifted with a Whatman-filterpaper (Cat no. 1001-085) and dried for 5min at 28°C. Meanwhile the screening solution was prepared (Table 13).

Table 13: Screening solution for α-Naphthylacetate assay

	Volume
α-Naphthylacetate	375µl
FastBlueB	175µl
Tris-HCl 0,1M pH 7	5ml

1ml of the screening solution was pipetted onto a glass plate and the filter with the colonies was placed, colonies upside, onto the solution. After 1-3min active colonies appear blue.

pH shift assay

Material:

- Biodyne® nitrocellulose membrane from PALL Life Sciences (A 0,45µm)
- Buffer (e.g. 10mM Tris-HCl pH = 8)
- Indicator phenolred
- Methyl acetate [25mM]

Principle:

The esterase cleaves its substrate („ester“) into 2 components, an alcohol and a (carboxylic) acid. Through the formation of an acid the pH is lowered. By the aid of a pH indicator (e.g. phenolred) the pH shift becomes visible by a color change, in the case of phenolred from red to yellow. At pH 6,4 phenolred shifts from red to yellow.

Screening Procedure:

The *P. pastoris* transformants were stamped onto a nitrocellulose membrane which was then placed onto MD or MM agar plates for 2 days at 28°C. For the screening the membrane was dried for 5min at 28°C. In the meantime the screening solution was prepared.

The screening solution consisted of the buffer, phenolred and 25mM methyl acetate. The optimal buffer conditions had to be determined first by testing several buffers which varied in concentration and/or pH (phosphate buffer and Tris-HCl buffer from 10mM up to 25mM and pH from 7 to 8). The best results were shown with 10mM Tris-HCl buffer pH 8 and 10g/L phenolred.

1ml of the screening solution was pipetted onto a glass plate. The membrane with the colonies, which was equilibrated before by incubating the membrane for 30min on a filter-paper saturated with the buffer used for the screening without phenolred, was then placed, colony side up, onto the screening solution containing phenol red. After some time active colonies appeared yellow.

RhodamineB assay

This assay is based on the paper “Specific and Sensitive Plate Assay for Bacterial Lipases” from Gisela Kouker and Karl-Erich Jaeger.

It is a simple plate assay to detect lipases (EC 3.1.1.3) in a medium containing a long chain fatty acid as substrate and the fluorescent dye rhodamine B. Substrate hydrolysis causes the formation of orange fluorescent halos around the colonies or the colonies itself show fluorescence visible upon UV irradiation (350nm). The mechanism is not quite clear but it is suggested that a complex formation between the cationic rhodamine B and the fatty acid ion causes the fluorescence [**Fehler! Verweisquelle konnte nicht gefunden werden.**].

<u>RhodamineB plates (1L)</u>

1,34% YNB

$4*10^{-5}$% biotin

0,5% dextrose

2% agar

1% substrate

0,001% rhodamineB

(200mM potassium phosphate buffer pH 7)

20g Agar were dissolved in 663ml H_2O_{dest} and autoclaved for 20min at 120°C. After the solution had cooled down to about 60°C, 100ml 10x YNB, 200ml 1M phosphate-buffer pH 7, 25ml 10x dextrose and 2ml 500x biotin were added and complemented with 10ml substrate and 1ml 1% RhodamineB.

Substrates:

- methyl oleate
- olive oil
- biodiesel

Colonies were directly grown on these plates. After 2 days of incubation at 28°C the fluorescence was detected under UV light.

Native Gel Electrophoresis and SDS Page

Fermentation conditions
Condition 1 and 2:

50ml BYPD were inoculated with a single colony and incubated over night at 28°C and 100rpm. The next day 125ml BMD (1% dextrose) were inoculated with the ONC to an OD_{600} of 0,01. They were again incubated at 28°C and 100rpm. After 24h the culture was pelleted through centrifugation (2500rpm, 5°C, 20min; Avanti^{TM} J-20 XP Centrifuge and JA-10 rotor from Beckman Coulter). For the main culture of condition 1 the pellets were resuspended in 125ml BMD and for condition 2 in 125ml BMS (1% sorbitol) and 0,5% MeOH. After 6h 0,2% MetOleat and after 24h/36h 0,5% MetOleat were added to condition 2.

Condition 3 and 4:

50ml YPD were inoculated with a single colony and incubated at 28°C and 100rpm. The main culture (125ml YPD, 3% dextrose) were inoculated with the ONC to an OD_{600} of 0,01.After 36h/44h 1% dextrose was added to condition 3 and 0,5% MetOleat to condition 4. After incubation at 28°C and 100rpm for further 12h the fermentation was finished.

Spheroplasting

Material:

- SED buffer
- STE buffer
- 1M Sorbitol
- zymolyase 20T (from A. luteus, Seikagaku Biobusiness, Code No. 120491)

The spheroplasting was performed according to the paper “Alternative pig liver esterase (APLE) – Cloning, identification and functional expression in *Pichia pastoris* of a versatile new biocatalyst” .

OD_{600} was measured with a Beckman Coulter DU® 800 spectrophotometer. The volumes of the samples were normalized corresponding to the lowest OD_{600}. The fermentation broth was centrifuged in an Avanti™ J-20 XP centrifuge from Beckman Coulter with a JA-10 rotor from Beckman Coulter at 2000 x g and 21°C for 20min. Supernatants were collected and stored at 4°C. Pellets were resuspended in an equal volume of SED buffer (1M Sorbitol, 25mM EDTA, 50mM DTT, pH 8) and again centrifuged at the same conditions in an Eppendorf Centrifuge 5810 R with an Eppendorf A-4-62 rotor. The supernatants were discarded and the pellets were washed with the same volume of 1M sorbitol. The suspensions were again centrifuged at the same conditions and the resulting pellets were resuspended in the same volume of STE buffer (1M sorbitol, 1mM EDTA, 20mM Tris/HCl, pH 7). Spheroplasting was performed by adding 0,5µg/ml zymolase and incubation at 30°C. After 30min 2,5µg/ml zymolyase were added and incubated for 60min at 30°C. To gain the supernatant which mostly contains the periplasmatic proteins, the suspension was centrifuged at 1000 x g. The filter sterilization of the supernatant was performed with a Filtropur S 0,2µm PAT US4900449 syringe filter from SARSTEDT. The supernatant with the periplasmatic proteins was stored at 4°C. The pellet was resuspended in 10mM Tris-HCl (pH 7) buffer and stored at 4°C.

Native polyacrylamide electrophoresis

This method was developed by Regina Leber as described in her paper "Visualization of hydrolytic activities and substrate preferences in gels by a highly sensitive zymogram technique" (Leber R., Bekerle-Bogner M., and Schwab H) which is not published yet.

This special zymogram technique is based on pH shifts using the fluorescent pH indicator HPTS (8-hydroxypyrene-1,3,6-trisulfonic acid). When excited at 458nm, HPTS fluorescence is known to increase on alkalinization and decrease on acidification. Through this method proteins can be separated in polyacrylamide gels and their hydrolyse activity can be determined directly in the gel.

The separation of the proteins takes place according to their own charge, due to the low Coomassie G250 concentration. The determination of the protein size is therefore impossible and basic proteins (pI higher than 8) get stuck between the stacking gel and the separation gel. ε-aminocaproic acid is a serine-protease inhibitor which avoids the degradation of the proteins during the electrophoresis.

Material:

- Hoefer SM 250 mini-vertical gel electrophoresis unit:
  - Glass Plates 8x10cm
  - Notched alumina plates
  - Spacer 0,75mm and Comb 0,75mm
  - Dual Gel Caster
  - Mighty Small II SE 250
  - Invitrogen Power Ease 500

Table 14: Preparation of native gels

	Separation gel 12% **(4 gels)**	**Stacking gel 4%** **(4 gels)**
Acrylamid (30%) from Serva	6ml	1,35ml
BN-Gelbuffer (6x)	2,5ml	1,67ml
H_2O	6,5ml	6,98ml
APS (10%) in H_2O from Roth	75µl	50µl
Temed from Roth	15µl	10µl

HPTS was purchased from SIGMA.

Loading buffer (5x):

150mM ε-aminocaproic acid (from Fluka)

50% (x/v) glycerol (from Roth)

0,025% Coomassie G250 (from Fluka)

BN-Gel buffer (6x):

3M ε-aminocaproic acid

0,3M Bis-Tris-HCl pH 7 (from Roth)

Cathode buffer (10x):

500mM Tricine (from Roth)

150mM Bis-Tris-HCl pH 7

Anode buffer (10x):

500mM Bis-Tris-HCl pH 7

Sample preparation:

The samples were mixed with the right amount of 5x loading buffer and loaded onto the gel. The supernatant was used in an unconcentrated and concentrated version. The concentration of the supernatant was performed by centrifugation with a Vivaspin concentrator tube from sartorius stedim (Product No. VS2001).

The electrophoresis was performed at 24mA and 200V for 90min at 4°C.

In-gel detection of hydrolytic enzymes with HPTS

Hydrolyases like esterases produce acids when they cleave the substrate and therefore a local pH decrease takes place in the gel. The fluorescence of the sensitive pH indicator HPTS decreases when it becomes acidic and active enzymes appear as a dark band on a bright background.

For the detection of pH shifts, the native polyacrylamide gel was rinsed with water after electrophoresis and washed for 10min with 1mM Tris-HCl buffer pH 7,4. After that the gel was incubated with 1mM Tris-HCl buffer containing HPTS (15mg HPTS per 300ml buffer). After 15min the gel was placed onto a glass plate and overlayed with the substrate-filter-paper or the substrate-membrane. The gel-filter or gel-membrane sandwich was incubated at 28°C in a dark moist chamber for at least 60min.

Substrates could either be used pure or diluted. Watersoluble substrates were diluted with 1mM Tris-HCl pH 7,4 or applied without dilution to a filter-paper from Whatman before overlaying the gel. Non-water soluble substrates were dissolved in aceton or used pure and applied to a Biodyne A membrane (0,45µm; 7,2 x 11,3cm; Pall Life Sciences). After evaporation of aceton, the gel was overlayed with the membrane.

Active bands were visualized as dark spots on a fluorescent background with the gel documentation system from Syngene (G:Box). Therefore the gel was placed without the filter or the membrane onto the blue plate for the geldoc system. The excitation was performed by the Transilluminator and the filter used was Sybr Gold. Pictures were taken in a series of 5 pictures with 15ms exposure time.

In-gel detection of esterases with α-Naphthylacetate

After native gel electrophoresis the gel was rinsed with water and then incubated with the screeningsolution described in 0. The principle is the same and active enzymes appear as a brownish violet band.

SDS Page

SDS Page (Sodium Dodecyl Sulfate Polyacrylamide Gelelectrophoresis) was used to separate and identify proteins because of their size. The SDS page was performed with the NuPAGE® System of Invitrogen.

Material:

- NuPage gel (12% Bis-Tris gel, 1mm, 12 slots, Invitrogen)
- NuPage system (Invitrogen)
- NuPage LDS Sample buffer 4x (Invitrogen)
- PageRuler Prestained Protein Ladder (Fermentas, SM0671)
- 1x MOPS running buffer (Invitrogen)

The samples were mixed with the right amount of 4x LDS sample buffer and heated for 10min at 70°C. They were loaded onto the gel and run for 2h at 200V and 25mA. The gel was stained with Coomasie Blue and discoloured with 10% acidic acid.

TCA Precipitation:

500µl of the periplasmatic fraction were mixed with 100µl of a 50% trichloroacetic acid. The reaction was stored over night at 4°C. The next day, the samples were centrifuged for 15min at 13000rpm and the pellets were resuspended in 15µl H_2O.

PageRuler Prestained Protein Ladder (Fermentas, SM0671) is given in the appendix (**Fehler! Verweisquelle konnte nicht gefunden werden.**).

HNL Assay

If the chosen enzymes are expressed and cleave the substrates, the released methanol should induce the P_{AOX1}-promoter and HNL should be expressed. The assay for the detection of HNL is based on the method published in the paper "Novel screening assay for hydroxynitrile lyases suitable for high-throughput screening" from B. Krammer, K. Rumbold, M. Tschemmernegg, P. Pöchlauer, and H. Schwab.

Principle:

The generation of cyanide from cyanogenic glycosides is a two step process involving first a deglycosylation resulting in a cyanohydrin. Finally the HNL catalyse the last step of cyanogenesis, i.e. the breakdown of the cyanohydrin to release the corresponding aldehyde or ketone and cyanide. The assay bases upon the detection of produced hydrocyanic acid, which makes it an all-purpose screening assay, without restriction regarding the substrate. Active colonies appear blue [**Fehler! Verweisquelle konnte nicht gefunden werden.**].

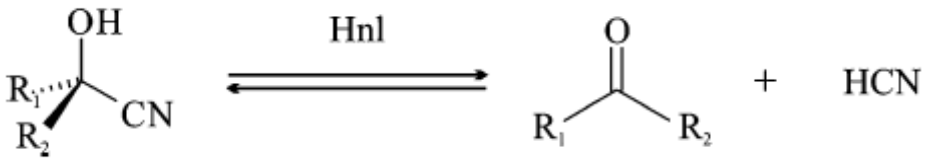


Figure 2: Reaction scheme: HNL catalyzed breakdown reaction of cyanohydrins to yield HCN [Fehler! Verweisquelle konnte nicht gefunden werden.**]**

Procedure:

5µl of the transformants from the glycerol stock were inoculated in 250µl BYPD (1% dextrose) and incubated for 24h at 28°C and 320rpm. After that a 1:10 and 1:100 dilution in 0,9% NaCl was prepared. The 1:100 dilution was stamped onto Biodyne A membranes (0,45µm; 7,2 x 11,3cm; Pall Life Sciences) and laid onto BMD (1% dextrose) agarplates. They were incubated for 24h at 28°C and then the membranes were transferred onto the induction plates and again incubated for 24h at 28°C. After 24h of induction the first HNL Assay was performed. The rest of the membranes were transferred onto new induction plates for another 24h.

Induction plates:

1. BM Sorbitol 1%
2. BM MeOH 1%
3. BM Methyl oleate 1% Triton 0,1%
4. BM Sorbitol 1% Biodiesel 0,2% Triton 0,1%
5. BM Biodiesel 1% Triton 0,1%
6. BM Sorbitol 1% Methyl acetate 0,2%
7. BM Triton 0,1%
8. BM Dextorse 1%
9. BM Methyl acetate 1%
10. BM Sorbitol 1% Methyl oleate 0,2% Triton 0,1%
11. BM Sorbitol 1% MeOH 0,2%

The BiodyneA membrane with the colonies had to be equilibrated by incubating the membrane for 30min on a filter-paper saturated with 100mM citrate-phosphate buffer pH 5,3. The membrane was then applied, colony side down, onto a filter soaked with substrate solution. This assembly was then covered with a permeable nylon tissue to separate it from the HCN sensitive detection paper. Finally, a plastic disk was put above the HCN detection paper to grant an equal reaction surface (Figure 3).

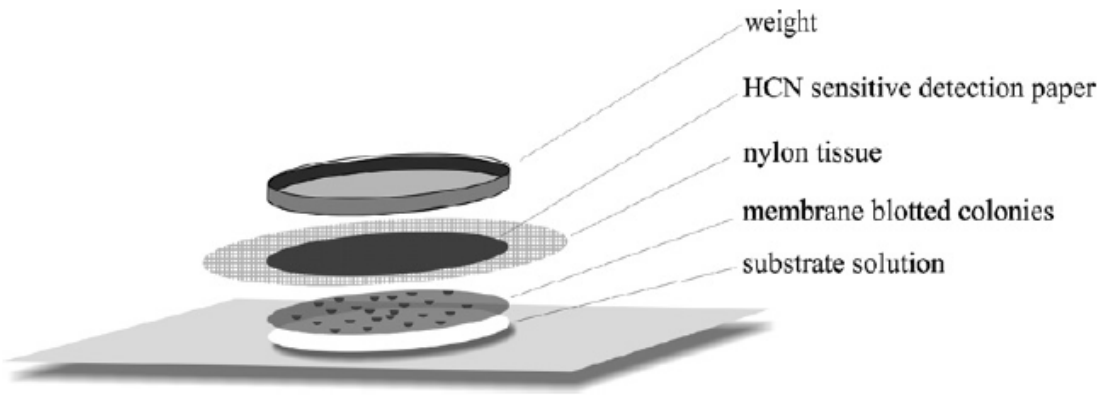


Figure 3: Assembly for detection of gaseous HCN released from HNL producing *P.pastoris* colonies

Detection filter:

A Whatman filter-paper was soaked with a chloroform solution, containing 0,5% (w/v) copper-(II)ethyl-acetoacetate and 0,5% (w/v) 4,4-methylenebis (N,N-dimethyl-aniline) ("Tetrabase").

Substrate filter:

A Whatman filter-paper was soaked with the substrate solution which consisted of 8µl mandelonitrile per 1ml 100mM citrate-phosphate buffer pH 3,5.

The reaction was stopped at two different time points (30sec and 2min).

SDS Page and Westernblot

Fermentation

25ml BYPD were inoculated with a single colony of each clone and incubated at 28°C and 130rpm for 24h. The main culture (50ml ¼ BYPD) was inoculated with the pre-culture to an DO_{600} of 0,01. The OD_{600} and the glucose concentration were measured at different time points. When the glucose was finished and the cells reached the stationary phase, the induction with methyl oleate, biodiesel or methanol was started. From that point the cells were incubated for 48h. During this 48h the OD_{600} was measured and small samples (max. 500µl) were taken at each breakpoint according to the lowest OD_{600}. The samples were centrifuged and the pellets were stored at -20°C. OD_{600} was measured with a Beckman Coulter DU® 800 spectrophotometer. Glucose concentration was measured with Diabur-Test® 5000 Test-stripes from ACCU-CHEK (Roche).

Table 15: Induction status of fermentation

Time point	Hours after induction	Methyl oleate/Biodiesel	Methanol
0	0	0,1%	1%
1	3	0,2%	
2	13	0,3%	0,5%
3	17		
4	21		
5	25	0,4%	1%
6	37		
7	41	0,4%	0,5%
8	45		
9	49		

SDS PAGE:

Sample preparation: The pellets were resuspended in the same amount of phosphate buffer (0,1M pH7) like it was used for the sampling. Half of the resuspended solution was given into a new Eppendorf vial which was weighted before. The suspension was pelleted by centrifugation and the pellet was weighted. Per g pellet 5ml YeastBuster and 50µl 100xTHP were used to resuspend it. To perform the breakage of the cells they were incubated for 30min and 500rpm in a thermocycler from Eppendorf. After that the suspension was centrifuged for 30min at 32000rpm. 20µl of the supernatant were given into a new Eppendorf vial and 2µl 1M DTT were added. It was again incubated for 30min at 300rpm. 3µl of the sample were mixed with 2µl 4x LDS buffer and heated for 10min at 70°C.

The gels were run, stained and discoloured like described in 0. All centrifugation steps were performed with an Eppendorf centrifuge 5415 R at 13000rpm and the rotor type was F45-24-11.

Western Blot

The Western blot is a technique used to detect specific proteins in a sample. The native or denatured proteins are separated by SDS-Page which was performed like described in point **Fehler! Verweisquelle konnte nicht gefunden werden.**. The proteins were then transferred to a membrane (nitrocellulose, Satorius) by electro blotting using the Tank Transfer Unit TE22 from Amersham Biosciences. To verify if the transfer of the proteins was successful, the membrane was coloured with PonceauS and then rinsed with water.

To saturate the unspecific binding sites of the membrane, it was blocked in 1x TBST milk buffer (1h RT, o.n. 4°C). To detect the desired protein, the membrane was incubated with the primary antibody which is specific against *Hb*_HNL. The primary antibody was diluted 1:5000 in 1x TBST milk buffer and the incubation took place at RT for 2h. The excess antibody was washed away with 1x TBST buffer (5x 5min). The membrane was incubated with the second antibody (anti-rabbit) which was diluted 1:30000 in 1x TBST milk buffer for 1h at RT. This secondary antibody is specific against the primary antibody and is covalently linked with an alkaline phosphatase (Sigma). To remove the excess secondary antibody the membrane is washed three times for 5min in 1x TBST and two times for 5min in 1x TBS buffer. The detection of bound antibody was performed by adding the substrate directly onto the membrane. The substrate BCIP®/NBT-Purple Liquid (Sigma) is cleaved by the alkaline phosphatase and a brownish colour appears.

Important parameter: Complete saturation of unspecific binding sites, right specificity of the primary antibody and complete removal of unbound antibody.

10x TBS buffer:

30,3g Tris and 87,6g NaCl were dissolved in 1L H_2O and the pH was adjusted to 7,5 with HCl.

1x TBST:

500µl Tween20 per L TBS

1x TBST milk:

5g milk powder in 100ml 1x TBST

20x Transferbuffer:

14,5g Tris and 72g Glycin were dissolved in 500ml H_2O.

1x Transferbuffer:

50ml 20x Transfer buffer were mixed with 200ml MeOH and 750 H_2O.

Levanase Assays of aox1 autoactivation project

Streak strains freshly on BYPD before use!

Use the following strains: Pp H567A, PpH567N, PpH567Q, PpN616A, double KO, aoxfunct.,aox stop, Retransf., CBS7435 wt

Preculture: Inoculate 10ml ½ BYPD with a single colony, incubate in a shaker (at least 100rpm) for 24h at 28°C.

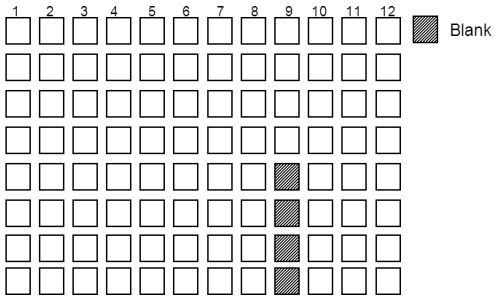
Main culture: Measure OD600 of preculture (1:20 dilution), inoculate 30ml of BYP0,1%D medium (100ml flask) to a starting OD of 0,2. Shake 24h at 28°C and 120rpm.

After 24h, centrifuge cultures in 50ml falcon tubes (2500rpm, 5 min) and resuspend the cultures in either BYP or BYP0.1%Sorbitol medium. Measure OD600.

Aliquote cultures into deepwell plates (1 row per clone = 8 replicates) -->Σ 200µl per well

Add substrate to a certain concentration. In case of formaldehyde seal deep well plates with sealing foil. In case of MeOHuse the normal lid. Shake on titrimax shaker at 28°C and 750-900rpm for certain time.

Different set-ups:



1	Pp funct. Aox1
2	Pp Aox1 stop
3	Pp Aox1 H567A
4	Pp Aox1 H567N
5	Pp Aox1 H567Q
6	Pp Aox1 N616A
7	Pp double KO
8	Pp Retransf. MT
9	Pp CBS7435 wt

Plate1: BYP + 0.5% MeOH

Plate 2: BYP0.1Sorbitol + 0.5% MeOH

Plate 3: BYP + 0.1% FA

Plate 4: BYP0.1%S + 0.1%FA

Plate 5: BYP

Plate 6: BYP0.1%S

Levanase activity assay:

- Measure OD600: 10µl culture + 190µl 0,9% NaCl
- Transfer culture from deep well into v-bottom microtiter plates, centrifuge at 3000rpm for 15 min. at 4°C (store at 4°C until all plates are centrifuged; proceed together).
- Meanwhile provide another v-bottom mt-plate with 20µl/well 1% sucrose
- Add 20µl of supernatant to the provided sucrose, mix by pipetting up and down
- Incubate for 20 min. at RT
- Meanwhile provide 190µl/well glucose-UV-reagent in a **UV**-mt-plate
- Add 10µl of the sucrose-SN-mixture to the UV-reagent, mix rapidly by pipetting up and down (just once or twice)
- Incubate for 10 min at RT
- Measure OD 340nm in a plate reader (UV!)

Wachstums-Screening der pPICZ_aox1 und pGAPZ_aox1 Transformanten (KW16):

Verwendet wird die Platte 2 von 30.3.2010:

Reihen 1-5: 1.17pPICZaox1 in double KO

Reihe 6: Kontrollen (wie Michi Tscherner Platten)

Reihen 7-11: 1.17pGAPZaox1 in double KO

Reihe 12: Kontrollen

Außerdem werden folgende Platten mitgemacht:

Kontrolle aox(Retrafo Genbankfragment)/aox stop

Eventuell H567A Platte mitmachen als Kontrolle

Tag 1 (Mittwoch)

Nach 24 h Inkubation oder aus dem Glycerolstock werden je 10 µl der Kultur in 250 µl BMD 1% überimpft.

Die Platten inkubieren nun 48 h im Schüttler (28°C, 320 rpm, 80% H2O)

Tag 2 (Donnerstag)

Herstellung der Buffered Minimal Inulin und Saccharose Platten (0,5%)

Für die Inulin Platten werden 2x 1,75 g Inulin von Dahlia Tubes in 35 mL Fresenius

Wasser unter Erhitzen im Wasserbad gelöst (Dauer ca. 3 min bis Inulin gelöst).

Es muss darauf geachtet werden, dass das Inulin nicht länger als notwendig

erhitzt wird, da es sonst hydrolisiert! Nachdem es gelöst ist, wird es sofort warm

steril filtriert.

Tag 3 (Freitag)

Herstellung von Buffered Minimal Methanol Platten (0,5% Methanol)

Nach 48 h Inkubation werden 25 µl der Kultur in 225 µl NaCl 0,9% in Mikrotiterplatten

verdünnt (1:10 Verdünnung) und davon die OD600 vermessen.

Anschließend werden 5 µl pro well auf Buffered Minimal Inulin und Methanol Platten aufgespottet und übers Wochenende 1x bei Raumtemperatur im Labor (und 1x bei 30°C im Brutschrank inkubiert).

Enterokinase treatment:

- Centrifuge 1ml of OD3 (better OD20) induced culture (harvested after 48h?) at 4000g for 5min at 4°C
- Wash twice with 10mM Tris/HCl buffer pH 7.8 containing 2mM CaCl2 and 50mM NaCl
- Finally resuspend pellet in 250µl of the same buffer containing 2u porcine enterokinase
- Incubate at 4°C for 4 hours
- Centrifuge at 4000g for 10 min at 4°C
- Store supernatant for SDS PAGE
- Wash cells twice with PBS
- Store at 4°C for immunofluorescence microscopy.

Immunofluorescence (Quick protocol) - adapted

(Pichia pastoris fermentation: cells were induced with Meoh – 48 h)

Fixation/permeabilization

- Precultures were grown in YPD or BMGY (48h)
- Main cultures: 50 mL BMMY (1% MeoH) in buffled flaskes; 500 µl of precultures were taken to inoculate main culture (Higher diluted cultures are better for IF)
- Induction with MeOH for 48 h (Addition of MeOH (1%) – 500µl - in morning and evening)
- An aliquot (around $1x10^8$ cells) should be taken (2 mL of cultures were taken and 200µl of Formalin (37% Formaldehyde solution) was added and incubated for 30 min (or several hours) at 30°C. (can be stored over night at 4°C) – *note: adapt amount of formalin volume to culture volume*
- Centrifugation at 2500 rpm, for 5 min
- Wash with 100 mM KPi buffer
- Add 100 mM KPi buffer/4% Formaldehyde (can be stored in the fridge)
- Wash with solution B
- Pellet is resuspended in solution B with 20 µl Zymolyase (5 mg/mL)
- Incubation at 30°C for 30 min (gentle shaking)
- Centrifugation at 1600 rpm, wash 1x with 1 mL solution C
- Resuspend the pellet in 1 mL solution C + 1 % SDS (50 µl of 20% SDS) (gently resuspended)
- Wash 2 x with solution C (gently)
- Can be stored at 4°C

Preparation of slides (Multiwell slides)

- Coat slide (each well) with 10-20 µl poly-L-lysine (0.1% in H2O)
- Incubate 10 min at RT
- Remove the poly-L-lysine , wash 5 x with H2O , remove the water and let the slides dry (10-15 min)
- 20 µl of fixed cells are pipetted into wells – leave it for 5- 30 min
- Remove cells and add 20 µl of "Blocking solution", incubate for 30 min
- Add 20 µl of primary antibody (1:100 dilution in PBS) – incubation for 30 min (or eventually over night, at 4°C in humid chamber)
- Remove 1st Ab,
- Wash 8x with 20 µl PBS Albumin
- Add 20 µl of secondary Ab (DyLight anibodies 1:500 diluted in PBS/Albumin) – Incubate 30 min – 1 h (in the dark, humid chamber, RT)
- Wash 5 x with PBS/Albumin
- *Optional (to stain nuclei): add 10 µl DAPI (10 µg/mL) -incubate 5 min (in the dark) – wash 5x with PBS /albumin*
- Add 3 µl Vectashield – cover slide with coverslip (40x50 mm) – fix coverslip on both ends with nailpolish – store at 4 °C in the dark (can be kept for month) – *note: use only little nailpolish, make sure it does not flow into wells*

SOLUTIONS:

100 mM KPi Buffer, pH 7.5	**400 mL:**	
	32 mL KH2PO4 (0.2M)	
	168 mL K2HPO4 (0.2M)	
	200 mL H2O	
Solution B		
1.2M Sorbit	Dissolve 10.93 g sorbit in 50 mL KPi	
100 MM KPi		
Solution C		
1.2 M sorbit	Dissolve 10.93 g sorbit in 50 mL H2O	
PBS		
100 mM KPi buffer, pH 7.5	Dissolve 0.1 g BSA in 5 mL buffer	
2 % BSA		
Blocking solution	**Stock**	10 mL
40 mM K2HPO4	0.2 M	2 mL
10 mM KH2PO4	0.2 M	0.5 mL
150 mM NaCl	5 M	0.3 mL
0.1 % NaN3		10 mg
0.1 %Tween 20	10 %	0.1 mL
2 % Molke		0.2 g

Material:

Poly-L –Lysine: Mw > 300 000, sigma P-1524

Slides: 8 wells a 6 mm # 10-5D, Dynatech (?)

Vectashield: # H-1000, Vector Laboratories (supplier:Immuno-Diffusion)

Secondary antibodies:

- Goat anti-Mouse IgG, DyLight 594 conjugated highly cross-adsorbed (Thermo scientific, #35511)

- Goat anti-Rabbit IgG DyLight 488 conjugated highly cross- adsorbed (Thermo scientific, # 35553)

Also available in our lab:

- Goat anti- Rabbit IgG DyLight 488 conjugated highly cross-adsorbed (thermo scientific, # 35503)
- Goat pAb to rabbit IgG (Cy3) – ab6939-100 (in freezer)
- Goat pAb to rabbit IgG (Cy5) ab652426 (in freezer)