Enhancing the expression of recombinant proteins in the methylotrophic yeast *Pichia pastoris*

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Enhancing the expression of recombinant proteins in the methylotrophic yeast *Pichia* pastoris

Abstract:

Methylotrophic yeast *Pichia pastoris* is an excellent platform for expressing recombinant proteins. By engineering these yeast cells further it is possible to elevate the expression levels of the recombinant proteins. I have focussed on three different aspects.

1) Yeast vacoules harbour different types of proteases, which may be released into fermentation media leading to proteolysis of the expressed recombinant protein. Carboxypeptidase Y is one such major protease. We have applied anti-sense RNA technology as a tool for downregulating the *PRC1* gene coding for CPY without disturbing the metabolic network of the cell.

2) Heterologous protein expression in *P. pastoris* is usually directed into secretory mode. Unfortunately, it has been observed that recombinant proteins often cannot surpass the cell wall boundary and accumulate within the periplasmic space. Inability of these proteins to cross the cell wall is generally attributed to size and confirmation of the protein expressed. To overcome this bottleneck instead of expressing the protein into secretory mode we planned for cytoplasmic mode of expression. For this purpose, model protein ATGL (Adipose triglyceride lipase) has been selected. Functional expression of ATGL is a challenging task since it contains multiple cysteins and structural information indicates that stability of ATGL depends on the formation of the proper disulfide bonds. Disulfide bond formation requires a relatively oxidising environment present in the ER lumen whereas cytoplasmic environment is relatively reducing. Activity assays on recombinant ATGL demonstrated that it was possible to express the recombinant protein in a functional manner presumingly by forming correct disulfide bonds in the cytoplasmic environment.

3) Furthermore, a general high throughput screening system was established which allows identification of clones expressing high levels of recombinant protein.

Verbesserung der rekombinanten Proteinexpression in der methylotrophen Hefe *Pichia pastoris*

Zusammenfassung:

Die methylotrophe Hefe *Pichia pastoris* bietet eine exzellente Grundlage zur rekombinanten Proteinexpression. Durch Weiterentwicklung dieser Hefezellen kann es gelingen die Ausbeuten an rekombinantem Protein weiter zu steigern. Wir haben uns auf 3 Aspekte konzentriert:

1) Hefe-Vakuolen enthalten verschiedene Arten von Proteasen, die ins Fermentationsmedium gelangen und dort rekombinante Proteine hydrolysieren können. Eine solche Protease ist Carboxypeptidase Y (CPY). Wir haben anti-sense RNA-Technologie eingesetzt, um das *PRC1* Gen, das für CPY codiert, herunterzuregeln ohne dabei den grundlegenden Metabolismus der Zellen zu stören.

2) Üblicherweise erfolgt heterologe Proteinexpression in *P. pastoris* auf dem sekretorischen Weg. Allerdings hat sich in zahlreichen Fällen gezeigt, dass rekombinante Proteine nicht durch die Zellwand dringen können und sich daher im Periplasma anreichern. Man nimmt an, dass dies hauptsächlich auf die Größe und Konformation der exprimierten Proteine zurückzuführen ist. Um dieses Nadelöhr zu umgehen, haben wir anstelle von sekretorischer auf intrazelluläre Proteinexpression gesetzt. Als Modellprotein haben wir uns dazu ATGL (Adipose triglyceride lipase) ausgesucht. Funktionelle Expression von ATGL ist aufgrund seiner multiplen Cysteine eine Herausforderung, weil strukturelle Informationen zu ATGL andeuten, dass die korrekte Ausbildung von Disulfid-Brücken für die Proteinstabilität maßgeblich ist. Die Bildung von Disulfid-Brücken erfordert üblicherweise das relativ oxidierende Milieu des ER-Lumens. Im Unterschied dazu ist das Cytoplasma von Hefe als reduzierende Umgebung anzusehen. Wie uns Aktivitätsassays von rekombinanter ATGL zeigten, gelang uns, gegen die Erwartung, die funktionelle Produktion der Enzyms im Cytoplasma; vermutlich durch korrekte Ausbildung von Disulfid-Brücken.

3) Weiters haben wir ein allgemeines Hochdurchsatz-Screening für *Pichia pastoris* etabliert, das die einfache Erkennung von Hochleistungs-Expressionsstämmen erlaubt.

General Introduction – *Pichia pastoris*

Background:

Yeast has been an integral part of human culture for immemorial time in grape wine as well as beer fermentation and baking. However, in parallel to the discoveries of cell biology revealing some of the secrets of basic construction and functions of a living cell, new branches of life sciences called molecular biology and biotechnology have developed. New dimensions in these novel areas have broadened the application of yeasts, i.e., unicellular fungi, to industrial applications and heterologous protein production. Yeasts are practically free of toxin production, oncogenic viral DNA and are used in food industry for years, which has led to certification of some yeasts with GRAS (Generally Regarded As Safe) status. Research on yeast for industrial process, and various bottle necks in applying *Saccharomyces cerevisiae* for commercial applications led to a quest for a novel yeast strain with outstanding characteristics. In the 1970s, a limited number of methylotropic yeasts of the genera Pichia*, Hansenula, Candida* and *Torulopsis* has come into light. Phillips Petroleum in the 70s developed protocols for growing *Pichia pastoris* for single cell production for animal feed. Increased oil price made it impossible to grow yeast on Petrol wastes furtheron. Later Phillips Petroleum contacted The Salk institute of Biotechnology/Industrial associates, Inc to develop *P.pastoris* as a platform for heterologous protein expression. Since then, *Pichia pastoris* has been both a model organism in basic research covering aspects of Peroxisome biogenesis, lipid transport and yeast physiology, and is applied in the field of biotechnology.

Methanol utilisation pathway (MUT pathway):

This MUT pathway is the biochemical pathway to metabolise methanol as carbon source for energy production. Metabolism of methanol in *P. pastoris* takes place both in peroxisome and cytosol (2, 1, 3). In the first step, methanol is oxidised to formaldehyde and hydrogen peroxide by alcohol oxidase (AOX). H_2O_2 is broken down into water and oxygen by the action of catalase. A portion of formaldehyde is transported into cytosol where it is further oxidised to formate and CO_2 by two cytoplasmic dehydrogenases, releasing energy in the form of NADH. Remaining formaldehyde is assimilated forming cellular constituents such by a cyclic pathway that starts with the condensation of formaldehyde with xylulose 5-

monophosphate. This reaction is carried by third peroxisomal enzyme dihydroxyacetone synthase (DAS). These two products enter cytoplasmic pathway that generates xylulose 5-monophosphate and, for every 3 cycles, one net molecule of glyceraldehyde 3-phosphate is generated.

AOX and DAS are expressed when yeast cells are grown only in the presence of methanol as C source (2, 3).

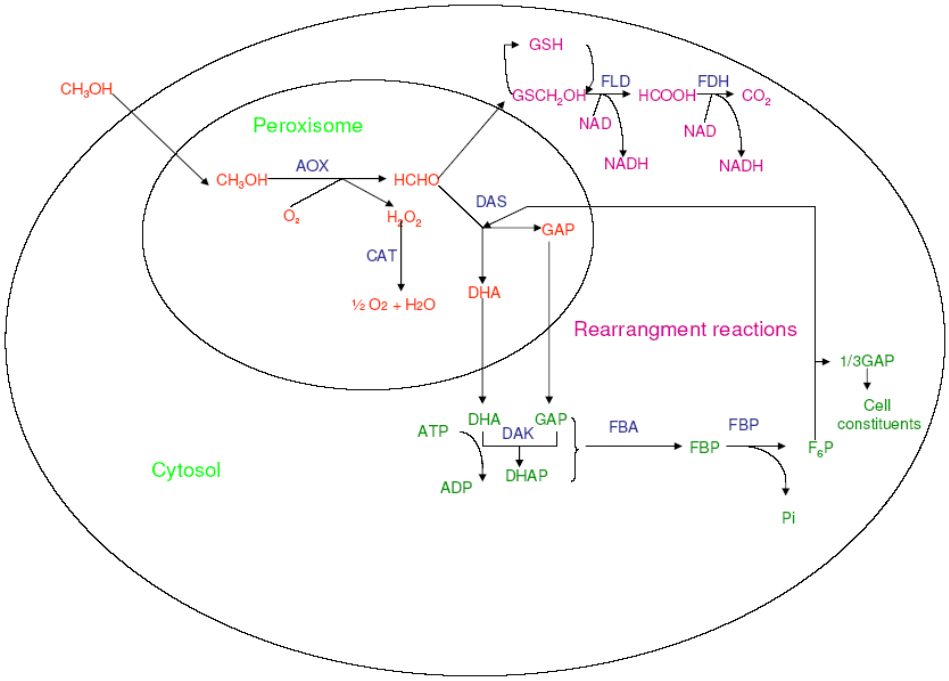

Fig 1: Methanol utilisation pathway in *P.pastoris*. AOX: alcohol oxidase, CAT: catalase, DAK: dihydroxy acetone kinase, DAS: dihydroxy acetone synthase, FBA: fructose-1,6-bisphosphate aldolase, FBP: fructose-1,6-bisphosphatase, FLD: formaldehyde dehydrogenase, FDH: formate dehydrogenase (2).

Pichia Expression system:

The success of *Pichia pastoris* yeasts as a host for heterologous protein expression can be attributed to many factors (2, 1, 3, 4, 5) some of them are 1) Strong and highly regulated promoters of the genes involved in methanol utilisation pathway 2) Simple techniques required for growth and transformation, ability to perform typical post translational modifications of proteins such as glycosylation and disulfide bond formation 3) Growth of the yeast cells to very high cell densities in the bioreactors resulting in high product yield 4) Ability of these cells to secrete even high molecular weight proteins into the external media 5)

Recently approaches in engineering the biochemical pathways in glycosylation patterns have opened the doors for the methlyotropic yeasts for the production of biopharmaceuticals such as therapeutic antibodies (6).

AOX1 promoter:

The *AOX1* gene product is the most important protein in initialising MUT pathway. The upstream sequence, i.e., promoter sequence of this gene is used in constructing expression vectors for heterologous protein expression. Advantages in using the *AOX1* promoter is

1) Transcription of foreign protein under the control of *AOX1* promoter is tightly regulated and controlled by a repression/derepression mechanism.
2) High levels of heterologous protein expression.
3) Repression of *AOX1* gene by most C sources other than methanol ensures high cell growth before gene expression.
4) Induction of transcription is easily achieved by the addition of methanol.

The disadvantages in using this promoter are as follows,

1) Online monitoring of methanol utilisation during fermentation is difficult.
2) Methanol is a fire hazard, storing large quantities of methanol is undesirable.
3) Methanol is a petrochemical product. Thus, its application in producing proteins for food industry is unsuitable.
4) Utilisation of two carbon source makes switching over from one to the other at a precise time point difficult (2, 1, 3, 4, 5, 7, 8, 9, 10, 11).

GAP Promoter:

GAP is acronym for glyceraldehyde-3-phosphate dehydrogenase gene encoding a NAD-dependent enzyme that forms tetramers. The 500 bp upstream sequence from the translation initiation codon ATG was used for constructing expression vectors. P_{GAP} is a constitutive promoter expressing constitutively on all carbon sources, including glucose, glycerol, ethanol and oleic acid. The *GAP* promoter can be used, instead of the *AOX1* promoter for the production of heterologous proteins in *P.pastoris*. Advantages of using the *GAP* promoter are that methanol is not required for induction and it is not necessary to shift culture from one carbon source to another. However, constitutive expression of heterologous toxic proteins could be toxic to the yeast cells (2, 1, 9, 12).

***FLD1* Promoter:**

Glutathione-dependent formaldehyde dehydrogenase (*FLD*) is a key enzyme required for metabolism of methanol as a carbon source and certain alkylated amines such as methylamine as nitrogen source. Its primary role appears to be protecting cells from toxic effects of formaldehyde, with a side benefit of yielding a net reducing power in the form of NADH. *P.pastoris FLD1* promoter sequence is used in constructing expression vectors, i.e., P_{FLD1} is a potential alternative to the routinely employed P_{AOX1} expression. P_{FLD1} is a highly regulated promoter that is capable of producing heterologous protein at levels or higher than those produced by P_{AOX1}. Application of P_{FLD1} provides a wider choice of conditions for induction including methanol, alkylated amines such as methyl amine or choline (2, 1, 13).

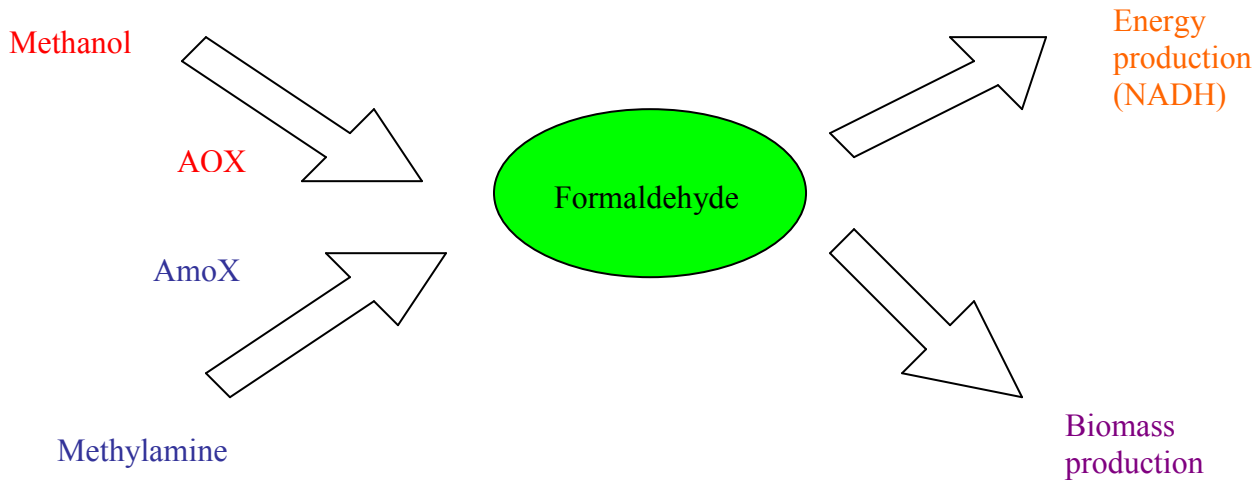

Fig 2: The intermediate formaldehyde is common to both the methanol and methylamine pathways in the yeast *Pichia pastoris* (1).

***ICL1* Promoter:**

Isocitrate lyase, catalyses the formation of succinate and glyoxalate from isocitrate, a key reaction in the glyoxylate cycle. *ICL1* gene is repressed in the presence of glucose and induced in the absence of glucose are in the presence of ethanol. Due to this characteristic, the use of this promoter has been an attractive alternative to the conventional P_{AOX1} promoter for the expression of foreign genes. No actual figures were offered and there is no detailed study on the use of *ICL1* promoter as an efficient promoter for constructing expression vectors (1, 14).

***PEX8* and *YPT1* promoters:**

AOX1, *GAP* and *FLD1* promoters may be too strong, expressing genes at too high a level, such that high level expression of proteins will block proper folding and post translation modification. There by a significant proportion of these proteins could be unprocessed or misfolded. Moderately expressing promoters might be desirable, such as P_{PEX8} directing Pex8p formation, which is a peroxisomal matrix protein that is essential for peroxisome biogenesis. It is expressed at a low but significant level on glucose and is induced modestly when cells are shifted to methanol. *YPT1* genes encode a GTPase involved in secretion, and its promoter provides a low but constitutive protein expression in media containing glucose, methanol or mannitol as carbon source (2).

Selectable markers:

P. pastoris has a few selectable marker genes for the molecular genetic manipulation as represented in Table 1. Generally, these markers originated from *S .cerevisiae* or *P. pastoris* biosynthetic pathway genes (2, 1, 15).

Table1: Marker genes for use with the *Pichia pastoris* expression system (1).

Gene	Origin	Function
HIS4 (histidinol dehydrogenase gene)	*P. pastoris or S. cerevisiae*	Biosynthetic pathway gene
ARG4 (argininosuccinate lyase gene)	*S. cerevisiae*	Biosynthetic pathway gene
Sh ble (Zeocin resistance gene)	*Streptoalloteichus hindustanus*	Confers resistance to Bleomycin-related drugs
bsr (Blasticidin S deaminase)	*Aspergillus terreus*	Confers resistance to drug blasticidin
ADE1 (N-succinyl-5-aminoimidazole-4-carboxamide ribotide synthetase)	*P. pastoris*	Biosynthetic pathway gene
URA3 (Orotidine-5'-phosphate decarboxylase)	*P. pastoris*	Biosynthetic pathway gene
sor^R (acetyl-CoA carboxylase)	*Sorangium cellulosum*	Confers resistance to the macrocyclic polyketide soraphen A
neo (aminoglycoside phosphotransferase)	Tn5 and Tn601	Confers resistance to geneticin

Host strains:

All host strains of *Pichia* for transformation are derived from NRRL-Y 11430. Most of the strains are auxotrophic mutants. Complementation of auxotrophies is used for selection of transformants. Prior to transformation host strains are grown on complex media supplemented with appropriate nutrient(s) for growth (2, 1).

Table 2: *P. pastoris* host strains. (Northern Regional Research Laboratories, Peoria, IL) (2).

Strain	Genotype
Auxotropic strains	
Y-11430	*Wild-type*
GS115	*his4*
GS190	*arg4*
JC220	*ade1*
JC254	*ura3*
GS200	*arg4 his4*
JC227	*ade1 arg4*
JC300	*ade1 arg4 his4*
JC301	*ade1 his4 ura3*
JC302	*ade1 arg4 ura3*
JC303	*arg4 his4 ura3*
JC304	*ade1 his4*
JC305	*ade1 ura3*
JC306	*arg4 ura3*
JC307	*his4 ura3*
JC308	*ade1 arg4 his4 ura3*
Protease-deficient strains	
SMD1163	*Pep4 prb1 his4*
SMD1165	*Prb1 his4*
SMD1168	*Δpep4:: URA3 his4 ura3*
SMD1168 kex1::SUC2	*Δpep4:: URA3 Δkex1::SUC2 his4 ura3*

Integration of expression vectors into the *P. pastoris* genome:

Expression vectors are integrated into the *P. pastoris* genome to maximize the stability of the heterologous expression construct. This can be done in two ways. Restricting the recombinant vector in a marker gene, e.g., *HIS4* by SalI. Alternatively, standard expression vectors can be digested in such a way that the expression cassette and marker genes are released from the vector and flanked by 5' and 3' *AOX1* sequences. To achieve high level protein expression

strains, transformants containing multiple copies of the expression cassettes are isolated. Such so-called multicopy strain may yield more heterologous protein than single copy strain (2, 16).

Methanol utilization phenotype:

Depending upon the extent of utilisation capacity of methanol there are three phenotypes of *P.pastoris*. Mut^+ or methanol utilisation plus phenotype cells have two functional alcohol oxidative enzymes, Aox1p and Aox2p, and require high feeding rates of methanol in large scale fermentations. Mut^S or methanol utilising slow phenotype cells carry a disruption of *AOXI* gene, which is the case e.g., in *KM71*. Since the cells depend on the weaker *AOX2* for methanol metabolism, a slower methanol utilising and, therefore, slower growing strain is produced. Mut^- or methanol utilisation minus phenotype cells is unable to grow on methanol, since these strains lack both *AOX* genes confering methanol utilisation. In general, Mut^+ or Mut^S strains, depending on the amount and type of proteins to be expressed, are used (2, 1, 3).

Protease deficient host strains:

Yeast cells are known to be stressed by starvation, change of carbon source, heat and pH change, or toxic chemicals. Proteins damaged by oxidative stress and heat-shock response also elicit a proteolytic response. Methanol metabolism demands high amounts of oxygen and yields hydrogen peroxide as by-product. Vacuoles are the primary source of proteases. After 72 h of induction, i.e., at the end of fermentations, vacuole size and number increases enormously. Cell lysis steps in down-stream processing liberate proteases from the vacuole leading to proteolytic cleavage of the recombinant proteins. Few preventive measures like addition of casamino acids and protease inhibitors are in application. Protease deficient strains have been developed and have been shown to be effective in reducing the degradation of some heterologus proteins. Unfortunately, these protease deficient strains are not as vigorous as wild-type strains. In addition to lower viability, they possess a slower growth rate and are more difficult to transform. Therefore, the use of protease-deficient strains is only recommended in situation where other measures to reduce proteolysis have yielded unsatisfactory results (2, 1, 17, 18, 19).

Co-over expression of chaperone proteins and PDI:

Multicopy strains or over-expressing strains of *P. pastoris* can result in misfolding of the proteins to non-native, suboptimal conformations and/or overloading of the host machinery.

This might be detrimental to the viability of the host cells. The endoplasmic Reticulum (ER) is the site for disulfide bond formation, protein folding and maturation. Disulfide bonds are crucial for the stability of the final protein structure, and the mispairing of cysteine residues can prevent proteins from attaining their native confirmation and lead to misfolding.

Co-over expression of *S .cervesiae* chaperones such as Kar2p, Sec63p, YDJIp, Ssalp and Protein disulfide isomerase (PDI) could be helpful. PDI is a 57 kDa protein involved in rearrangement of incorrect disulfide pairings by isomerase activity of CGHC domains in active site. In this reaction, cysteine residue in the active site of PDI forms a transient disulfide bond with the misfolded protein. While this bond exists, the protein resumes a proper confirmation there by developing new correct disulfide link, the bond with PDI is then broken. Ssalp and Kar2p are homologs of Hsp70s in the ER. Kar2p has been suggested to affect events even on the cytosolic face of the ER (14); Kar2p helps in maintaining the nascent polypeptide in intermediate confirmation thereby allowing PDI access to the disulfide bonds. Ssa1p, the cytoplasmic chaperon, helps to maintain the polypeptide in an ER-translocation competent or unfolded conformation. Expression of these chaperons individually increased secreted expression 4 - 7 times. When co-expressed few combinations such as YPJ1p/PDI, YPJ1p/Sec63 and Kar2p/PDI have synergistically improved the secretion levels of the reporter proteins (20, 21, 22).

Secretion signals and *GAS1* gene disruption:

P.pastoris expresses heterologous proteins extracellularly or intracellularly. Extracellular expression of proteins is most advantageous because it eliminates the need for cell breakage, removal of cell debris, etc, as whole cells can easily be removed by centrifugation or filtration. Foreign proteins can be expressed extracellularly by cloning the gene of interest with natural signal sequence. Alternatively *S. cerevisiae* α-factor prepro-peptide or the *P.pastoris* acid phosphatase (*PHO1*) signal sequences can be applied.

Once a protein has been released from the ER - Golgi system and secretory vesicles have fused with the plasma membrane, the protein has to penetrate the cell wall for extracellular expression of protein. The cell wall often is not a limiting barrier, but high molecular weight proteins might not be able to pass it. *GAS1* gene encodes a cell wall bound 1, 3-β-glucanosyltransferase involved in the formation and maintenance of 1, 3-β-glucan, which is the major polysaccharide of the cell wall. *gas1* null mutation weakens the cell wall, generates mutant cells that are abnormally round, have reduced viability and increased sensitivity to

elevated temperatures. It has been observed that the amount of recombinant protein that is released into fermentation supernatant is very high in *Pichia* cells disrupted for *GAS1* (23).

Glycosylation:

For many therapeutic proteins such as Interferon, erythropoietin, monoclonal antibodies to attain complete biological activity, glycosylation plays an important role. *P. pastoris* is capable of performing both N - and O- linked glycosylation (18). In mammals, O- linked oligosaccharides are composed of a variety of sugars including N-acetylglucosamine, galactose (Gal), and sialic acid (NeuAc). Very little is known about O- linked glycosylation in *P. pastoris* but it is mainly composed of mannose (Man) residues. Basically it contains α-1,2-mannans containing dimeric, trimeric, and tetrameric and pentameric oligosaccharides, but no α-1,3 linkages were detected.

N-linked glycosylation:

N-linked glycosylation is an important post translational modification. For instance, in therapeutic proteins this glycosylation constitutes a small fraction of the molecular mass of a given protein, yet it contributes significantly to its hydrodynamic volume and, therefore, to its pharmacodynamic behaviour. N-linked glycosylation begins in the ER with the transfer of a oligosaccharide core unit, $Glc_3Man_9GlcNAc_2$ (Glc = glucose; GlcNAc = *N*-acetylglucosamine) to aspargine at the recognition sequence Asn-X-Ser/Thr. This oligosaccharide unit is trimmed to $Man_8GlcNAc_2$. At this point glycosylation patterns start to differ between the lower and higher eukaryotes. The Golgi apparatus performs a series of trimming and addition reactions that generate oligosaccharides composed of $Man_{5-6}GlcNAc_2$. Secreted protein of *P.pastoris* is decorated by $Man_8GlcNAc_2$ or $Man_9GlcNAc_2$. Finally, *P.pastoris* oligosaccharides appear not to have any terminal α-1,3-linked mannosylation which makes recombinant proteins unsuitable for pharmaceutical use in humans. However *Pichia* has been engineered to mammalian N-glycosylation pathway as shown in Figure 3 (2, 1, 6).

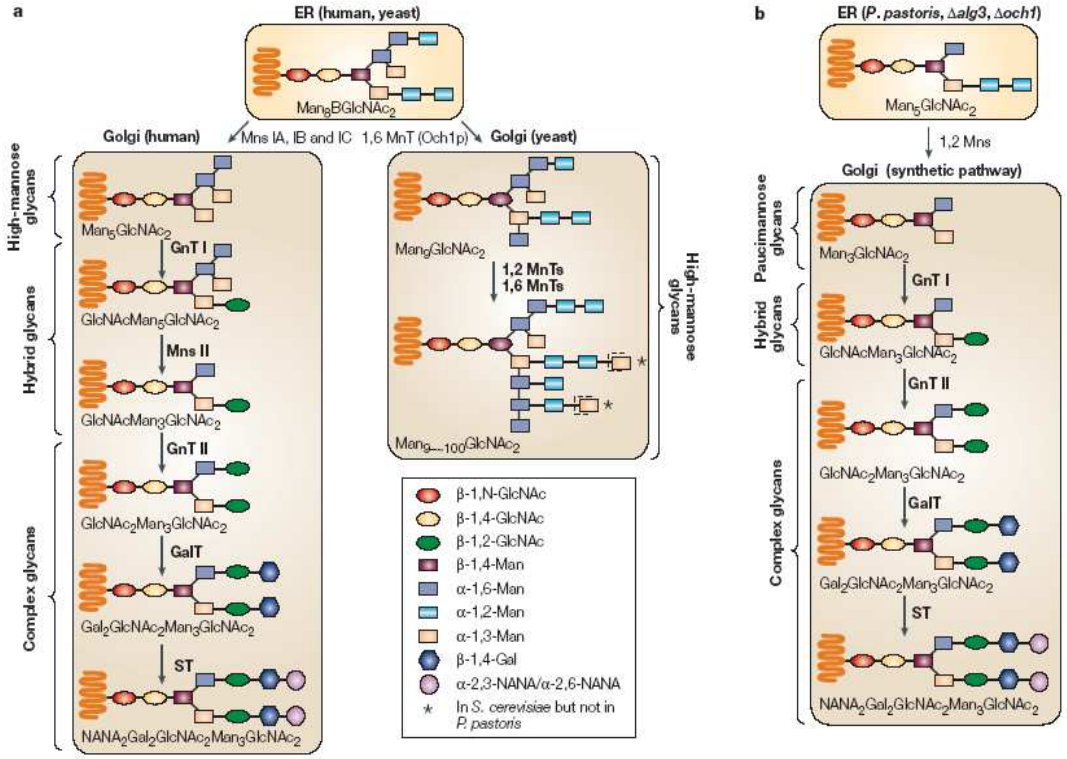

Fig 3: Major *N*-glycosylation pathways in humans and yeast. a) Representative N-glycosylation pathways in humans (left) provide a template for humanising N-glycosylation pathways in yeast (right). b) Early oligosaccharide assembly mutants can be used to create synthetic glycosylation pathways that lead to complex N-glycosylation pathways. ER, endoplasmic reticulum; GalT, galactosyltransferase; GnT I, N-acetylglucosaminyl transferase I; GlcNAc N-acetylglucosamine; GnT II, N-acetylglucosaminyl transferase II, Man, mannose; MnsII, mannosidase II; MnTs, mannosyltransferase; NANA, GnT l, N-acetylneuramic acid; ST, sialytransferase (6).

Heterologous protein expression:

Pichia pastoris has been accepted as an excellent platform for protein expression. A wide range of proteins from different sources ranging from plant to humans have been expressed which have been listed in the Table 3 (2, 1).

Table 3: Summary of a range of foreign proteins produced using the *Pichia* expression system. (2)

Protein	Amount	Mode and Signal sequence
Bacteria		
Bacillus licheniformis α-amylase	2.5 g l^{-1}	S, SUC2
B. stearothermophilus D-alanine CPY	100 mg l^{-1}	S, native
Staphylococcus aureus Staphylokinase	50 mg l^{-1}	S, α-MF
Streptococcus equisimilis streptokinase	77 mg l^{-1}	I
Fungi		
Aspergillus awamori glucoamylase	400 mg l^{-1}	S, native
S. cerevisiae Ktr1p	400 mg l^{-1}	S, PHO1
Schizophyllum commune vit B2-aldehyde-forming enzyme	120 mg l^{-1}	S, α-MF
Plants		
Hevea brasiliensis hydroxynitrile lyase	22 mg l^{-1}	I
Invertebrates		
Drosophila melanogaster angiotensin I-converting enzyme	160 mg l^{-1}	S, α-MF
Spider dragline silk protein	663 mg l^{-1}	I
Humans		
Insulin-like growth factor	600 mg l^{-1}	S, α-MF
Monoclonal single-chain Fv	50 mg l^{-1}	S, α-MF
Viruses		
Hepatitis B virus surface antigen	400 mg l^{-1}	I

S=secreted, I=intracellular, Signal sequences: α-MF= *S. cerevisiae* α-mating factor, PHO1= *P. pastoris* phosphatase, SUC2= *S. cerevisiae* invertase.

Conclusions:

P. pastoris expression system has been accepted as a successful platform for heterologous protein expression both academically and industrially. A wide range of proteins from different origins has been expressed including human pharmaceuticals which are in a phase of clinical trials. The contributing factors for the success of *Pichia* are many-fold, such as wide range of selectable markers, secretion signals, selection markers, availability as commercial kit, simple techniques required for genetic manipulation, etc.

Moreover, engineering of glycosylation pathways to human like glycosylation made it possible to express pharmacologically active proteins. Pathways for releasing the heterologous protein directly outside the yeast cell, establishing novel screening systems for high-through put screening (18, 24), search for novel markers (15) and new promoters, setting up new versatile protocols for optimising fed batch fermentations (25, 19, 24), isolation of factors affecting *AOX* promoters (11), designing new plasmids for better expression of foreign proteins and, studying in detail the physiology of yeast cells during heterologous protein production (4, 26) are the current areas of research in *Pichia*.

The successes in these areas make *Pichia pastoris* a much more user friendly organism for protein expression.

Future prospects:

Inspite of many advantages of using *Pichia pastoris*, still there are many bottlenecks in using yeast for protein expression, e.g., developing strain with reduced protease activities. Strategies for humanising the glycosylation pathways of this yeast are not completely achieved. Developing high throughput screening systems for a large number of clones selecting for a single clone expressing better than the average on a single platform, are not yet described. Analysis of *Pichia* genome is far from being complete as the number of nucleotide sequences in NCBI does not even exceed 300 entries (27). Expression of mammalian membrane proteins has been limited because of fungal membrane lipid biodiversity and over-expression problems leading to incorrect protein folding (28).

Protease system of *Pichia* has not yet been studied completely like in the *S. cerevisiae* especially vacoular proteases. The biochemical reactions in the MUT pathway make it clear that vacuoles play an important role in metabolising methanol; there number also increases

during this process proportionately (2, 1, 3). It has been shown that some of these proteases will be released into the culture supernatant during the process of fermentation and some during the cell lysis step of protein purification. Due to this event much of the protein expressed is degraded. Application of protease deficient strains is not advisable since these strains are not as active as wild type strains in growth and transformation concluding that these enzymes play an important role in house keeping activity of the cell metabolism (2, 1, 3). So we want to suppress the expression of one of these proteases at the level of protein with out disturbing the metabolic network. For this purpose we have selected one of the major vacoular protease carboxypeptidaseY (CPY) which is expressed by PRC1 gene and its sequence is known. CPY is a C-terminal exopeptidase involved non specific protein degradation in the vacuole. It is involved in bulk degradation of non-specific cytoplasmic proteins, mislocalized proteins from the secretory pathway, misfolded proteins, proteins delivered by autophagy in the vacuole (29). Therefore this enzyme in one way involved in cleaning of cell vacuole and also in recycling the aminoacids produced during degradation with in the cell. There fore PRC1 deletion may be harmful to the cell, so for shut down of PRC1 expression we have used antisense mRNA technology where antisense RNA has been harboured to the PRC1 mRNA there by inhibiting its translation.

Translational coupling is a process where a collection of genes *(Polycistronic mRNA)* transcribed (*by a single ribosome*) on the same mRNA strand. Generally this type of gene expression is the characteristic feature of the prokaryotes i.e. bacteria. This phenomenon has been described in eukaryotes (30). We want to apply this phenomenon in yeast cells to develop a better screening system for screening a single clone ideally expressing high amounts of protein of interest from a pool of clones.

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Functional expression of Adipose Triglyceride Lipase in *Pichia pastoris*

Abstract:

Adipose Triglyceride Lipase (ATGL) catabolizes triglycerides liberating free fatty acids and is, thus, of utmost importance in cellular energy metabolism. ATGL is a member of patatin domain containing proteins. The patatin domain is unique in that it contains an α/β/α protein sandwich architecture and an active serine in the lipase consensus motif GXSXG. Sequence homology searches showed that lipases containing a patatin domain are also found in *S. cerevisiae,* i.e. Tgl3p and Tgl4p, further underscoring the conservation and importance of these enzymes. Biochemical characterization and structural/functional studies require sufficient quantities of native ATGL which is a bottle neck, because large scale expression and purification of this enzyme from tissue cultures is not feasible. Prokaryotic expression platforms, e.g. *E. coli*, yielded largely inactive ATGL protein, presumably due to inclusion body formation. We selected the methylotrophic yeast *Pichia pastoris* as a most promising platform for large scale heterologous expression of mouse ATGL because of its capacity to grow to high cell densities and its strong, inducible *AOX1* promoter. Aiming for cytosolic expression we obtained protein fractions which harbored specific ATGL activities similar to recombinant ATGL expressed in mammalian cells. Furthermore, the lipase activity of recombinant ATGL expressed in yeast was enhanced by addition of CGI-58, very much like mouse ATGL produced in mammalian cell culture. Thus, we have a promising microbial expression system at hand that may aid in elucidating the structural and functional properties of ATGL on a molecular level.

Key words: *Pichia pastoris*, heterologous expression, ATGL

Introduction:

Lipids are stored as triacylglycerols (TAG) in the adipocyte and are a most important energy source in eukaryotes. On requirement, TAG are broken down to fatty acids (FFA) and diacylglycerol (DAG), monoacylglycerol (MAG) or glycerol. Hormone sensitive lipase (HSL) was suggested to catalyse the rate-limiting step in the hydrolysis of TAG (1). However, HSL knock-out mice exhibited normal body weight without any traces of fat accumulation (2). Secondly, these animals retained a marked basal isoproterenol-stimulated lipolytic capacity in adipose tissue. Thirdly, lipolysis in the absence of the HSL led to accumulation of diacylglycerol in the fat cells. Together these results suggested that there must be at least one additional unidentified triglyceride lipase catabolizing triglyceride in the absence of HSL, which was later discovered as ATGL (Adipose Triglyceride Lipase) (2).ATGL is predominantly expressed in the white adipose tissue (WAT) and is a member of patatin domain containing proteins. The patatin domain is unique in that it contains an α/β/α protein sandwich architecture and an active serine in the lipase consensus motif GXSXG (2).

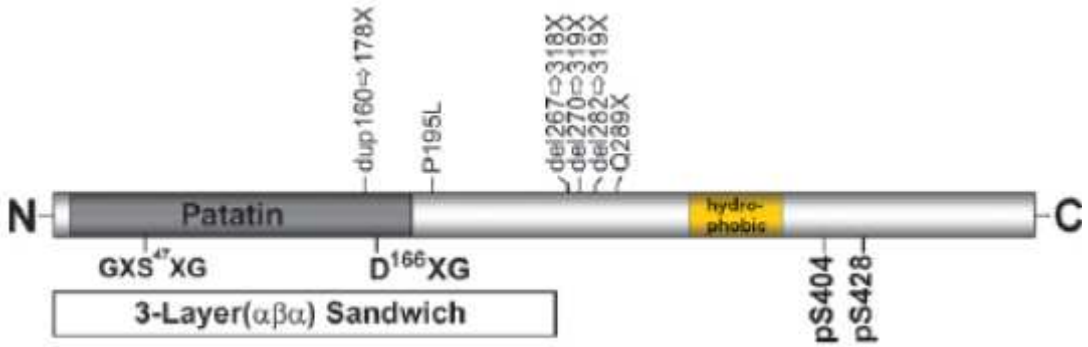


Figure 1: Representation of the structural domain of the human Adipose Triglyceride Lipase protein. Important components for the enzyme activity have been highlighted which include patatin domain, α/β hydrolase fold, active site serine (S47), putative aspartic acid (D166) in the catalytic dyad, probable lipid binding domain (hydrophobic) and two confirmed phosphorylation sites (S404, S428). Mutations leading to neutral lipid storage disease have also been indicated (2).

Sequence homology searches showed that lipases containing a patatin domain are also found in *S. cerevisiae,* i.e. Tgl3p and Tgl4p, further underscoring the conservation and importance of these enzymes (3). Structurally ATGL and Tgl3p belong to a large group of α/β hydrolases containing a patatin domain. A phylogenetic tree to determine the evolutionary relationship

between ATGL and Tgl3p shows that in early eukaryotic evolution a duplication event gave rise to ATGL and Tgl3p families (3).

CGI-58 belongs to the esterase/thioesterase/lipase subfamily of proteins structurally characterised by the presence of α/β hydrolase folds. In the esterase/lipase motif GXSXG the serine is replaced by asparagine, CGI-58 binds to intracellular lipid droplets by interacting with perillin and enhances the lipolytic activity of the ATGL (4).

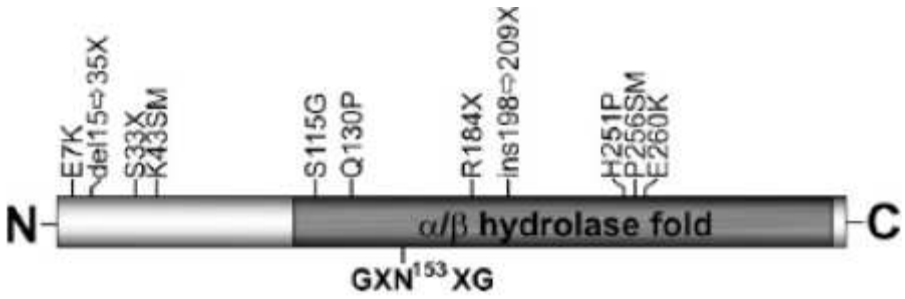


Figure 2: Representation of the structural domains of CGI-58, the α/β hydrolase region and the replacement of serine by aspargine within the esterase/lipase motif GXSXG. Two mutations Q130P, E260K within CGI-58 leading to neutral lipid storage disease with ichthyosis are shown(2).

Biochemical characterization and structural/functional studies require sufficient quantities of native ATGL which is a bottle neck, because large scale expression and purification of this enzyme from tissue cultures is not feasible. Prokaryotic expression platforms, e.g. *E. coli*, yielded largely inactive ATGL protein, presumably due to inclusion body formation (Achim Lass, personal communication). We selected the methylotrophic yeast *Pichia pastoris* as a most promising platform for heterologous expression of mouse ATGL because of its capacity to grow to high cell densities and its strong, inducible *AOX1* promoter. The origin, function and its amino acid composition clearly shows that this enzyme is highly hydrophobic and, most likely, is stabilized by disulfide bonds.

The molecular weight of ATGL is 53.7 kDa; making it relatively big for expression in the secretory mode. Recombinant protein may be blocked within the periplasmic space instead of being secreting from the cell (5). Moreover, native ATGL is cytoplasmic in mammalian cells. In *Pichia pastoris*, recombinant ATGL was directed towards cytoplasmic expression. Functional expression of this lipase is a challenging task since it contains multiple cysteins

and structural information indicates that stability of ATGL depends on the formation of the proper disulfide bonds (6). Disulfide bond formation requires a relatively oxidising environment present in the ER lumen whereas cytoplasmic environment is relatively reducing (7, 8).

Both murine ATGL full length and truncated versions (N- terminal 283 amino acid), were designed for expression in *P. pastoris*. Truncated ATGL shows high levels of lipase activity when compared to its full length counter part (9). It has been argued that the truncated version will overcome damage caused to the protein by proteases. To understand the expression behaviour of this lipase in *P. pastoris* and to facilitate purification of the recombinant protein, eight different constructs of ATGL were designed including both untagged and tagged versions. They are:

1) Full-length ATGL without tag
2) Full-length ATGL with N- terminal GST tag
3) Full-length ATGL with C- terminal His-tag
4) Full-length ATGL with both N- terminal GST and C-terminal His-tags
5) Truncated ATGL (N- terminal 283 amino acids)
6) Truncated ATGL with N- terminal GST tag
7) Truncated ATGL with C- terminal His-tag
8) Truncated ATGL with both N- terminal GST and C-terminal His-tags

Constructs 4 and 8 were double tagged. Presence of two tags should help in performing protein purification employing the two tags in a sequential manner and, thus, avoiding the presence of any incomplete proteins ending up finally in the pure protein with the expected size.

Materials and methods:

Site specific mutagenesis of ATGL:
Nucleotide sequence of the murine ATGL is available in NCBI as nucleotide entry AK031609. ATGL expression plasmid specific for mammalian cell lines (pcDNA4/HisMax C) was used as template for PCR (6). ATGL coding sequence sequence contains a recognition site for *Eco*RI which was required for cloning into the plasmid pHIL-D2. A site specific

mutagenesis reaction was performed to eliminate this restriction site. Mutagenesis reactions were performed using the single primers separately in a total volume of 50 µl with 2.5 U of *PfuUltra*™ high-fidelity DNA polymerase (Stratgene, Texas, USA), 1X PfuUltra buffer, 100 ng of template, 0.3 µM of single primer and 0.2 mM of dNTPs. After 4 cycles, 25 µl of each reaction were intermixed and remaining 21 cycles were completed. PCR conditions used for mutagenesis were 1 min at 95°C, followed by 25 cycles of (50 s 95°C, 50 s 60°C, 22 min 70°C) and 7 min at 70°C.

Table 1: Primers used for site specific mutagenesis

S.no	Primer Sequence	Primer name
1	CAGCCTTATAGAAAAGATC***GCATTC***TAGAGCACCTGCCTGCC	A to C FP
2	GGCAGGCAGGTGCTCTA***GAATGC***GATCTTTTCTATAAGGCTG	T to G RP

Primers are given in the 5' to 3' orientation with the original *Eco*RI site in bold and italicized. The point mutation to be introduced has been underlined.

Mutagenesis reaction mixture was digested with *Dpn*I restriction enzyme to get rid of the template. In a total volume of 30 µl, restriction reactions were done taking 25 µl of the mutagenesis reactions, 20 U of *Dpn*I restriction enzyme (Fermentas, Burlington, Canada) and, 1X tango (Yellow) buffer. Samples were incubated for 2 h at 37°C. After restriction was completed samples were desalted and transformed into electro-competent *E. coli* TOP10 cells following standard protocols (10). After regeneration 200 µl portions of the LB broth with transformants were plated onto LB ampicillin plates and incubated o/n. Randomly a few transformants were selected for plasmid isolation. Purified plasmids were sent for sequencing (AGOWA genomics, Germany). Plasmid pcDNA4/HisMax C ATGL *Eco*RI$^-$ was served for further work

PCR synthesis of ATGL coding sequences:

PCR synthesis of eight different ATGL coding sequences full length or truncated and (un-) tagged, were done using Phusion™ DNA polymerase (Finnzymes, Espoo, Finland) and plasmid pcDNA4/HisMax C ATGL *Eco*RI$^-$ as a template. All PCR synthesis reactions were performed according to Phusion High-Fidelity DNA Polymerase Manual in a total volume of 50 µl with 1 U Phusion DNA polymerase, 1X HF buffer, 10-20 ng of template, 0.2 mM of dNTPs and 0.4 µM of each primer.

While His-tags were introduced through the reverse PCR primers, the GST tag sequence was amplified from the pGEX6p-2 plasmid (GE Healthcare Life Sciences, Uppsala, Sweden) and fused to the ATGL coding sequences by overlap extension PCR. PCR conditions for GST amplification were 30 s at 98°C, followed by 25 cycles of (10 s 98°C, 30 s Tm°C, 30 s 72°C) and 10 min at 72°C. Tm°C refers to the primer annealing temperature, and varies from reaction to reaction. Different primers used for synthesis of eight different constructs, their sequences and primer annealing temperatures are described in the tables below.

Table 2: Different PCR synthesis reactions and primer pairs used

Tm	PCR product and its length	Primer pair	Immediate application
60°C	Full length ATGL without tag (1471 bp)	pHIL-D2 ATGL FP ATGL common RP	Restriction digestion and ligation
55°C	Truncated ATGL without tag (819 bp)	pHIL-D2 ATGL FP pHIL-D2 ATGL truncated RP	Restriction digestion and ligation
70°C	Full length ATGL with C-terminal His tag	pHIL-D2 ATGL FP His full length ATGL RP	Restriction digestion and ligation
65°C	Truncated ATGL with C-terminal His tag	pHIL-D2 ATGL FP His truncated ATGL RP	Restriction digestion and ligation
70°C	Full length ATGL with C-terminal His tag	GSTATGLFP His full length ATGL RP	For overlap extension
65°C	Truncated ATGL with C-terminal His tag	GSTATGLFP His truncated ATGL RP	For overlap extension
65°C	Full length ATGL with N-terminal GST tag	*EcoR*I GST FP ATGL common RP	Restriction digestion and ligation and for overlap extension
55°C	Truncated ATGL with N-terminal GST tag	*EcoR*I GST FP pHIL-D2 ATGL truncated RP	Restriction digestion and ligation and for overlap extension
65°C	Full length ATGL with both N-terminal GST and C-terminal His	*EcoR*I GST FP His full length ATGL RP	Restriction digestion and ligation
55°C	Truncated ATGL with both N-terminal GST and C-terminal His	*EcoR*I GST FP His truncated ATGL RP	Restriction digestion and ligation
65°C	GST tag	*EcoR*I GST FP GST ATGL RP	For overlap extension reaction generating GST tagged ATGL

Tm = primer annealing temperature, Primers used for the overlap extension PCR have been highlighted in red colour, Primers used for the synthesis of GST tag have been highlighted in turquoise colour.

After the PCR reactions, aliquots were analyzed by agarose gel electrophoresis.

Table 3: Primer sequences

Primer name	Primer sequence
pHIL-D2 ATGL FP	ACGAGGAATTCATGTTCCCGAGGGAGACCAAGTGG
ATGL common RP	GGCGAATTCTCAGCAAGGCGGGAGGCCAGG
pHIL-D2 ATGL truncated RP	GCGAATTCTCACTCTTCCTGGGGGACAACTGG
His full length ATGL RP	AAGGCGAATTCTCAATGATGATGATGATGATGCTCGACGGCGCT ATTGCAAGGCGGGAGGCCAGG
His truncated ATGL RP	AAGGCGAATTCTCAATGATGATGATGATGATGCTCGACGGCGCTATT GCAAGGCGGGAGGCCAGG
GSTATGLFP	ATGTTCCCGAGGGAGACCAAGTGGAACATCTCAT
*EcoR*I GST FP	ACGAGGAATTCATGTCCCCTATACTAGGTTATTGG
GST ATGL RP	TCCACTTGGTCTCCCTCGGGAACATGGGCCCCTGGAACAGAACTTC

Primers used in the synthesis of different ATGL inserts are given in 5' to 3' orientation with *EcoR*I site in red colour, stop codon in blue colour. Sequences initially annealing to ATGL template are in green colour, Linker region connecting His tag and ATGL sequence is in pink colur, His tag is in brown colur and sequences initially annealing to GST template are in turquoise colour.

Cloning of ATGL inserts:

pHIL-D2 plasmid and ATGL PCR products to be cloned were digested with FastDigest *EcoR*I (Fermentas, Burlington, Canada) restriction enzyme as recommended by the supplier (Table 4).

Table 4: Restriction reactions with *EcoRI*

S.no	Contents	pHIL-D2	PCR products
1	DNA concentration	2 µl (1 µg)	10 µl (0.2 µg)
2	Fast digest enzyme	1 µl	1 µl
3	10 x fast buffer	2 µl	2 µl
4	Water	15 µl	17 µl
Total volume		20 µl	30 µl

After restriction inserts were purified over PCR purification columns to get rid of the buffer and enzyme. Linearized plasmid samples were purified by gel extraction kit (QIAGEN, Hilden, Germany). Dephosphorylation of the linearized plasmid DNA was performed using calf intestinal alkaline phosphatase (Roche, Basel, Switzerland) using 1 U of the enzyme per µg of the plasmid DNA. After 2 h of incubation at 37°C, alkaline phosphatase was inactivated by heating the sample to 80°C for 10 min.

Ligation:

Concentrations of purified vector backbones and inserts were determined by agarose gel electrophoresis. T4 DNA Ligase (Promega, Madison, USA) was used in ligation reactions of 1:2 molar ratio of vecotor: insert and a total DNA concentration of 200 ng. Samples were incubated over night at 16°C.

Table 5: Ligation reactions

S.no	Contents	Volume in µl
1	Plasmid backbone	10 (174 ng)
2	ATGL inserts	6 (26 ng)
3	10x Ligase buffer	2
4	T4 DNA ligase	2

Ligation reactions were desalted and transformed into electro-competent *E. coli* TOP10 cells (10). Regeneration was done in LB broth for 1 h at 37°C and moderate shaking at 600 rpm. 200 µl portions of the LB broth with transformants were spread on LB ampicillin plates and incubated o/n at 37°C. Eight randomly selected transformants per construct were restreaked for plasmid isolation and sequenced to confirm correct orientation of the insert (AGOWA genomics, Germany).

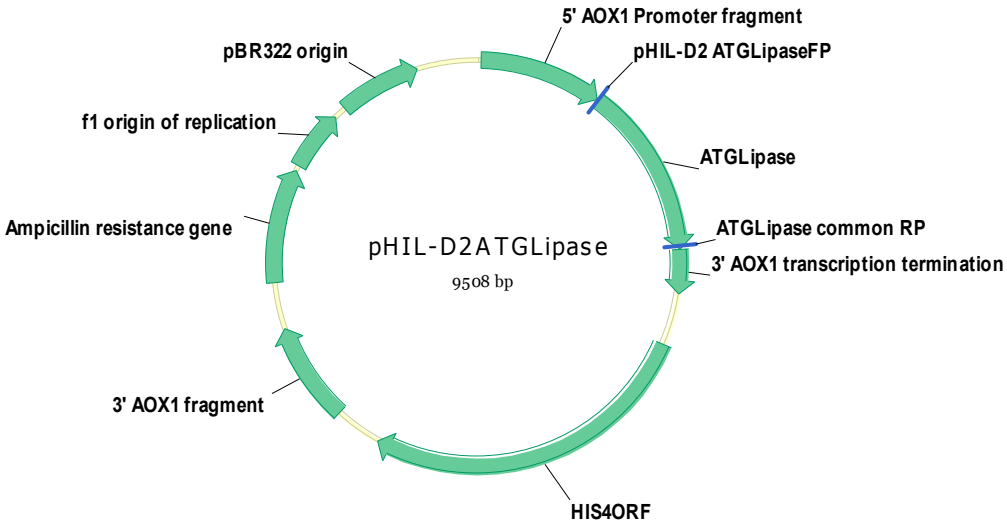


Figure 3: Schematic representation of one of the recombinant expression plasmids for ATGL in yeast.

Transformation of the *P. pastoris* GS115 cells:

Plasmid DNA required for yeast transformation was isolated in large scale i.e., 4 µg of the plasmid DNA. Before transformation into yeast plasmid DNA was linearized with *Sal*I in the

HIS4 region of the plasmids. Ethanol precipitation of linearized DNA was performed to pool the DNA in a small volume of 10 µl as required for transformation into *P.pastoris*.

Electrocompetent cells and transformation into yeast cells were performed in a similar manner as explained in chapter 1. Yeast transformants were regenerated in YPS for 1 h at 30°C. Aliquots of 200 µl of regeneration medium were plated onto selective regeneration dextrose agar plates (refer to appendix for the composition of regeneration dextrose agar plates).

Screening for ATGL expression:
High-throughput screening of ATGL expressing clones by a simple colorimetric assay was not possible. Thus a screening system for ATGL expression was set up based on Western blots employing an antibody against ATGL (kindly provided by Achim Lass). A few transformants (n = 48) were randomly selected for fermentation in deep well plates. Transformants were inoculated and grown for 48 h in 300 µl 4/5 concentrated BYPD medium to stationary phase. Induction of ATGL expression was carried out for 48 h under control of the *AOXI* promoter. Primary induction of the culture was done by adding 300 µl of buffered minimal methanol medium (1.5% methanol) and incubation for 8 h. Then for the night interval, yeast cultures were induced with 1.5% v/v absolute methanol. Methanol induction was carried on for 48 h in total.

Whole cell lysates of *Pichia* were obtained by centrifuging 100 µl aliquots of the cell suspensions from deep well plates and heating cell pellet to 95°C for 10 min in 50 µl of SDS-loading buffer supplemented with 10 mM DTT. Western blotting was performed as described in chapter 1.

Shake flask fermentation for ATGL expression:
Selected yeast clones expressing the highest levels of lipase were grown in shake flasks for quantifying expression levels and isolating the enzyme for activity assays. Growth and induction of ATGL expression was performed following the same line and media compositions as used for deepwell fermentation during the screening round. However 125 ml of the 4/5 BYPD were employed. After the induction phase of 48 h, harvesting of the cells was carried out by centrifuging the cells for 10 min at 4°C and 3,500 rpm in a JA 10 rotor (Beckman coulter, California, USA). Cells were washed with sterile ice cold distilled water once.

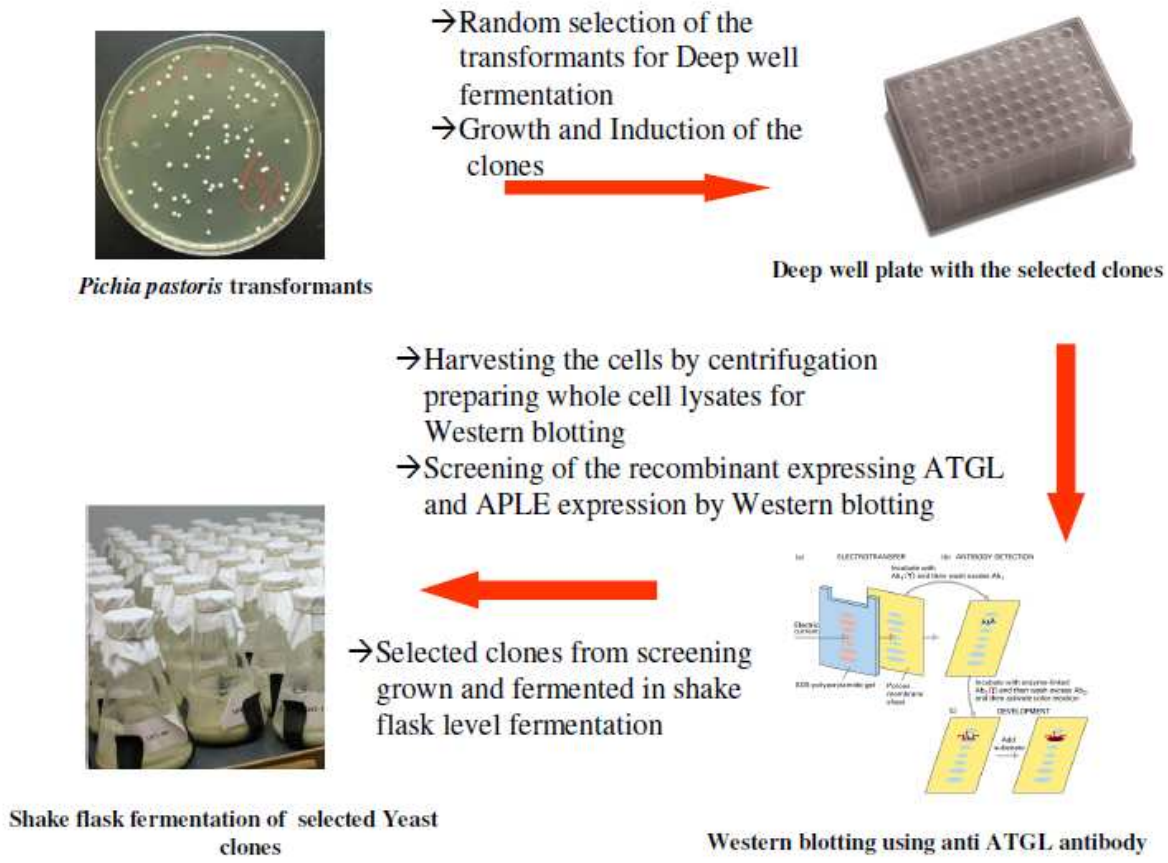


Figure 4: Screening system for ATGL expression clones based on Western blotting.

Colony PCR:

A small colony from a fresh culture was taken with a sterile toothpick and dispersed uniformly in 50 µl of water in an eppendorf tube. A small amount of glass beads was added and samples were heated at 95°C for 10 min followed by chilling on ice for 10 min. To complete cell lyses for releasing genomic DNA, small scale glass bead lyses of the cell suspension was performed for 5 min using the cell lyser (Retsch GmbH, Haan, Germany). Upon lysis, samples were centrifuged for 10 min at 4°C and maximum speed in a tabletop centrifuge. Different volumes of supernatant were taken as template for PCR reactions using standard 5' *AOXI* and 3' *AOXI* sequencing primers. PCR amplification was been performed in a total volume of 50 µl with 1 U of Phusion DNA polymerase, 1X HF buffer, 0.2 mM of dNTPs, and 0.4 µM of each primer. PCR conditions were 30 s at 98°C, followed by 25 cycles of (10 s 98°C, 30 s 50°C, 30 s 72°C) and 10 min at 72°C. PCR products were analysed by agarose gel electrophoresis.

Cell lysis:

Cell lysis was been carried out by two different modes, namely (1) spheroplasting of the yeast cells and sonication of the spheroplasts or (2) Glass bead lysis using the Merckenschlager system. For spheroplasting harvested cells were resuspended in 20 ml 1 M sorbitol solution and the resulting cell suspension was distributed equally into three falcon tubes of 50 ml volume each. These resuspended cells were centrifuged once again at 4°C for 10 min at 2465 x g. Supernatants were decanted and cells were resuspended in spheroplasting solution. Spheroplasting was carried out by incubating the samples for 1 h at 37°C and 177 rpm. Spheroplasts were washed twice, primarily with 1 M sorbitol and secondarily with PBS buffer. Washed spheroplasts were resuspended in 30 ml of PBS^{++} buffer and sonication was done at 5% output, 50% duty cycle for 5 minutes at 4°C. For glass bead lysis by Merckenschlager, cells were resuspended in ~ 20 ml of PBS^{++} buffer. Cell suspensions were transferred into prechilled bottles filled with glass beads. Cell disruption was performed in the Merckenschlager (B.Braun Biotech International GmbH, Melsungen, Germany) shaker for 15 min with intermittent cooling by CO_2 for every 10-15 s. Glass beads, intact cells and yeast cell debris were separated from cell extract by a low speed centrifugation at 3000 x g for 10 min at 4°C. Supernatants with the soluble proteins were either centrifuged by ultra centrifugation for 1 h at 100,000 x g at 4°C or centrifugation at maximum speed JA 25.50 (Beckman coulter, California, USA) 75,600 x g for 1 h at 10°C. The membrane fractions were resuspended in 5 ml of the PBS^{++} buffer. Protein concentrations of the membrane and cytosolic fractions were estimated by the Bradford method (Bio-Rad, Munich, Germany).

Western blotting for recombinant ATGL:

Expression of ATGL was confirmed by Western blotting. 80 µg of total cytosolic protein and 1 µl of the membrane fractions were heated at 95°C in SDS-loading buffer supplemented with 10 mM DTT before loading onto the gel. Western blotting was performed as described in the chapter 1.

ATGL activity assay:

Triglyceride lipase activity of ATGL was assessed with commercially available triolein. 60 µl of triolein (100mg/ml in toluene; Sigma Aldrich), 30 µl of Phosphatidylcholine/phosphatidylinositol mixture (3:1; 20 mg/ml in chloroform, sigma Aldrich) and 50 µl of ^{3}H-triolein (glycerol-tri-[9, 10-^{3}H (N) oleate] in heptane, 1 µCi/µl, Amersham Biosciences) were mixed and brought to dryness with N_2. 2 µl of 0.1 M potassium

phosphate pH 7, was added and sonication was performed with a Virtis Virsonic 475 at 750 W and 20% output three times for 30 s there by generating micelles. One ml of 0.1 M potassium-phosphate, pH 7, was added and once again sonication was performed as described above for 30 s two times. In order to stabilise the micelles 1 ml of 20% fat free BSA dissolved in 0.1 M potassium phosphate pH 7.0 was added. The substrate was kept on ice until use (11).100 µl of the cytosolic or membrane fractions were added to100 µl of substrate mixture in 15 ml falcon tubes and samples were incubated for 1 h at 37°C in a shaking water bath at 60 rpm. Reactions were terminated by adding 3.25 ml of stop solution, i.e. 1 ml 0.1 M potassium carbonate pH 10.5 saturated with boric acid for adjusting pH. Falcon tubes were vortexed for 30 s and the samples were spun at 2061 x g for 10 min at 4°C. One ml aliquots of the aqueous supernatants were mixed with 8 ml of Ultima Gold scintillation cocktail (Perkin Elmer, USA) and subjected to scintillation counting.

GST-purification of recombinant ATGL:

One ml of a fresh portion of the 50% glutathione sepharose slurry (GE Healthcare Life Sciences, Uppsala, Sweden) was transferred into a sterile 2 ml eppendorf tube. To get rid of the supernatant, resin was spun at 500 x g for 2 min. Supernatant was removed carefully by pipetting without loosing resin. Thereafter the resin was washed three times with 1 ml of cold lysis buffer each, making the resin ready for binding of tagged protein. Cytosolic fractions were transferred along with the washed resin into a 50 ml falcon tube and kept overnight at 4°C with gentle shaking for binding GST-tagged ATGL. Unbound material was separated from the resin by centrifugation and preserved. Resin along with the bound protein was washed 5 times with cold lysis buffer. ATGL was eluted from the resin upon transferring the resin into a 2 ml eppendorf tube. Twice the resin volume of elution buffer was added and samples were kept overnight at 4°C with gentle shaking. Then the supernatant containing GST-ATGL was carefully transferred into a new eppendorf tube and stored at -80°C for further use.

Results:

Site specific mutagenesis of ATGL:

ATGL expression plasmid PcDNA4/HisMax C specific for mammalian cell lines was modified by site specific mutagenesis to remove an intrinsic *EcoR*I site. The first A within the GAATTC sequence was replaced by a C with out changing the amino acid sequence of

ATGL. After introducing the point mutation by PCR template plasmid was degraded by *Dpn*I and the mutated plasmid was transformed into the *E. coli*TOP10. Randomly, seven transformants were selected for plasmid isolation and sequencing. Plasmids that resisted restriction by *EcoR*I were sent for sequencing to confirm the presence of the desired mutation A → C (Fig 5). This plasmid template was named pcDNA4/HisMax C ATGL *Eco*RI$^-$ and it was used for further work.

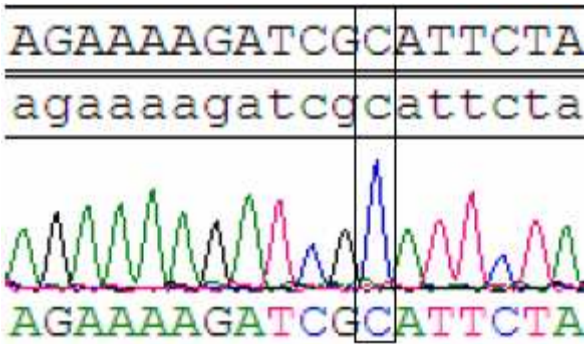


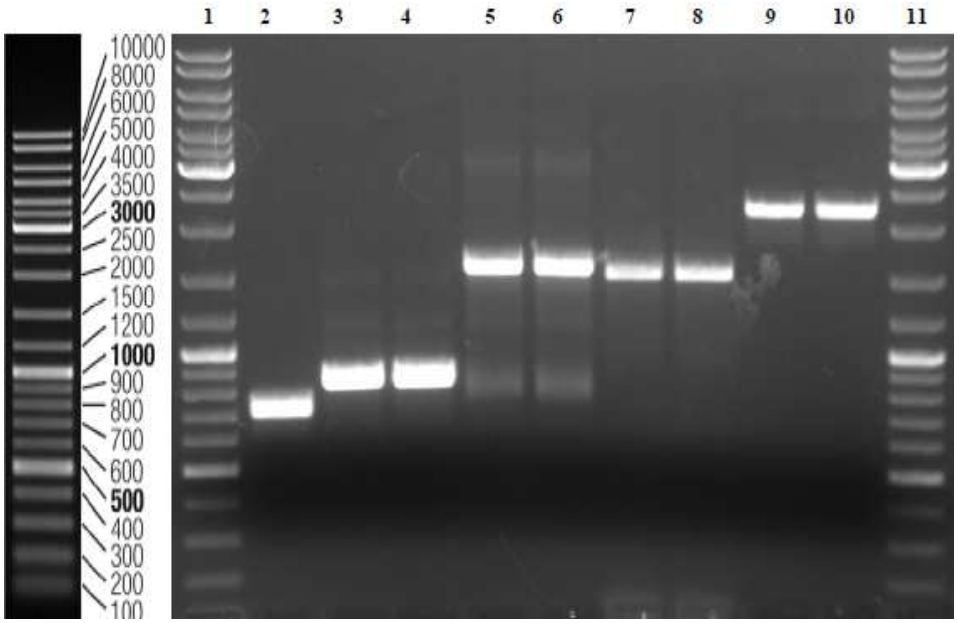
Figure 5: Results from DNA sequencing confirmed nucleotide exchange removing *EcoR*I site. 891 (GAATTC → GCATTC) nucleotide position in ATGL coding sequence

GST- and His- tagging of ATGL:

Plasmid pGEX-6P-2 was used as template for amplifying the coding sequence of the GST tag. Analysis of the PCR products by agarose gel electrophoresis showed DNA fragments around 684 bp coinciding with the GST sequence (Fig 6B).The amplified GST tag cassette was used later for overlap extension PCR with full length and truncated ATGL sequences for N-terminal GST tagging.

Plasmid pcDNA4/HisMax C ATGL *Eco*RI$^-$ was taken as a template for amplifying the different ATGL sequences. While the GST tag was introduced by overlap extension PCR, the His tag was inserted by the reverse primer used in PCR. Analysis of the PCR products by agarose gel electrophoresis showed DNA fragments in their respective sizes (Fig 6B).

Eight different PCR products and pHIL-D2 plasmid were digested with *EcoR*I. Inserts and plasmids were purified. Linearized plasmids were dephosporylated and the concentrations of vector and inserts was estimated by gel electrophoresis. Ligation reactions were set with T4 DNA ligase and samples have been incubated over night at 16°C. Ligation reactions were desalted and transformed into electro-competent *E. coli* TOP10 cells.



(A) (B)

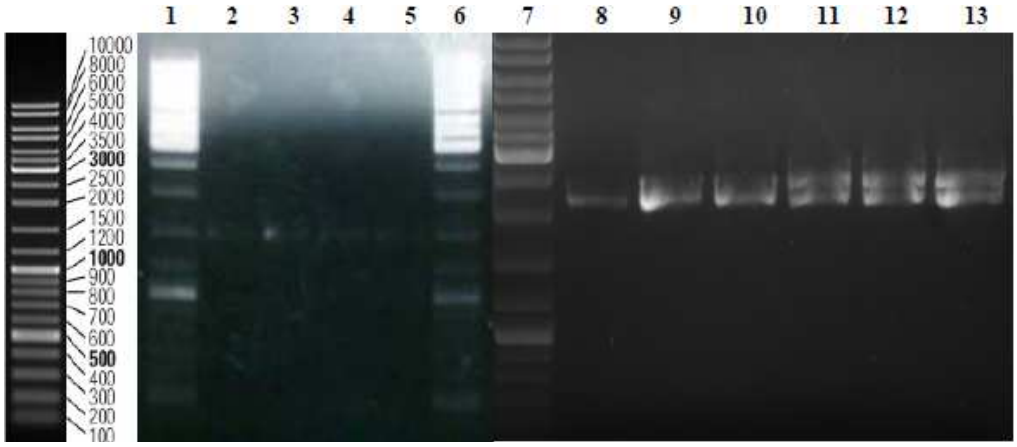
Figure 6: DNA gel photograph of the different PCR products used for constructing recombinant plasmids. (A) Gene Ruler DNA Ladder mix; (B) DNA Ladder mix (1,11), GST tag (684 bp) (2), truncated ATGL (819 bp) (3), truncated ATGL His (852 bp) (4), truncated GST ATGL (1504 bp) (5), truncated GST ATGL His (1537 bp) (6), full length ATGL (1471 bp) (7), full length ATGL His (1494 bp) (8), full length GST ATGL (2156 bp) (9), full length GST ATGL His (2179 bp) (10).

Eight transformants per construct were selected for plasmid preparation. Plasmids isolated and sequenced. Sequencing results confirmed correct integration of the (un-) tagged ATGL inserts. ATGL expression plasmids which had been found correct from the sequencing were linearized by *Sal*I and transformed into yeast cells expecting the recombination event to take place in the *HIS4* locus of *P. pastoris*. Transformants were selected for His^+ prototrophy on selective regeneration dextrose agar plates.

Screening for ATGL expression:

Screening for ATGL expression was based on Western blotting. Therefore, a few transformants were randomly selected for deep well plate fermentation. Whole cell lysates were made from deep well cultivated cells and load onto SDS gels for Western blotting. After development of the blot, yeast cells giving rise to specific bands coinciding with ATGL were selected for shake flask fermentation. Presence of the ATGL expression constructs in

yeast strains was confirmed by colony PCR. Analysis of the PCR products by agarose gel electrophoresis showed bands at 1500 bp for the full length ATGL clone, and 2280 bp for the full length GST-ATGL clone (Fig 7).



(A) (B) (C)

Figure 7 (A): Gene ruler DNA ladder mix, (B): PCR on the recombinant plasmid (Positive control) (2), PCR results using colony PCR of the full length ATGL clone (1500 bp) (3, 4, 5), (C): PCR on the recombinant plasmid (Positive control) (8), PCR result using colony PCR of the full length GST ATGL clone (2280 bp) (9, 10, 11, 12, 13).

ATGL activity of recombinant protein from yeast:

Triglyceride lipase activity of the recombinant ATGL was assessed with radioactively labelled triolein. Recombinant ATGL cleave the triglyceride into diglyceride and free fatty acids. The amount of radioactivity in free fatty acids released during the assay was detected by scintillation counting. The biological activity of the enzyme was expressed in nmol of free fatty acids released per h to the mg of the protein. ATGL expressed in yeast showed significant triglyceride lipase activity even in comparison to mammalian ATGL. Basal activities of ATGL, and increased lipase activities in the presence of its agonist CGI-58, were documented for several ATGL expression strains in duplicate.

GST- purification of tagged ATGL was monitored by Western blotting using anti-ATGL antibody (Figure 11). To further confirm the efficiency of the protocol, 10 µl of the cytosolic fractions, flow-through, five wash fractions and eluate were loaded. Upon developing the blot unbound GST- ATGL was observed both in the flow-through and wash fractions. Eluate showed partially purified ATGL.

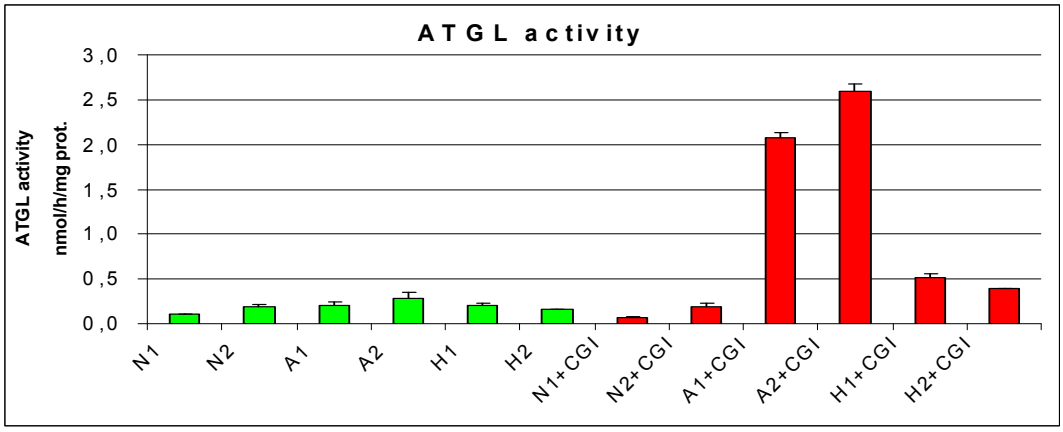
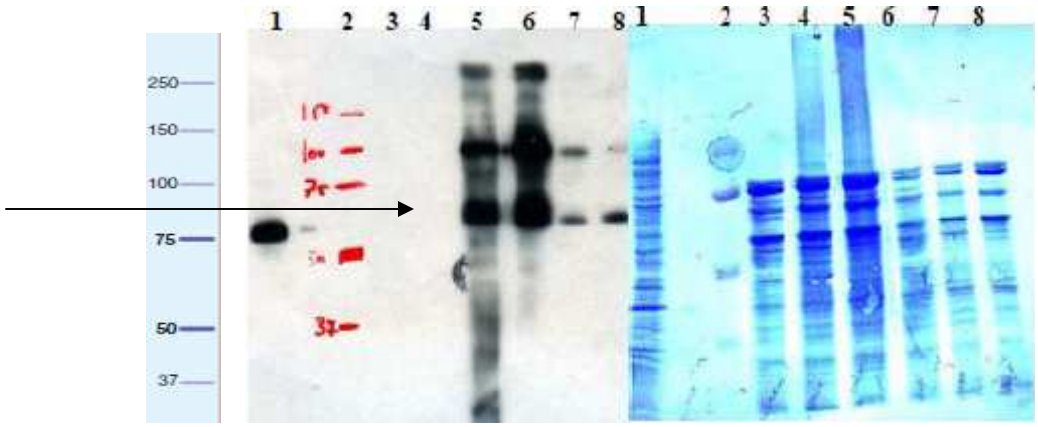


Figure 8: ATGL activity assay from full-length ATGL without tag. ATGL activity assays on Negative control GS115 (N1, N2), Active clone (A1, A2), Clone with low activity (H1, H2) were done in duplicate.



(A) (B) (C)

Figure 10 Precision plus protein standard from BIORAD (A) ATGL expression documented by Western blotting (B) and CB staining (C). ATGL His tagged (Positive control from mammalian cells) (1), Precision plus protein standard from BIORAD (2), Cytosolic fractions, wild type GS115 strain (Negative control) (3,4), Cytosolic fractions, high level ATGL (full-length with out any tag) expressing strain (5,6), Cytosolic fractions, low level ATGL (full-length with out any tag) expressing strain (7,8). Arrow indicates ATGL (53.7 kDa).

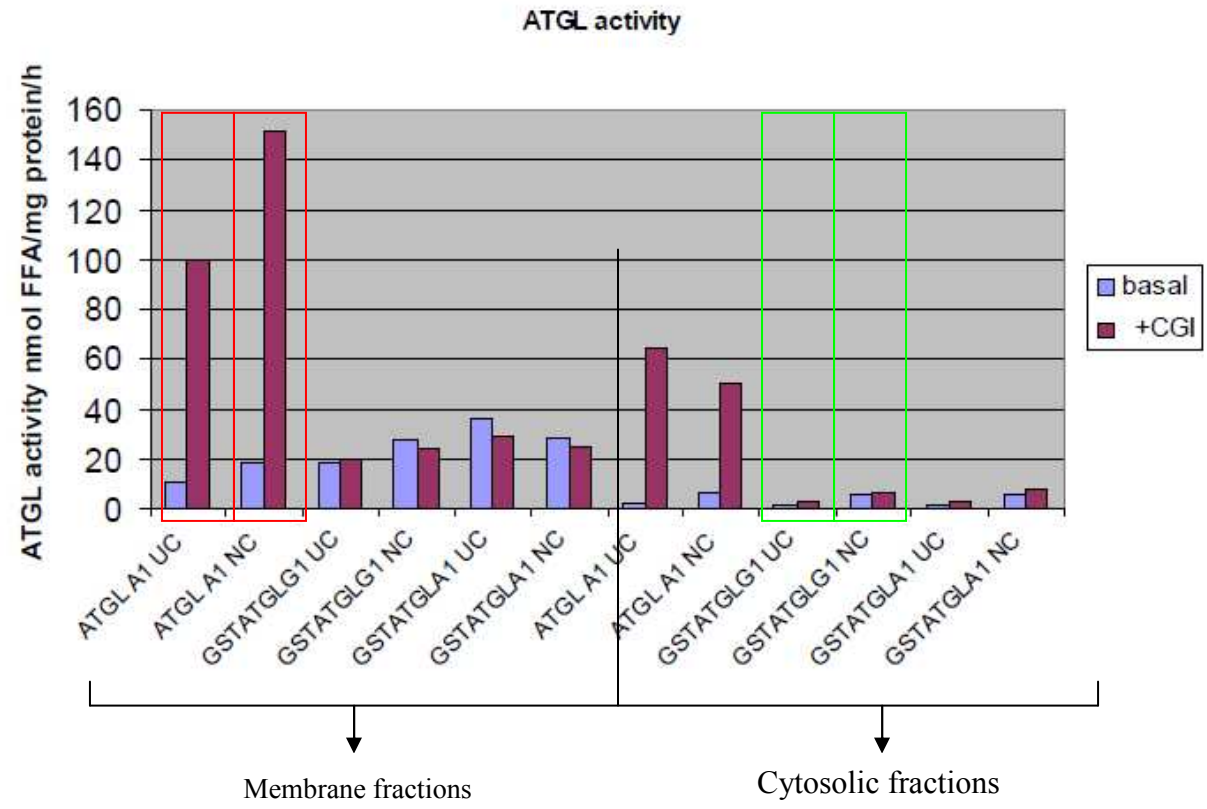
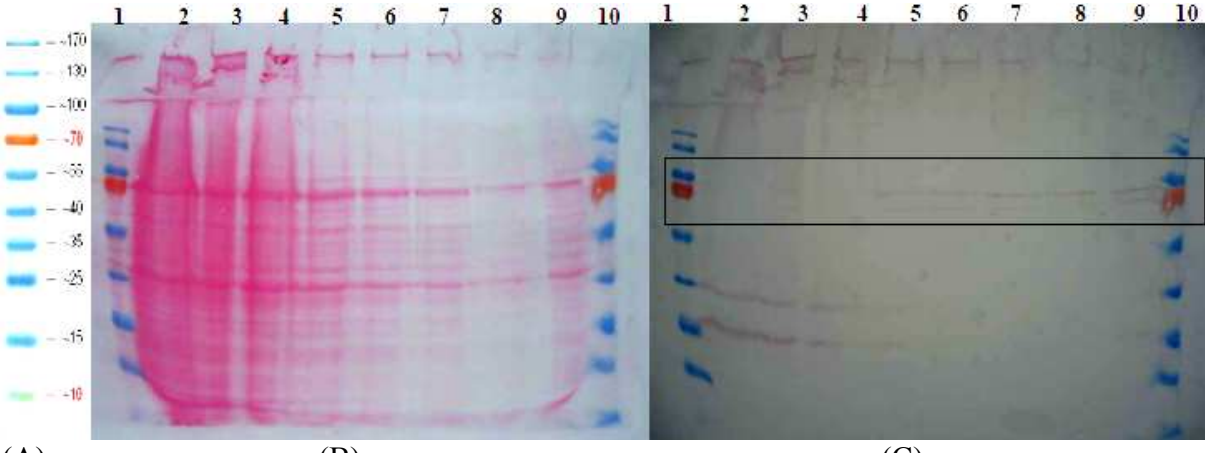


Figure 9: ATGL activity of full length ATGL (untagged version highlighted in red) and Full length GST-ATGL (tagged version highlighted in green) proteins. Recombinant ATGL showed lipase activity in the absence (basal activity) and presence of the CGI-58 protein. Samples were prepared by ultracentrifugation (UC) or high (= normal) speed centrifugation.



(A) (B) (C)

Figure 11 (A): Prestained protein ladder from FERMENTAS, (B): Ponceau S image of the blot, (C): Finally developed image of the Western blot. Prestained protein ladder (1, 10), Cytosolic fraction of GST-ATGL (full length) (2), Flow through fraction during protein purification (3), Wash fractions during protein purification (4, 5, 6, 7, 8), partially purified full length GST-ATGL at 80.2 kDa (9).

Discussion:

Adipocytes act as a store house for triglycerides. Over-accumulation of triglycerides within adipocyte is linked to obesity. Until 2004 HSL was ascribed a central role in the catabolism of triglycerides (2,12). Therefore HSL knock-out mice should have been obese. On the contrary, knock out mice were lean and healthy and accumulated diglycerides instead of triglycerides (2,12).This clearly suggested the presence of an undiscovered triglyceride lipase and revised the position of HSL in lipid metabolism as a potential diglyceride lipase. The search for this triglyceride lipase ended with the discovery of ATGL. To further demonstrate the function of ATGL, knock-out mice were created. These showed the expected phenotypes accumulating lipids in multiple tissues, particularly the heart, and suffered from cardiac failure. Amino acid composition of ATGL indicates that it is a member of patatin domain containing protein family. The patatin domain is unique as it contains an $\alpha/\beta/\alpha$ protein sandwich architecture with an active serine in the lipase consensus motif GXSXG (2).

CGI-58 is an activator of ATGL enhancing lipolytic activity (4). In vitro studies showed that CGI-58 itself does not have lipase/esterase activity. CGI-58 belongs to esterase/thioesterase/lipase subfamily of proteins structurally characterised by the presence of an α/β hydrolase folds. In the esterase/lipase motif GXSXG of CGI-58 serine is replaced by asparagine. In order to determine the importance of CGI-58 in lipid metabolism an attempt to create a knock-out for this gene was undertaken. Knock-out mice showed Chanarian Dorfman Syndrome (4). Affected individuals cannot break down triglycerides, and this fat accumulates in organs and tissues, including skin, liver, muscles, intestine, eyes, and ears. People with this phenotype also have dry, scaly skin (ichthyosis), which is usually present by birth. Additional features of this condition include an enlarged liver (hepatomegaly), clouding of the lens of the eyes (cataracts), difficulty with coordinating movements (ataxia), hearing loss, short stature, muscle weakness (myopathy), involuntary movement of the eyes (nystagmus), and mild intellectual disability (4).

Biochemical characterisation and performing biophysical studies such as protein crystallisation demands a lot of purified native ATGL. However, its isolation from tissue culture at such high levels is impossible. Recombinant expression of the lipase in *E. coli* yielded mainly inclusion bodies (Achim Lass, personal communication). *P. pastoris* could be

a promising alternative platform for large scale expression of ATGL. This methylotrophic yeast performs many post-translational modifications making it similar to higher eukaryotes.

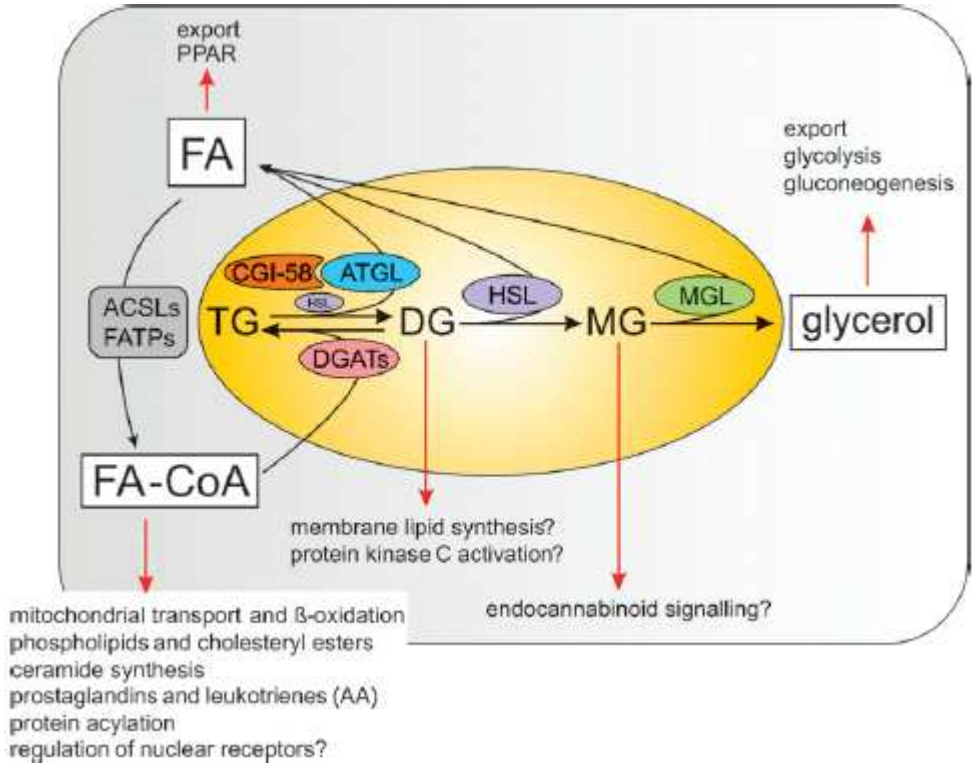


Figure 11: Revised model of the lipolytic process introducing newly discovered ATGL. Metabolic intermediates and potential utilization pathways of these intermediates are indicated (2).

ATGL is cytoplasmic in adipocytes, thus we planned for cytoplasmic protein expression in *P. pastoris*. Native amino acid composition of the ATGL made us assume that the final 3-D structure of the functional protein probably is stabilised by the formation of multiple disulfide bond. Disulfide bond formation requires relatively oxidising environment present in the ER lumen, whereas the cytoplasmic environment is relatively reducing. Activity assays on recombinant ATGL demonstrated that it is possible to express the recombinant protein in a functional manner, presumingly by forming correct disulfide bonds in the cytoplasmic environment (**7**,8).

In the endeavour to express the described lipase, eight different recombinant plasmids (un-) tagged for ATGL expression were constructed. ATGL truncated at amino acid position 283 showed increased lipase activity when compared to its full length counterpart (9). Untagged

versions of ATGL were supposed to help in understanding the expression behaviour in *P. pastoris,* whereas the tagged versions will help in purification of the expressed protein. Out of the 8 recombinant plasmids constructed, only those coding for full length ATGL with and without N-terminal GST tag have been transformed into *P. pastoris* so far and the respective transformants were characterised by Western blotting and activity assays.

Protein purification of the GST ATGL using glutathione sepharose resin showed partial purification of ATGL with significant amounts of the protein lost in the washing steps. Standardisation of the protocol has to be done to prevent loss of protein during washing steps. Yeast clones expressing high and low levels of full-length ATGL have been isolated and characterised. Activity assays showed relatively high levels of basal activity when compared to ATGL expressed in the bacterial platform (Fig 12). Secondarily, ATGL activity was significantly stimulated on interacting with CGI-58. These assays indicated that yeast derived specific ATGL activity compared well with native ATGL from *Mus musculus*.

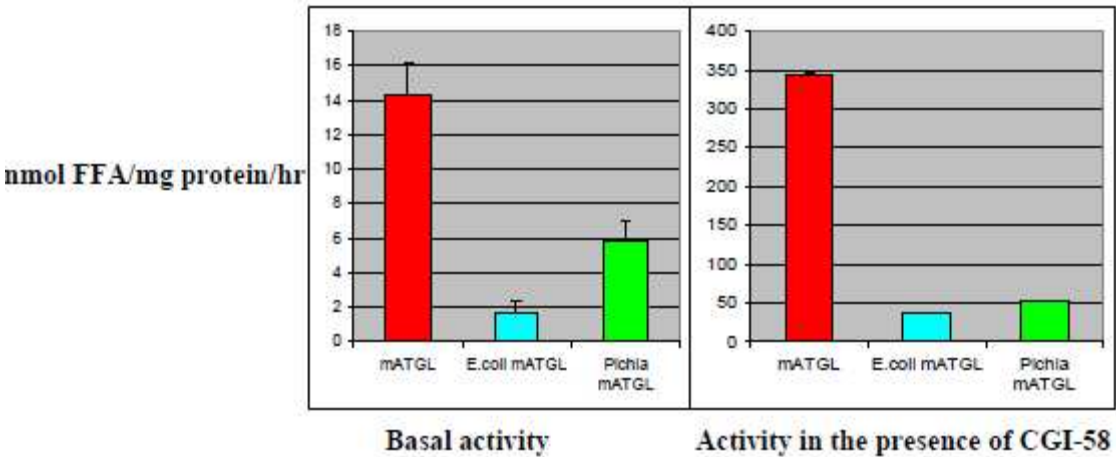


Figure 12: Comparative of lipase activity of the native ATGL from *Mus musculus* (Red colour histogram, mATGL) with the recombinant ATGL expressed in *E. coli* (Turquoise colour histogram, *E.coli* mATGL) and in *P. pastoris* (Green colour histogram, *Pichia* mATGL).

Activities derived with GST-ATGL were clearly lower than found for untagged ATGL (Fig 9).This could be due to a detrimental affect of the tag on expression level and/or specific activity of ATGL. It has been argued that GST tagging doesn't have any inhibitory affect on enzyme activity (Monika Oberer, personal communication). Primarily, it has been observed in the Western blot for monitoring GST-ATGL purification (Fig 11, lane 2), lane 2 loaded with

cytosolic fractions showed significant amounts of protein degradation products with in the range of 15 kDa and 25 kDa speculating negative affect of GST tagging on the expressed protein with in the cell. It has been shown that GST tag provides only moderate pure protein from *E. coli* extracts. Predominant protein contaminants in purified protein, at low molecular weight range originate from GST degradation, larger molecular weight contaminants likely correspond to *E. coli* proteins interacting with GST (13).

To obtain high-level expression of tagged constructs is a challenging goal which is probably not possible with the established vector design and screening systems. New constructs should be constructed ligating the ATGL coding sequence into the plasmid pPICZαA, and selecting for yeast transformants bearing multiple copies of the plasmid by screening the strains for resistance to high concentrations of zeocin (14). Western blotting based screening system doesn't allow screening large number of clones because of huge red tapism in the screening protocol. Alternatively systems for screening a plenitude of yeast transformants may be set up.

High throughput screening system towards p-nitrophenyl oleate (PNPO) is a possibility. Due to the esterase/lipase activity of the ATGL PNPO is cleaved releasing *p*-nitrophenol and oleic acid. Molar absorption spectrum coefficient for *p*-nitrophenol can be used for quantification (5,15,16). Further, more oleate esters of HPTS (Hydroxy pyrene trisulfonic acid) may be used for the same purpose. HPTS moiety creates micellar system for the lipase. Due to the ATGL activity, oleate may be released with oleic acid changing the pH of the reaction, thereby changing the fluorescent property of HPTS. Consequently change in the fluorescent property can be detected using a plate reader confirming enzyme activity present in the reaction system (17). Screening for high level expressing ATGL clones based on new constructs, application of high throughput screening system will speed up the process. Yeast clones expressing High levels of ATGL will be used for isolating recombinant protein and latter for protein purification. Purified protein collected will be applied in conducting protein crystallization studies and performing enzyme assays.

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Post Transcriptional Gene Silencing (PTGS) in *Pichia pastoris* by Antisense RNA technology.

Abstract:

Antisense RNA technology has been applied for down regulation, e.g. knock-down, of essential genes. This approach involves expression of artificial antisense RNA which hybridizes with the target mRNA. The RNA duplex thus formed will prevent the interaction of ribosome with target mRNA inhibiting translation.

Ripening of fruit is a complex phenomenon involving multiple changes in the biochemistry, physiology and gene expression. The role of cell wall hydrolase, polygalacturonase (PG) during tomato ripening was investigated by constitutive expression of anti-PG RNA. Antisense RNA expression led to reduction in PG enzyme activity in ripe tomato between 5-50% that of normal with out affecting other ripening parameters. It has been seen that down regulation of the PG inhibits depolymerisation of pectin there by delaying softening of ripe tomatoes (1, 2, 3).

Methylotrophic yeast *Pichia pastoris* is a widely applied platform strain for protein expression. Yeast cells contain vacuoles which harbour several types of proteases with important roles in protein turnover. It has been found that proteases may leak out into yeast culture supernatant leading to proteolysis of the expressed recombinant proteins, thereby reducing total protein yield in fermentation. Carboxypeptidase Y (CPY) is one such major protease localized both in vacuoles and culture-supernatant during fermentation.

We have applied antisense RNA technology as a tool for down-regulating CPY expression without disturbing metabolic networks of the cell. *P. pastoris* proved to be an attractive experimental model for gene regulation studies.

Key words: Antisense RNA, down regulation, *Pichia pastoris*, Vacuoles, CPY

Introduction:

Gene silencing techniques are major area of research for suppressing the expression of unwanted genes in research applications; particularly in the largest genes have an essential function either during the whole life cycle of the host organism or at least during parts thereof. In post transcriptional gene silencing (PTGS) translation of mRNA is inhibited thereby silencing expression. PTGS has been first discovered in plants in 1928, but the detailed study on this phenomenon has been started only in 1990 (4). PTGS has been primarily applied in higher plants and animals when constructing gene knock-outs is not possible. Though there are many different approaches for gene silencing techniques, only two major techniques have proven important i.e. 1) Small interfering (si RNA) and 2) Antisense RNA technologies.

In si RNA technology, long double-stranded RNAs (dsRNAs), i.e. typically >200 nt can be used to silence the expression of target genes in a variety of organisms and cell types, e.g., worms, fruit flies, and plants. Upon introduction, long dsRNAs enter a cellular pathway that is commonly referred to as the RNA interference (RNAi) pathway (5). First, the dsRNAs get processed into 20-25 nucleotide (nt) siRNAs by an RNase III-like enzyme called dicer (initiation step). Then, the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process. The siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the cognate RNA (effector step) (5). Cleavage of cognate RNA takes place near the middle of the region bound by the siRNA strand. In mammalian cells, introduction of long dsRNA (>30 nt) initiates a potent antiviral response, exemplified by non-specific inhibition of protein synthesis and RNA degradation. The mammalian antiviral response can be bypassed, however, by the introduction or expression of siRNAs (Fig 1).

mRNAs are single stranded RNA molecules that are translated by the ribosome into polypeptide chains. In the presence of complementary, i.e. antisense RNA, mRNA forms duplexes similar to DNA. Formation of duplex RNA prevents ribosome assembly on target mRNA inhibiting gene expression. This complementary RNA is called antisense RNA (Fig 2). This type of gene suppression has been seen in the different organisms like fungi plants, flies, worms, and even in *E. coli*.

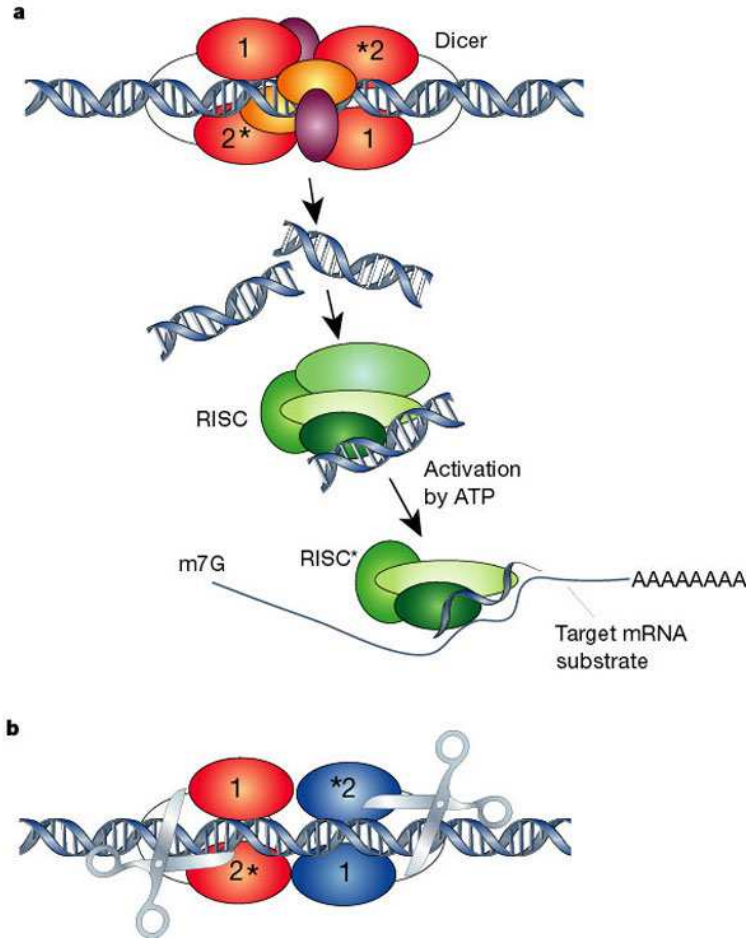


Fig 1: **a**, RNAi is initiated by the dicer enzyme (two dicer molecules with five domains each are shown), which processes double-stranded RNA into ~22-nucleotide small interfering RNAs. Based upon the known mechanisms for the RNase III family of enzymes, dicer is thought to work as a dimeric enzyme. Cleavage into precisely sized fragments is determined by the fact that one of the active sites in each dicer protein is defective (indicated by an asterisk), shifting the periodicity of cleavage from ~9–11 nucleotides for bacterial RNase III to ~22 nucleotides for dicer family members. The siRNAs are incorporated into a multicomponent nuclease, RISC (green). Recent reports suggest that RISC must be activated from a latent form, containing a double-stranded siRNA to an active form, RISC, by unwinding of siRNAs. RISC then uses the unwound siRNA as a guide to substrate selection. **b**, Diagrammatic representation of dicer binding and cleaving dsRNA (for clarity, not all the dicer domains are shown, and the two separate dicer molecules are coloured differently). Deviations from the consensus RNase III active site in the second RNase III domain inactivate the central catalytic sites, resulting in cleavage at 22-nucleotide intervals (Taken from 5).

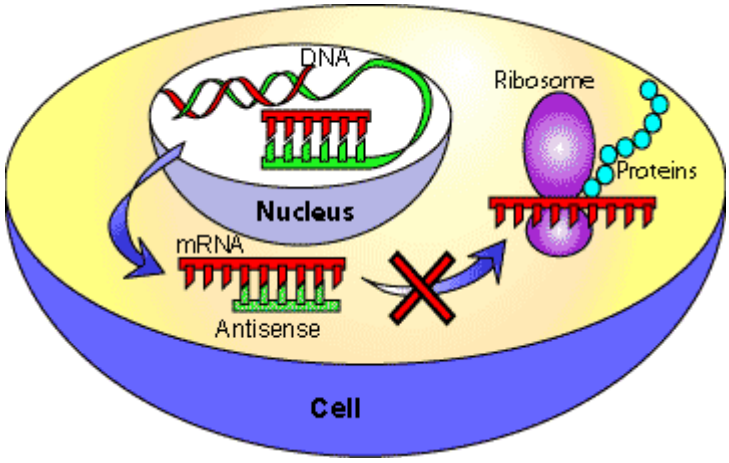
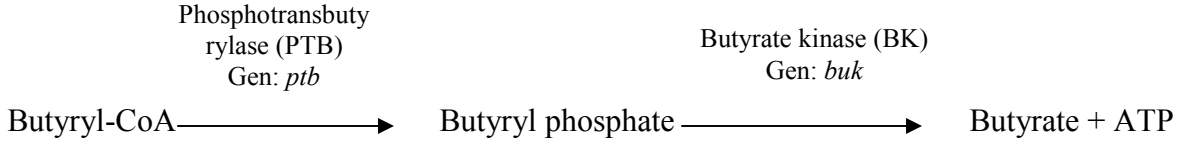


Fig 2: Antisense RNA transcribed from a sense DNA strand dimerises with the complementary mRNA preventing the binding of ribosome to mRNA and inhibiting expression (Taken from www.odec.ca).

E. coli cells undergo stress response on exposing to ethanol, heat shock and while overexpressing recombinant proteins. Hallmark of this response is rapid accumulation of σ^{32} factors. Stress response due to recombinant protein expression activates many stress proteases and chaperones. These chaperones in association with other proteins with unfoldase activity change confirmation of expressed proteins into protease susceptible forms there by degrading expressed proteins. On expressing antisense RNA against σ^{32}, levels of stress induced chaperones decreased with in bacterial cells. On expressing Organophosphorus Hydrolase (OPH) during antisense RNA production it showed six fold-higher specific activity in comparison to non-antisense producing cultures (6).

In *E. coli* mRNA degradation influences protein synthesis. RNasE initiates degradation of mRNAs in *E. coli* at the same time RNasE is essential for cell viability. Antisense RNA has been expressed for down regulating RNasE with out affecting the cell growth. Western blot analysis showed that expression of RNasE has been reduced to 35% (7).

Clostridium acetobutylicum is a gram-positive, sporulating, obligate anaerobe capable of fermenting wide varieties of sugars to acids and solvents. Antisense strategy has been applied to reduce the levels of enzymes responsible for butyrate formation as shown below



Two genes *ptb* and *buk* are arranged in single operon. Antisense RNA expressed against *buk* not only down regulates (lower by 85 - 90%) BK activity but also (lower by 45 - 50%) PTB activity when compared with control strain. Antisense RNA expressed against *ptb* gene showed reduced levels of both *ptb*, *buk* by 70 - 80% (8).

A *lacZ* expressing *Schizosaccharomyces pombe* strain was constructed and modified to express a set of antisense RNAs complementary to various regions of the target *lacZ* mRNA. β-Galactosidase activity in cells expressing long antisense RNA, short antisense RNA to the 5' region or short antisense RNA to the 3' region of the *lacZ* antisense RNAs showed reduced activity by 45%, 20% and 10% respectively (9).

Success of the antisense RNA technology appears to depend on 2 factors, i.e. 1) The length of Antisense RNA fragments and, 2) the positioning of the Antisense RNA to the functional mRNA. It has been found that dsRNA mediates 4 different modes of gene suppression comprising 1) Destruction of mRNA, 2) Inhibition of transcription, 3) Inhibition of translation, 4) Chromosomal rearrangements (10).

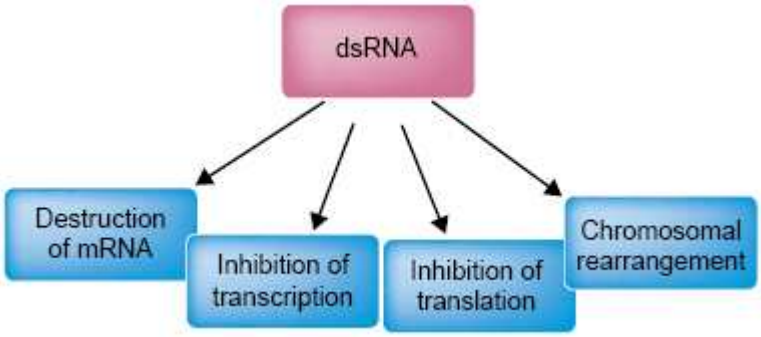


Fig 3: Different modes of gene silencing by dsRNA (10).

Pichia pastoris is a methylotrophic yeast and an excellent platform for expressing a broad range of heterologous proteins from various sources. But there are a few bottlenecks when expressing recombinant proteins. Particularly during the phase of induction with methanol i.e. when methanol utilisation (MUT) pathway is activated peptidases normally stored in vacuoles may be formed as well. During the induction phase and when cells are lysed for isolating recombinant proteins, these proteases are released leading to partial lysis of expressed recombinant proteins (11). Knock-out strains deficient in the peptidases have been developed for recombinant protein expression. However, it has been noticed that these knock-out strains lack vigour and vitality when compared with wild-type strains which is detrimental to their

application as expression hosts. These results suggested that the proteases play an important role in metabolic networks and survival of the yeast cells (12).

Therefore, we wanted to apply antisense RNA technology to downregulate the expression of one of such vacoular protease, carboxypeptidase Y (CPY) without disturbing the house-keeping metabolic activities it usually has within the cells. It was our goal to develop expression strains that would grow to high cell density with functional CPY before methanol induction but would have impaired CPY activities once recombinant protein was formed. In *P. pastoris* for the first time antisense RNA approach has been applied for downregulating a house keeping gene.

CPY is a serine carboxypeptidase encoded by *PRC1* gene in yeasts. The open reading frame of *P. pastoris PRC1* gene consists of 1569 bp encoding a protein of 523 amino acids (aa). The molecular weight of the unprocessed protein without glycosylation is calculated to be 54.4 kDa. CPY is composed of 20 aa pre (signal)-peptide, 87 aa pro-peptide and 416 aa mature peptide with 4 potential sites for N-glycosylation. Nucleotide sequence of this gene has been published in the EMBL Nucleotide Sequence Databases under the Accession Number X87987 (13). Serine proteases have a Ser-Gly-Gly motif in the active site. It is a broad-specific C-terminal exopeptidase not only involved in non-specific protein degradation in vacuole but also hydrolyses peptide esters, and also release ammonia from N-blocked C-termini (14). Monomeric structure of yeast serine carboxypeptidase from *S. cerevisiae* is available in protein data base under DOI**:**10.2210/pdb1ysc/pdb (Fig 4). Standard crystallographic *R*-factor is 0.162 observed between 20.0- and 2.8- Å resolution (14) .

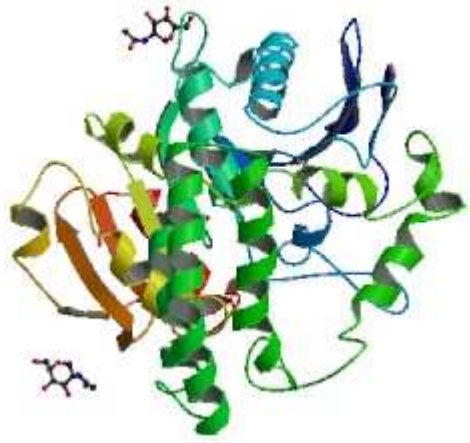


Fig 4: Structure of yeast serine carboxypeptidase (14)

PRC1 genes of several yeasts are highly homologous. *S. pombe* CPY shows 54.4% identity with the CPY of *S. cerevisiae*, 57.3% identity with CPY of *C. albicans*, 56.9% identity with the CPY of *P. pastoris* (15).

Hansenula polymorpha CPY has a molecular weight of 60 kDa on deglycosylation it reduces to 47kDa. CPY knock-out strain in these yeast cells does not show any impact on growth both in the presence of glucose or methanol indicating CPY is not a major protease involved in proteolytic degradation of peroxisomal constituents during selective degradation of the organelle (16).

Glycosylation of CPY in yeast is important for efficient intracellular transport, but not for vacuolar sorting, in vivo stability, or activity (17). Modulating the level of CPY synthesis through *PRC1* gene dosage experiments it has been demonstrated that proper transport of this glycoprotein to the vacuole can be overloaded (18). CPY transport to the vacuole is mediated by a saturable component such as a receptor Vps10. Yeast receptor would require protein determinants for recognition but not the glycosylation (19). In strains over expressing CPY the protein does not undergo glycosylation characteristic feature of a typical secreted yeast glycoproteins. These results indicate that glycosyl modifications in yeast may be dictated by the structural properties inherent in the protein undergoing the modifications, and not merely by exposure to the transferases (18).

For down regulation of *PRC1* we have planned two constructs 1) Antisense construct I (AS I) covering the whole *PRC1* mRNA and 2) Antisense construct II (ASII) covering 80% of the whole *PRC1* mRNA. Antisense RNAs expressed were quantified by RT-PCR using Taqman assays and down regulation of CPY protein was analysed by Western blotting.

Materials and methods:

Isolation of genomic DNA from *Pichia pastoris*:
Genomic DNA was isolated from *P. pastoris* GS115 grown overnight in 100 ml of YPD broth in a 500 ml shake flask with baffles. After checking for the absence of any potential contamination by microscopy, yeast cells were pelleted at 2168 x g for 10 min at 4°C (Beckman coulter, California, USA). After decanting the supernatant, yeast cells were lysed with the aid of liquid nitrogen using pre chilled motor and pestle. 200 µl of cell lysis powder

were collected into 2 ml eppendorf tube equilibrated in liquid nitrogen. 400 µl Phenol and 800 µl of H-buffer (250 µl 8M LiCl, 800 µl 0.25M EDTA, 200 µl 1M Tris (pH7.5), 500 µl 20% SDS, 18.25 ml sterile distilled water) were added to the cell lysis powder and thoroughly vortexed. Samples were heated for 5 min at 60°C in a thermomixer (Eppendorf, Hamburg, Germany). Later 400 µl of chloroform: Isoamylalcohol (24:1 v/v) were added and the samples were thoroughly mixed before spinning at 15700 x g for 1 min in a table top centrifuge. The upper aqueous phase containing DNA was carefully withdrawn and placed in a new eppendorf tube for ethanol precipitation. Therefore, 1/10 volume of 3 M sodium acetate and 2 volumes of ice-cold ethanol were added and samples were incubated at -70°C for 45 min. To precipitate DNA, samples were centrifuged for 10 min at 15700 x g and 4°C in a table top centrifuge. Supernatants were decanted and the DNA pellets were washed with 70% ice-cold ethanol. Centrifugation and washing steps were repeated twice. Finally ethanol was decanted and DNA pellets were dried at 37°C for 30 min.

Subsequently DNA was dissolved in 50 µl of TE buffer, pH 8 and 1 µl each of RNase A (10 mg/ml) and Proteinase K (33 µg/ml) (Fermentas, Burlington, Canada) were added before overnight incubation at 4°C. Then 400 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA), pH 8 and 500 µl of PCI solution (Phenol: Chloroform: Isoamylalcohol 25: 24:1 v/v) were added and samples were centrifuged for 1 min at 15700 x g in a table top centrifuge. The supernatants were transferred into new eppendorf tubes and ethanol precipitation of the genomic DNA was performed as described above. After 15 min of incubation at -70°C, samples were spun at 4°C and 15700 x g for 10 min. Upon decanting, the DNA pellet was washed twice with 70% ice-cold ethanol and dried at 37°C for 30 min. Finally, the DNA pellet was dissolved in double distilled water overnight at 4°C. Integrity of isolated genomic DNA was analyzed by agarose gel electrophoresis.

Construction of antisense RNA expression vectors:

Antisense inserts I and II were amplified in a final volume of 50 µl with 1 U Phusion DNA polymerase, 1x HF buffer, 40 ng of template, 0.2 mM of dNTPs and 0.4 µM of each primer according to the Phusion High-Fidelity DNA polymerase manual (Finnzymes, Espoo, Finland). PCR conditions were 30 s at 98°C, followed by 25 cycles of (10 s 98°C, 30 s 60°C, 1 min 72°C) and 10 min at 72°C. However after 10 cycles primer annealing temperature was raised to 75°C. Antisense inserts I and II were amplified using forward primers Insert 1 FP

and Insert 2 FP, respectively and common reverse primer HNLPRC1 RP which also introduced 50 bp long *Hb HNL* DNA for RT-PCR (see fig 5).

Table 1: Primer sequences for isolating the ASI and II

S.no	Primer Sequence	Primer name
1	TCGAAGGATCC***TACGTA***TTAATTTACTGACTATTTGCAATTCAGC	Insert 1 FP
2	TCGAAGGATCC***TACGTA***ATTGCAAGATTCATAGGTATCGACCTCG	Insert 2 FP
3	TAATTAAG***CGCGGCCGC***TCAATTATAGGTATCAGCCACCTCTTCGAGAATT TCAGCGATCTCCTTAGCTATAGATAGTTTCATACTATTTACAAG	HNLPRC1 RP

Primers used in the synthesis of different antisense expressing DNA inserts are given in 5' to 3' orientation with *Not*I site in red colour bold and italicized, *SnaB*I in pink colur bold and italicized. 50 bp long *Hb HNL* DNA sequence to be introduced is in blue colour, bold and underlined. Sequences initially annealing to template are in green colour bold.

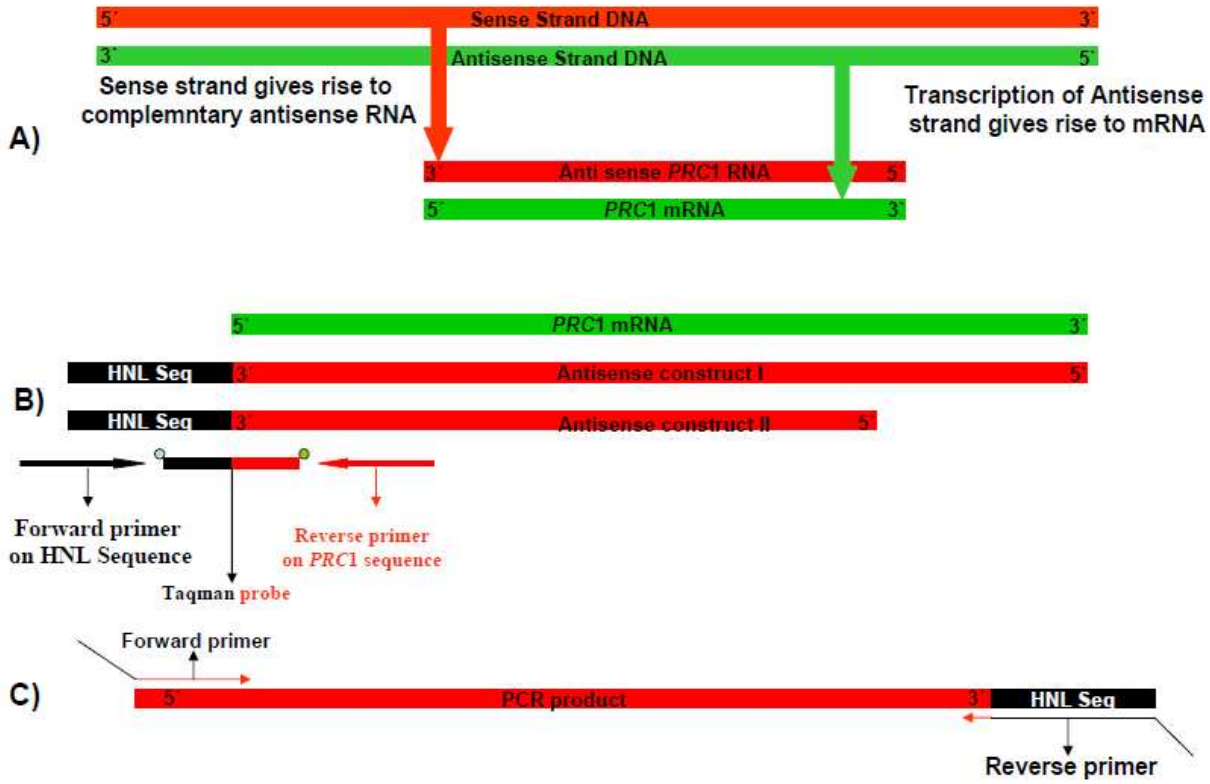


Fig 5: A) Origin of mRNA and antisense RNA from *PRC1* locus B) Representation of two Antisense RNA constructs complementary to *PRC1* mRNA and primer set up for detection of Antisense RNA by RT-PCR C) Alignment of PCR primers on the PCR product .

Anti sense inserts I and II were ligated into pPIC3.5 under the control of AOX1 promoter (Fig 6) using restriction enzymes *Not*I and *SnaB*I (*Eco*105I) (Fermentas, Burlington, Canada) sequentially. First, restriction reactions using *Not*I were incubated overnight at 37°C (Table 2)

and were purified by gel extraction (QIAGEN, Hilden, Germany). Upon estimating DNA concentrations, *Not*I linearized plasmids and antisense inserts were digested with *SnaB*I for

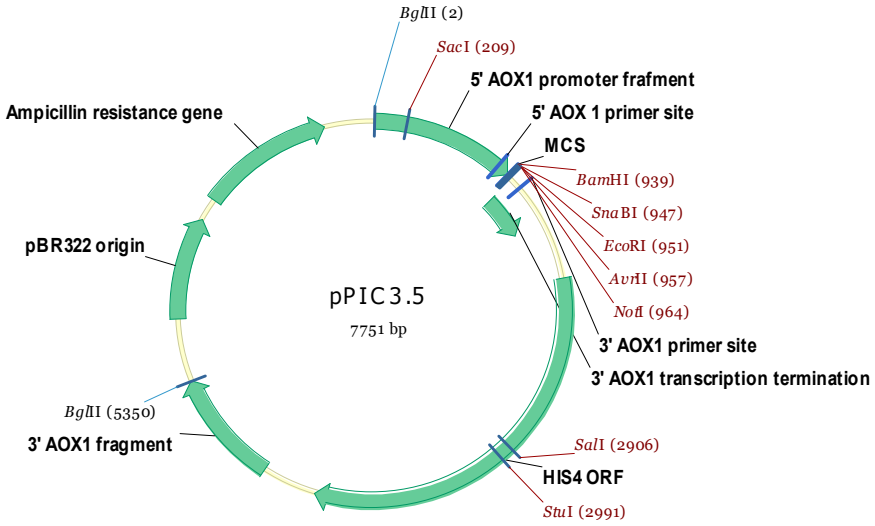


Fig 6: Plasmid pPIC3.5

Table 2: Protocol for *Not*I digestion of pPIC3.5 and antisense Inserts

S.no	Contents	Vol in µl	Contents	Vol in µl
1	pPIC3.5 (500 ng)	10	AS I and II (0.45 µg)	9
2	'O' buffer	1.5	'O' buffer	2
3	*Not*I	1	*Not*I	2
4	Double distilled water	2.5	Double distilled water	7

Table 3: Protocol for *SnaB*I digestion of pPIC3.5 and antisense Inserts

S.no	Contents	Vol in µl	Contents	Vol in µl
1	*Not*I pPIC3.5 (0.45 µg)	15	AS I and II (0.1 µg)	20
2	10 x Tango buffer	2	10 x Tango buffer	2.5
3	*SnaB*I	1.5	*SnaB*I	2
4	Double distilled water	1.5	Double distilled water	0.5

8 h at 37°C (Table 3). Following gel extraction plasmid backbone and insert concentrations were estimated from agarose gels finally. Calculating a 1:3 molar ratio of vector: insert,

ligation reactions were performed in a final volume of 20 µl and a final DNA concentration of 200 ng (Table 4). Ligation reactions were incubated overnight at 16°C.

Table 4: Assembly of the Ligation reaction

S.no	Contents	Vol in µl
1	Double digested pPIC3.5	9
2	Antisense Insert I or II	2
3	Ligation buffer	2
4	T4 DNA Ligase	2
Water to make final volume to 20 µl		5

Ligation reactions were desalted by placing the reaction mixtures on millipore filter discs (0.25 µm) (Millipore, Tullagreen, Ireland) themselves floating on sterile double distilled water for 30 min. After desalting, complete ligation mixtures were transferred into ice-cold electroporation cuvettes (Biozyme Scientific GmbH, Oldendorf, Germany) followed by 40 µl of electro-competent *E. coli*TOP10 cells. Electroporation cuvettes containing bacterial cells and DNA were chilled on ice for 5 min before transformation of the recombinant plasmids occurred by an electroporator (Bio-Rad, California, USA) with preset pulse program EC2 (2.49 kV, 6.10 ms). Transformants were regenerated for 30 min in 1000 µl of LB broth at 500 rpm and 37°C. After regeneration, 200 µl aliquots of regeneration culture were spread on LB ampicillin plates and incubated overnight at 37°C. Randomly 8 transformants per construct were selected for plasmid isolation. Correct orientation of the inserts was verified by *Bgl* II restriction, before plasmids were sequenced.

Transformation into *P. pastoris*:

Recombinant plasmids were linearized by *Sal*I, in a final volume of 20 µl containing 16 µg of plasmid DNA, 20 U of *Sal*I, and 1 x 'O' buffer. After linearization, Plasmid DNA was concentrated by ethanol precipitation and dissolved in double distilled water to a final volume of 10 µl. In parallel, *P. pastoris* cells were rendered competent for transformation. A single colony of GS115 strain from freshly inoculated YPD plates was inoculated into 30 ml of YPD broth for overnight culture at 28°C and 120 rpm. Subsequently to confirming purity of the culture by microscopy, 3 ml of the over night culture were inoculated into 100 ml of the YPD

preculture. After 8 h of incubation under the same conditions, 900 µl of the preculture were inoculated into 250 ml YPD main culture 1. After reaching OD_{600}= 1, 70 ml of main culture 1 were inoculated into 250 ml YPD main culture 2 and grown at 28°C and 120 rpm to OD_{600}= 1. Then yeast cells were harvested at 4,500 rpm and 4°C for 10 min in a Beckman JA10 rotor (Beckman coulter, California, USA). Cell pellets were washed twice with sterile, ice-cold distilled water and once with prechilled 1 M sorbitol. Sorbitol washed yeast cells were resuspended in 1000 µl of 1 M sorbitol. 80 µl of competent cell suspension were used per transformation event. Transformation of *P. pastoris* cells was performed by electroporation at 1.99 kV, for 6.10 ms. Yeast transformants were regenerated in 2 ml of YPS for 1 h at 30°C without shaking. After 1 h of regeneration, 100 µl portions of these regeneration cultures were plated onto regeneration dextrose agar plates.

Pichia pastoris is capable of integrating multiple copies of transforming DNA via recombination into the genome at sites of sequence homology. Linearised plasmid DNA for the expression of antisense RNA can be integrated both into the AOX1 or his4 locus. If the plasmid integrates into the his4 locus, transformants have the capacity to metabolise methanol very efficiently through AOX1 p and are denoted by Mut^+ (Methanol utilization positive) strains. Alternatively, if the plasmid is integrated in to *AOX1* locus these strains utilise methanol slowly and are termed Mut^S (Methanol utilisation slow) strains. To identify His^+Mut^+ strains from the pool of transformants, Mut^+/Mut^S screening was performed by streaking transformants along with Mut^+/Mut^S control strains onto buffered minimal methanol medium plates and MD agar plates and monitoring growth, i.e. rise of colonies on methanol containing plates.

Integration of expression constructs coding for antisense RNA was confirmed by PCR on isolated genomic DNA. For this purpose, 10 transformants per antisense expressing construct were selected and, DNA was isolated from these clones as discussed previously. PCR was performed using standard 5' *AOX1* and 3'*AOX1* primer. In a final volume of 50 µl PCR was performed with 1.25 U of Dream Taq DNA polymerase, 1x Dream Taq Buffer, 0.4 µM of each primer, 40 ng of genomic DNA, 0.2 mM of dNTPs. PCR conditions were 3 min at 95°C, 25 cycles of (30 s 95°C, 30 s 55°C, 2 min 72°C) and 15 min at 72°C.

Induction of antisense RNA expression:

Transformants verified by PCR were selected for shake flask cultivation and induction. Small colonies from a fresh YPD plate were inoculated into 200 ml BYPD broth and grown to stationary phase. After 24 h of growth, 50 ml of the culture was collected for RNA isolation (= before induction control), and induction of the yeast cultures was started by adding 150 ml of BMM (1.5%). After 8 h of primary induction, yeast cells were further induced for the night interval with 1.5% v/v absolute methanol. After 24 h of induction, 50 ml of the culture was collected for RNA isolation and the remaining 200 ml of yeast culture were induced with 0.75 % and 1.5% methanol (v/v) for another 24 h. Finally, after 48 h of induction, 50 ml of the cell broth were harvested for isolating RNA. Yeast cells were pelleted at 3,500 rpm and 4°C for 10 min in a Beckman JA10 rotor (Beckman coulter, California, USA) and the supernatants were decanted.

RNA isolation:

Total RNA was isolated from yeast cells using the standard protocol from Trizol (Invitrogen, Carlsbad, USA). Cells were disrupted in the presence of liquid nitrogen with the help of mortar and pestle. Approximately 100µl of yeast cell powder were collected into Eppendorf tubes prechilled in liquid nitrogen. For every 100 µl of cell lysis powder 1000 µl of trizol were added and immediately vortexed. To dissociate the nucleoprotein complexes in the samples completely tubes were incubated for 5 min at room temperature. 200 µl of chloroform were added before shaking the samples vigorously. Phases were separated centrifuged at 12,000 x g and 4°C for 15 min and the aqueous upper phases containing RNA were transferred into new eppendorf tubes. RNA was precipitated with 500 µl of isopropanol at room temperature for 10 min. Then RNA was pelleted at 12,000 x g and 4°C for 10 min and washed with 75% ethanol. Upon decanting the supernatants RNA pellets were briefly air-dried at 37°C to get rid of ethanol. After drying, RNA was dissolved in 100 µl DEPC water at 65°C for 10 min and preserved at -70°C until further use. To asses the quality of isolated RNA samples were analysed in a 1% w/v agarose gel made in 1x MOPS buffer. On the one hand, integrity was evaluated by the presence of two intact bands of 28S and 18S RNA, on the other hand RNA concentration was determined using a nano drop1000 spectrophotometer (Thermo Scientific, Wilmington, USA).

Quantifying antisense RNA expression – RT-PCR:

Superscript III Platinum Two-Step qRT-PCR kit (Invitrogen, Carlsbad, USA) was used for both cDNA synthesis and the RT-PCR reaction. RNA isolated from various samples was diluted to get a uniform concentration of 0.1µg/µl in a final volume of 50 µl. To eliminate any possible DNA background DNaseI treatment of RNA samples was performed. For 5 µg of the RNA 1 U of DnaseI in 1 x reaction buffer was employed at 37°C for half hour. Reactions were terminated by the addition of 1 µl of 25 mM EDTA followed by heating the sample at 65°C for 10 min. DNA-free RNA was used as a template for cDNA synthesis in a total volume of 25 µl with 1 µg RNA, 1x RT reaction mix, and 2µl RT enzyme mix. cDNA synthesis was performed in a thermocycler by incubating it at 25°C for 10 min and at 42°C for 50 min, finally before terminating the reaction by heating the samples at 85°C for 5 min. RNA in the cDNA sample was degraded by adding 2 U of *E. coli* RNase H and incubation at 37°C for 20 min. Until further use samples were preserved at -20°C.

Antisense RNA which was supposed to inhibit translation of functional *PRC1* mRNA was quantified by RT-PCR. For this purpose, qPCR was performed in the 96 well plate format for samples from uninduced cells and samples after 24 and 48 h of the methanol induction respectively. Totally, 6 independent RT-PCR reactions were executed considering 2 different lengths of antisense RNA constructs. RT-PCR reactions were done in a final volume of 25 µl with 12.5 µl of Platinum Quantitative PCR supermix-UDG, 5 µl of cDNA synthesis reaction mixture, 0.2 µM of each primer, 0.1 µM of Taqman probe, and 500 nM of ROX reference dye using standard cycling program designed for ABI 7300 RT-PCR instruments (Applied Biosystems, California, USA).PCR conditions were 2 min at 50°C (UDG incubation) and 2 min at 95°C followed by 40 cycles of (15 s 95°C, 30 s 60°C).

All quantitative RT-PCR data were normalized for the expression of the *P. pastoris ACT1* gene used as house-keeping gene. Based on information from the primary round of qPCR, a secondary qPCR was performed on several clones exhibiting different levels of antisense expression in the primary experiment. This secondary PCR was performed to quantify both antisense and house keeping gene expression on a single 96 well plate for all the 3 induction situations, i.e. 0, 24 and 48 h of methanol induction.

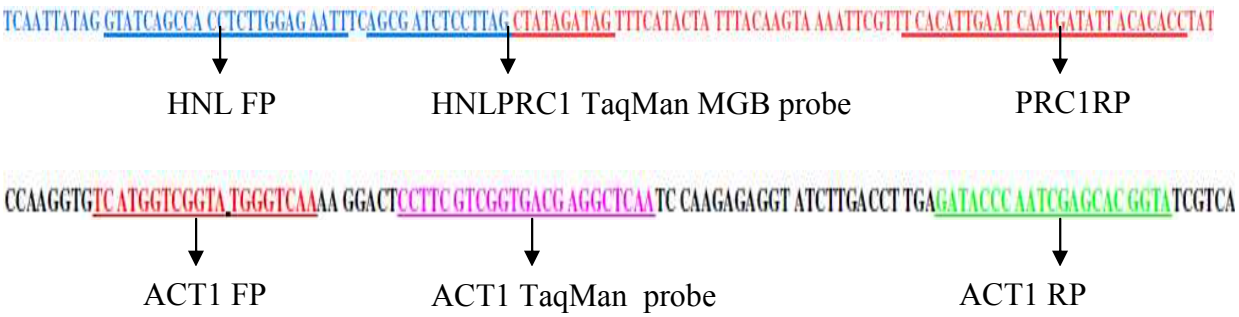


Fig 7: Primers for RT-PCR were designed under guidance of Dr. Peter remler using primer express software from Applied Biosystems. Primers designed for RT-PCR is aligned on cDNA sequences. HNL FP and PRC1 RP will bind to their respective HNL and PRC1 regions on the antisense cDNA, HNLPRC1 is a TaqMan minor grove binding probe with 5' 6-FAM and 3' MGBNFQ (Minor groove binding non fluorescent quencher) (Applied Biosystems, Cheshire, UK).This TaqMan probe binds on the junction covering both HNL and PRC1 regions. ACT1 FP, ACT1 RP and ACT1 TaqMan probe designed for ACT1 bind to its cDNA. ACT1 TaqMan probe contains 5' ABI-FAM and 3' ABI-TAMARA (Ingenetix, Vienna, Austria).

Western blotting:

Mature CPY protein had been expressed in *E. coli*BL21-Gold (DE3) cells as insoluble protein in inclusion bodies (20). Proteins were resolved in a preparative SDS-PAGE gel and mature CPY was electroeluted from gel slices by Anja Egger supported by Tamara Wriessnegger. This purified CPY protein was used for immunization of rabbits for generating anti-*CPY* antibodies. Serum fractions from immunized rabbits were collected and checked for the titer and specificity of anti-CPY antibodies in Western blotting.

Whole cell lysates of *Pichia pastoris* GS115 and *PRC1* knock-out strain were prepared by heating cell pellet to 95°C for 10 min in 50 µl of SDS-loading buffer supplemented with 10 mM DTT and loaded onto an SDS-PAGE gel. After Western blot transfer, anti-CPY antibody was diluted 1:5000 for the recognition of CPY and membranes were incubated with primary antibody for 1 h at room temperature. To get rid of un-specifically bound antibodies membranes were washed 5 times 5 min with 1x TBST buffer. A secondary antibody fused with reporter enzyme alkaline phosphatase was used for the detection of antigen-antibody complexes. Presence of the secondary antibody was recognized using NBIT-BCIP solution

(Calbiochem, La Jolla, California, USA) specific for alkaline phosphatase. Images were taken using Canon Digital IXUS 400 camera (Canon, Tokyo, Japan).

Correlating antisense RNA expression and CPY accumulation:
Over night cultures of antisense RNA *Pichia* clones were inoculated into 200 ml of BYPD. After 24 h of growth 50 ml and 1000 µl of the yeast cultures were collected for RNA isolation and Western blotting respectively. Cultures collected for RNA isolation and western blotting cells were pelleted by centrifugation at 3220 x g and 4°C for 10 min, after decanting the supernatants cell pellets have been freezed in Liquid nitrogen and preserved at -80°C. After collecting the samples cell cultures were induced with 150 ml of BMM (1.5%), simultaneously in other conical flask after collecting samples cell cultures were diluted with Buffered minimal sorbital (1% sorbitol) for derepression of *AOX1* promoter. Methanol induction and sample collection have been done at 4 (0.5 % v/v), 8 (0.5 % v/v), and 12 h (1 % v/v) respectively. After derepression of *AOX1* promoter with sorbitol samples were collected 4, 8, and 12 h respectively. Final samples were collected after 24 h of induction and derepression of *AOX1* promoter.

Results:

Generation of antisense RNA expression constructs and transformation into yeast:
To develop antisense RNA expression constructs, genomic DNA of *P. pastoris* GS115 was isolated as template for PCR amplification. Integrity of genomic DNA was verified by gel electrophoresis yielding a distinct, high molecular weight DNA band confirming the successful isolation of high quality DNA as template for the amplification of the *PRC1* locus. (data not shown). As the requirements regarding the length of antisense RNA for efficient down-regulation of target mRNAs/genes in *P. pastoris* were unknown at the start of this project, 2 different constructs were generated. One covers the whole *PRC1* mRNA length, the other about 75% thereof (See figure 5 B). Employing forward and reverse primers to introduce *Not*I site and 50 bp of *Hb HNL* coding sequence upstream of the *PRC1* coding sequence and *SnaB*I sites towards the 3' end or downstream of the *PRC1* coding sequence (See figure 5 B and C), *PRC1* locus was amplified and made accessible for generating antisense RNA expression cassettes. 5 µl of the PCR products were analyzed by agarose gel electrophoresis (Fig 10). By cloning the PCR products into the *SnaB*I and *Not*I sites of pPIC3.5 expression of antisense RNA can be driven from the strong, methanol-inducible

AOX1 promoter. Double-digested pPIC3.5 plasmid and AS inserts I and II were loaded onto an agarose gel to estimate their concentrations. Upon ligation and transformation into *E. coli*, eight clones per construct were analyzed by restriction with *Bgl*II and sequencing. Clones with desired sequences were prepared for transformation into *P. pastoris*. Antisense expression plasmids were linearised by *Sal*I for transforming into the his4 locus of the GS115 yeast strain. Transformants were regenerated for 1 h in YPS and plated onto regeneration dextrose agar plates. After two days of incubation minute colonies of the transformants were seen and randomly 100 clones per construct were selected for Mut^+/ Mut^s screening. All clones were found to be Mut^+. A restricted subset of transformants was checked for cassette integration by PCR.

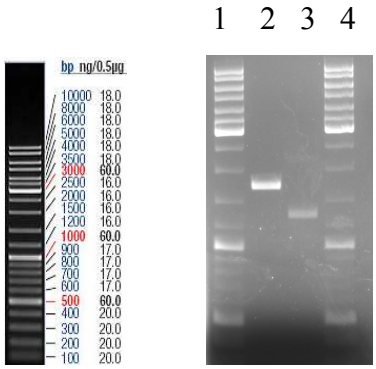


Fig 8: Gel Photograph of the antisense DNA PCR product. Lane 1 and 4 - DNA ladder mix, Lane 2 - 1720 bp long AS I insert, Lane 3 - 1308 bp long AS II insert.

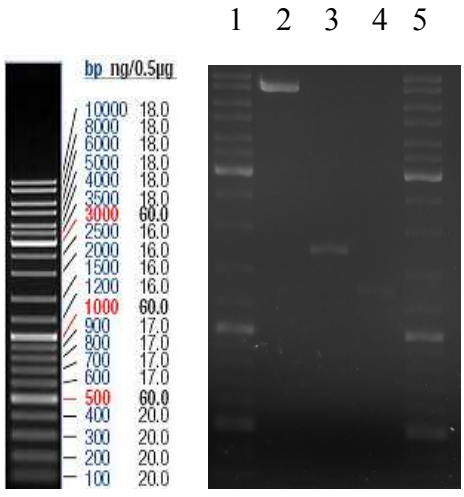
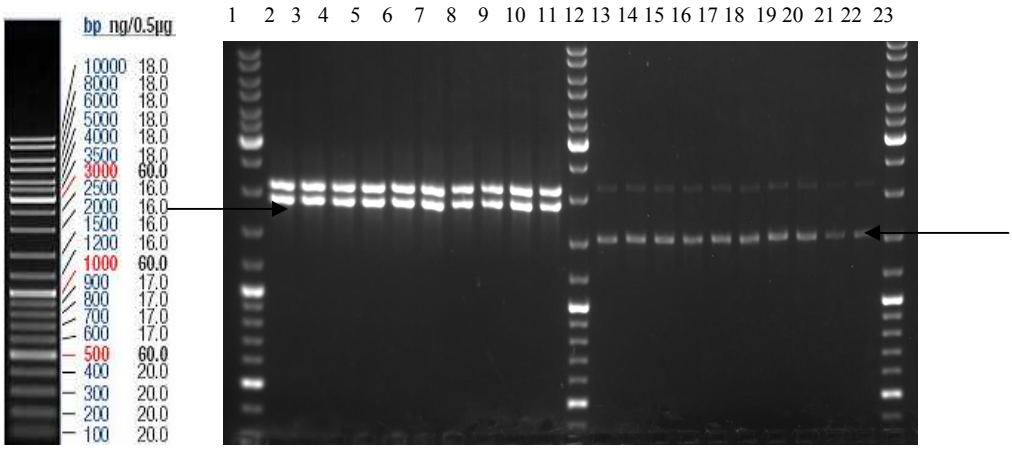


Fig 9: DNA Ladder mix from FERMENTAS, gel electrophoresis of Plasmids and Inserts for concentration estimation. Lane 1&5 - DNA ladder mix, Lane 2 - double digested pPIC3.5, Lane 3 - double digested AS I, Lane 4 - double digested AS II. DNA concentrations estimated from the gel electrophoresis were pPIC3.5 24 ng/µl, AS I insert 8 ng/µl, and AS II insert is 2.4 ng/µl.

Using isolated DNA from the transformants as template, presence of the antisense expression constructs was confirmed by bands at the expected sizes of 1916 bp and 1516 bp for ASI and ASII clones, respectively (Fig 10 b). PCR results confirmed integration of the antisense constructs in each case.



(a) (b)

Fig 10: (a) DNA Ladder mix from FERMENTAS, (b) PCR of ASI and ASII clones. Lane 1, 12, and 23 - DNA ladder mix from FERMENTAS, Lane 2 to 11 - ASI C1 to C10 PCR products (1916 bp product indicated by arrow), Lane 13 to 22 - ASII C1 to C10 PCR products (1516 bp product indicated by arrow)

Induction of antisense RNA expression and RT-PCR analysis:

Pichia pastoris cells which had been harvested at 3 different time points, i.e. before and after 24 h and 48 h of induction were subjected to total RNA isolation using Trizol.To judge integrity and overall quality of a total RNA preparation, gel electrophoresis was performed in 1% MOPS buffer. After gel electrophoresis two distinct bands coinciding with 18S and 28S ribosomal RNA bands were clearly visible indicating integrity of the RNA samples isolated (Fig 13).

Then, RNA concentrations were estimated using a Nanodrop TM 1000 Spectrophotometer (Table 7). To compensate for unequal RNA concentrations in different samples, all of them were uniformly diluted to 500 ng/µl for cDNA synthesis. Contaminating DNA was removed by Dnase I treatment of the RNA samples before reverse transcription. cDNA synthesis and RT-PCR reactions were performed according to the manual of SuperScript III Platinum Two Step Quantitative RT-PCR (Invitrogen, Carlsbad, USA).

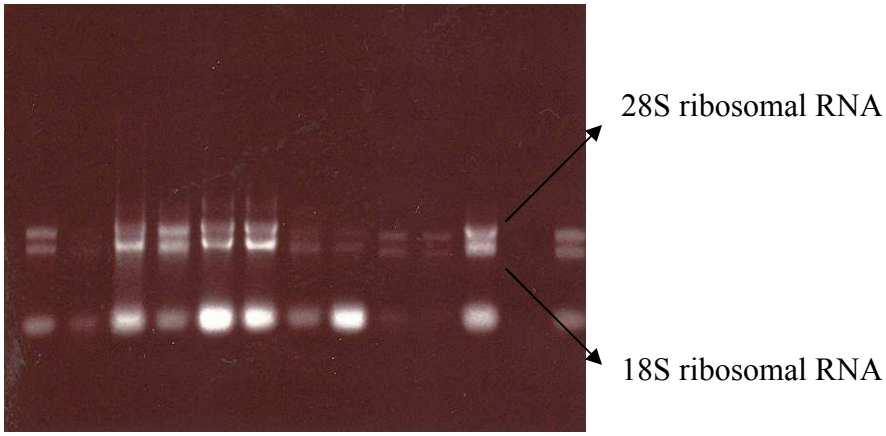


Figure 13: RNA Gel electrophoresis of 13 randomly selected transformants. 5 µl of new DNA loading dye from FERMENTAS were added to 25 µl of RNA samples from randomly selected transformants and loaded onto the gel.

RT-PCR was done in two tiers. In the primary round, antisense RNA expression and expression of housekeeping gene *ACT1* was quantified for the 3 time points on separate plates with suitable positive and negative controls on each of the plates. In the second round, of RT-PCR selected clones covering the different ranges of antisense RNA expression were analysed both for antisense RNA expression and for expression of housekeeping gene *ACT1* on a single plate. This was done to avoid possible experimental variation among experiments conducted with independent plates (Table 8 and 9).

Correlating antisense RNA expression to CPY production:

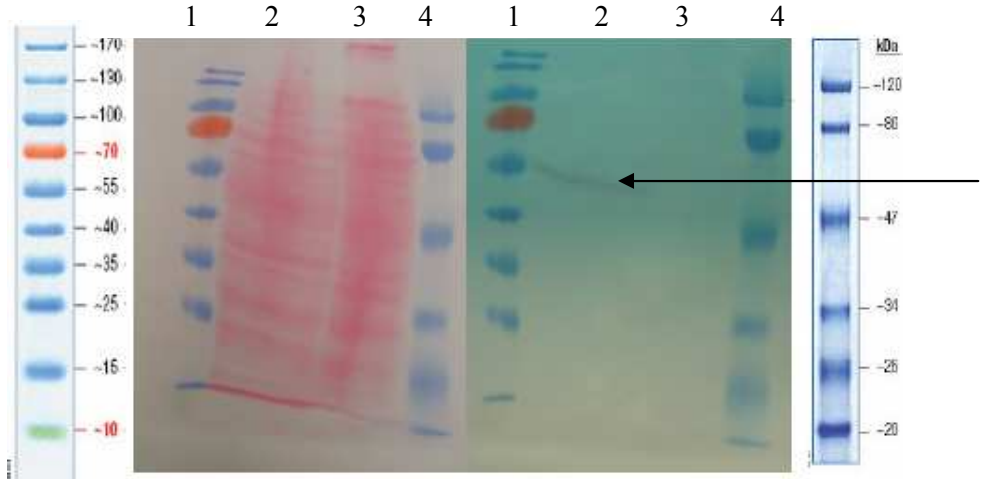
As the second round of RT-PCR had shown reproducible results for the housekeeping gene *ACT1* indicating that RNA isolation and experimental setup of RT-PCR were reliable, the observed differences in antisense RNA expression, e.g. between ASII C2 and ASII C9 (Table 9), suggested that same antisense clones, e.g. ASII C2 might be efficiently depleted of CPY. Therefore, the amount of CPY produced in those strains was monitored using an anti-CPY antiserum in Western blotting experiments. Mature CPY had been produced in *E. coli* (20), purified by SDS-PAGE and electroelution, and had been used to raise antibodies in the rabbit. Titer and specificity of antisera from different bleedings were tested on cell extracts from wild-type GS115 and a *PRC1* knock-out strain in the same background. It turned out that serum from the fourth bleeding showed a clear band indicating CPY at blunt 58 kDa in the wild-type (lane 2), but not in the knock-out strain (lane 3)(Fig 14).

Table 7: Concentrations of the isolated RNA samples

S.no	Sample ID	RNA Concn (original) ng/µl	Sample ID	RNA Concn (original) ng/µl
1	ASI C1 0 h Induction	2280.4	ASII C1 0 h Induction	1144.1
2	ASI C2 0 h Induction	1643.1	ASIIC2 0 h Induction	1773.5
3	ASI C3 0 h Induction	2329.7	ASII C3 0 h Induction	925.7
4	ASI C4 0 h Induction	1696.4	ASII C4 0 h Induction	1146.9
5	ASI C5 0 h Induction	1504	ASII C5 0 h Induction	618.15
6	ASI C6 0 h Induction	1854.2	ASII C6 0 h Induction	1396.1
7	ASI C7 0 h Induction	2086.8	ASIIC7 0 h Induction	1121.8
8	ASI C8 0 h Induction	1429.9	ASII C8 0 h Induction	2641
9	ASI C9 0 h Induction	1725.7	ASII C9 0 h Induction	867.94
10	ASI C10 0 h Induction	2506	ASII C10 0 h Induction	753.08
11	ASI C1 24 h Induction	1400.4	ASII C1 24 h Induction	1985.4
12	ASI C2 24 h Induction	2025.7	ASII C2 24 h Induction	2997
13	ASI C3 24 h Induction	504.91	ASII C3 24 h Induction	2610.4
14	ASI C4 24 h Induction	1254	ASIIC4 24 h Induction	1931.5
15	ASI C5 24 h Induction	1308.4	ASII C5 24 h Induction	1926.6
16	ASI C6 24 h Induction	706.37	ASII C6 24 h Induction	1937
17	ASI C7 24 h Induction	1419.7	ASII C7 24 h Induction	1740.3
18	ASI C8 24 h Induction	585.06	ASII C8 24 h Induction	1687.7
19	ASI C9 24 h Induction	1377.1	ASII C9 24 h Induction	1496.4
20	ASI C10 24 h Induction	1443.1	ASII C10 24 h Induction	1817
21	ASI C1 48 h Induction	706.28	ASIIC1 48 h Induction	1655.4
22	ASI C2 48 h Induction	2109	ASII C2 48 h Induction	1546.4
23	ASI C3 48 h Induction	1457	ASII C3 48 h Induction	2326.4
24	ASI C4 48 h Induction	714.52	ASII C4 48 h Induction	2137.4
25	ASI C5 48 h Induction	797.35	ASII C5 48 h Induction	640.15
26	ASI C6 48 h Induction	1093	ASII C6 48 h Induction	1591.8
27	ASI C7 48 h Induction	656.22	ASII C7 48 h Induction	677.97
28	ASI C8 48 h Induction	526.29	ASII C8 48 h Induction	389.3
29	ASI C9 48 h Induction	735.70	ASII C9 48 h Induction	885
30	ASI C10 48 h Induction	719.21	ASII C10 48 h Induction	898
31	Wild-type strain as control		GS115 0 h Induction	1287.3
32			GS115 24h Induction	1773.3
33			GS115 48h Induction	1177

Two clones, ASIC1 and ASIIC2, which had shown the highest expression of antisense RNA in second round of RT-PCR, were selected for the Western blotting experiment with the wild-type strain as control. These three strains were grown to stationary phase in BYPD and continuously induced with methanol. Samples were collected at 0 h, 4 h, 8 h, 12 h and 24 h of induction and down regulation, of CPY was assessed by Western blotting against CPY.

Western blot images suggested that before methanol induction GS115 and clone ASIIC2 showed no or only low level production of CPY, whereas ASIC1 contained a substantial amount of CPY (Fig 15).



(a) (b)

Figure 14: (a) Ponceau S Image of Western blot and (b) Membrane after developing for alkaline phosphatase activity. Lanes 1 and 4, Prestained protein ladder (FERMENTAS); Lane 2, *P. pastoris* GS115 whole cell lysate, Lane 3, *PRC1* Knock-out whole cell lysate.

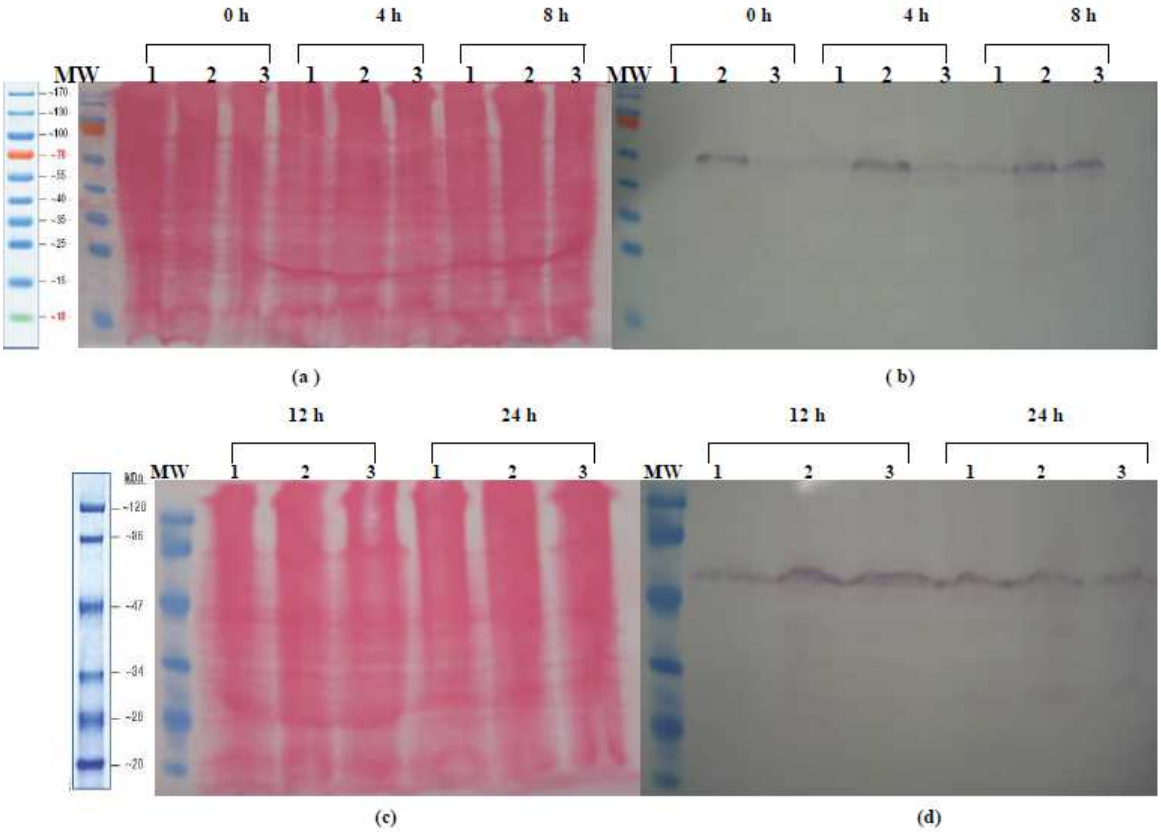


Figure 15: (a, c) Ponceau S Image of western blot, (b, d) and Western blotting membranes developed for alkaline phosphate activity. Prestained molecular weight markers (MW, FERMENTAS) and whole cell extracts of wild-type GS115 (1), ASI C1 (2), ASII C3 (3) derived at different time points of methanol induction, i.e. 0 h, 4 h, 8 h, 12 h, and 24 h were applied for Western blotting.

Table 8: Results from the Primary round of RT-PCR

S.no	Sample name	50h Growth BYPD (= 0 h Induction)		24 h Induction		48 h Induction	
Mean of the two Ct values per each reaction (RT-PCR reactions were run in duplicate)		Antisense Ct	Act1 Ct	Antisense Ct	Act1 Ct	Antisense Ct	Act1 Ct
1	ASI C1	20,185	14,135	22,9	17,045	19	13,855
2	ASI C2	27,795	14,525	27,535	14,945	28,13	13,86
3	ASI C3	20,415	13,89	23,29	18,54	19,755	14,24
4	ASI C4	21,06	14,03	22,69	15,06	21,045	14,21
5	ASI C5	19,295	14,15	22,6	17,205	18,545	14,03
6	ASI C6	19,435	14,315	21,895	16,895	21,02	14,245
7	ASI C7	20,45	14,145	22,615	19,58	19,66	14,19
8	ASI C8	31,505	15,335	29,485	17,435	31,44	16,275
9	ASI C9	21,735	15,545	29,76	16,74	21,265	14,36
10	ASI C10	20,535	14,79	22,23	18,425	20,535	14,74
11	ASII C1	17,94	14,17	10,95	15,82	18,21	14,725
12	ASII C2	19,6	14,34	26,9	15,975	18,31	14,735
13	ASII C3	20,125	14,95	22,065	16,645	18,35	14,475
14	ASII C4	19,41	14,53	21,375	16,25	18,67	14,67
15	ASII C5	18,505	17,8	22,02	14,955	19,865	13,91
16	ASII C6	19,13	14,55	21,31	15,205	18,42	14,68
17	ASII C7	21,245	16,59	21,005	16,31	19,9	14,21
18	ASII C8	20,29	15,87	21,395	16,12	20,345	14,905
19	ASII C9	34,245	17,09	36,245	14,735	30,55	14,315
20	ASII C10	22,095	15,61	21,16	14,825	20,15	14,125
21	*Pichia pastoris* GS115 (Negative control)	36,4	15,295	37,455	15,87	35,85	14,185
22	ASI plasmid 10 plasmids/µl (Positive control)	29.19	-NA-	29.19	-NA-	30.62	-NA-
23	NTC (No Template Control)	undet	35.32	undet	38.55	undet	37.98

Table 9: Results from the Second round of RT-PCR

S.no	Sample name	50h Growth BYPD (= 0 h Induction)		24 h Induction		48 h Induction	
Mean of the two Ct values per each reaction (RT-PCR reactions are run in duplicate)		Antisense Ct	Act1 Ct	Antisense Ct	Act1 Ct	Antisense Ct	Act1 Ct
1	ASI C1	18.55	14.32	20.85	16.60	18.76	15.18
2	ASI C2	24.08	14.60	25.01	14.48	26.26	14.20
3	ASI C8	29.69	15.56	31.3	19.95	26.68	15.84
4	ASII C2	19.25	14.32	20.01	15.42	20.14	14.38
5	ASII C9	31.50	15.59	28.29	14.25	31.61	14.11
6	ASII C10	23.98	17.12	19.94	14.87	21.5	14.30
7	*Pichia pastoris* GS115 (Negative control)	32.89	14.89	31.62	15.52	34.01	14.87
8	ASI plasmid 10 plasmids/µl (Positive control)	14.80	-NA-	14.80	-NA-	14.80	-NA-
9	NTC	undet	undet	undet	undet	undet	undet

After 4 h of methanol induction, which has been supposed to down regulate CPY levels actually the adverse affect was observed. While the amount of CPY remained low in GS115 and unexplicably high in ASI C1, in case of ASII C2 up regulation of CPY was obviously initiated to yield a higher amount of CPY in the latter strain than in wild-type strain GS115. The trend was confirmed by the enhanced amounts of CPY after 8 h of induction while GS115 slightly enhanced expression of CPY, CPY levels were more or less at the same level in ASIC1 and ASIIC2 clones, By 12 h of methanol induction, CPY expression was uniform in all strains. This situation was preserved at 24 h of induction. While antisense RNA expression driven from the methanol inducible AOX1 promoter had been expected to decrease the level of cellular CPY, the opposite affect was observed and underscored by the time course experiment (Figure 15). In fact, microarray studies performed by Maria Freigassner in parallel to this project suggested that methanol induction upregulates CPY expression. Comparing *PRC1* expression in dextrose grown *P. pastoris* cells to cells further induced with methanol for 6 h, she found *PRC1* upregulated by a factor of 3 upon addition of methanol. As this regulatory event might have been stronger than downregulation achievable by antisense RNA constructs, we tested whether derepressing the AOX1 promoters of the antisense expression cassettes might efficiently downregulate CPY expression.

Therefore, another round of experiments was done derepressing the AOX1 promoter by growing the cells on sorbitol. Studies on the AOX1 promoter showed that sorbitol does not inhibit promoter function like dextrose (21, 22). So the above experiments were repeated with the same clones. But instead of inducing the clones with methanol cells were fed with 1% buffered minimal sorbitol medium after 24 h of growth on BPYD. Sorbitol was expected to be a carbon source derepressing the AOX1 promoter; thereby triggering antisense RNA expression without simultaneously affecting *PRC1* transcript levels for the change in the carbon source. There was no clear difference in CPY levels among GS115, ASIC1, and ASIIC2 strains after growth on dextrose only (Fig 16). Unfortunately, also derepressive conditions did not lead to a down-regulation of CPY, but rather to an increased amount of the protease in all of the tested strains. There even seemed to be slightly more CPY in ASI C1 than in wild-type and ASII C2 strains. As CPY down-regulation did not mark out with the antisense constructs driven from the AOX1 promoter, this project was terminated at this point.

Discussion:

Vacoules, the yeast equivalent to mammalian lysosomes, occupy a major fraction of the intracellular space and are of central importance to yeast physiology and proliferation. They contain several proteases such as Proteinase A (PrA), Proteinase B (PrB), Carboxypeptidase Y (CPY) , Carboxypeptidase S, Aminopeptidase I, Aminopeptidase Co and dipeptidyl aminopeptidase B (23). Vacuoles of the yeast play an important role in the degradation of unwanted proteins and other cellular compartments and thereby help in the recycling of amino acids and other building blocks for house keeping activities of the cells.

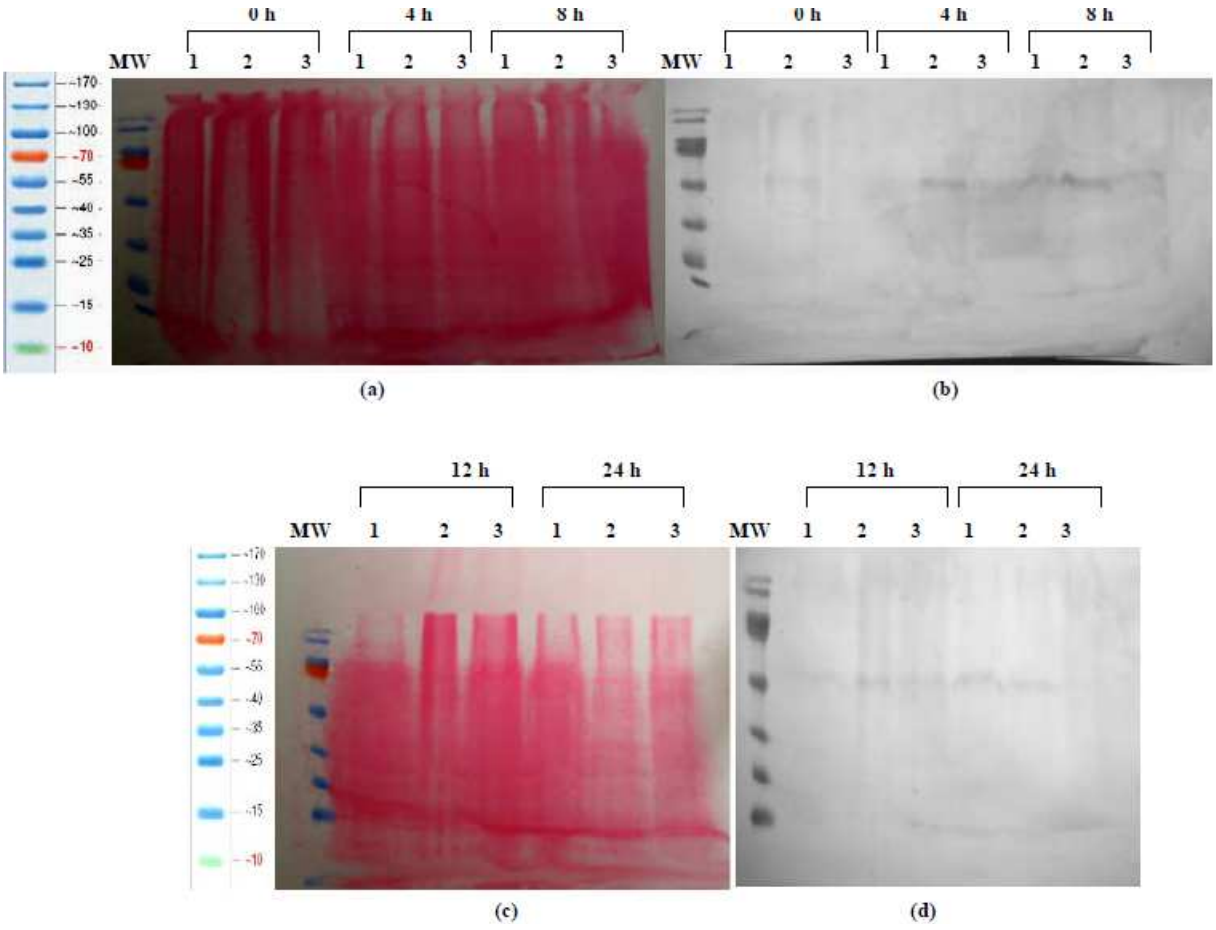


Figure 16: (a, c) Ponceau S Image of western blot, (b, d) and Western blotting membranes developed for alkaline phosphate activity. Prestained molecular weight markers (MW, FERMENTAS) and whole cell extracts of wild-type GS115 (1), ASI C1 (2), ASII C3 (3) derived at different time points of derepression in BMS medium , i.e. 0 h, 4 h, 8 h, 12 h, and 24 h were applied for Western blotting.

Vacuolar proteases of *S. cerevisiae* have been studied and characterised rather well. In case of methylotrophic yeast *Pichia pastoris* this type of studies has not been done. The best studied protease of *Pichia pastoris* is encoded by the *PRC1* gene. *PRC1* has been cloned, recombinantly expressed and characterised. The nucleotide sequence of *PRC1* has been published more than a decade ago and the molecular mass of the protein has been found to be 58 kDa with four possible sites for glycosylation (13).CPY belongs to the family of serine carboxypeptidase which is characterised by highly conserved serine and histidine residues in the active site. It is an exopeptidase that acts on the C-terminal region of proteins and peptides. CPY has been most widely used reporter protein for understanding the protein secretion pathway in yeast but, it has also been used in studies of protein glycosylation (17).

CPY has also been identified and characterised in other methylotrophic yeasts such as *Schizosaccharomyces pombe* and *Hansenula polymorpha* (15,16). In case of *Hansenula polymorpha*, CPY-deficient strain has been constructed. Growth experiments did not show any significant affect of *PRC1* deletions on growth. Interestingly, analysis of major peroxisomal enzymes such as alcohol oxidase, catalase and dihydroxyacetone synthase showed normal proteolytic degradation in the knock-out strain, indicating that CPY was not a major proteinase in this pathway. Additionally, peroxisome proliferation in these cells was virtually unaffected, suggesting that deletion of CPY did not affect the synthesis of normal peroxisomes (16).

In case of *P. pastoris* few protease knock-out strains have been constructed, e.g. SMD1168 (Δ *pep4:: URA3 his4 ura3)*, SMD1168 (Δ*pep4:: URA3* Δ*kex1::SUC2 his4 ura3*) that have been applied affectively to reduce degradation of some heterogeneously expressed proteins therein. Unfortunately, these knock-out strains, particularly PEP4Δ, grow less vigorously and have a lowered transformability as compared to the wild-type background. This clearly indicated that knock-out of a gene coding for a central protease may affect the metabolic network and house keeping activities of the cell (12). Considering the reduced viability of *P. pastoris* Δ PEP4 knock-out strains, we wanted to follow an alternative approach that should not only bring efficient down-regulation of CPY at defined time-points, but should also not excessively affect house keeping activities of the yeast cells. We wanted to apply antisense RNA technology for this purpose. In contrast to higher eukaryotic cells, where the RNAi technology is widely used, Antisense RNA technology is rather unexplored in yeasts. Particularly in plants, Antisense RNA technology has been used for down regulating gene

expression where construction of knock-out organisms is not possible. Antisense RNA down-regulates gene expression at the level of translation. Antisense RNA can impair protein synthesis by any one of the following mechanisms, i.e. 1) Double stranded RNA inhibiting ribosomal binding, 2) double stranded RNA being degraded by RNA-specific RNases, 3) Premature termination of mRNA translation (10).

We have applied the antisense RNA approach to down-regulate CPY expression in *P. pastoris* with the expectation that there should be sufficient CPY for performing house keeping requirements but as little CPY as possible to prevent degradation of recombinant protein. Therefore, antisense constructs were placed under the control of AOX1 promoter. Thus, *Pichia* clones should be able to grow to high cell densities with normal CPY levels and only upon expression of heterologous protein through methanol induction via the AOX1 promoter, CPY levels would be concomitantly down-regulated by antisense RNA. Two antisense RNA expression constructs were designed covering the full length of *PRC1* mRNA, which was named Anti sense I, and a second construct covering ¾ of *PRC1* mRNA, which named Anti sense II. To detect the expression of antisense RNA by RT-PCR, 50 bp of *Hb HNL* coding sequence were added to the 3' end of the antisense DNA. The Taqman probe for RT-PCR was designed in a manner that the primer sequence covered both *HNL* and antisense *PRC1* RNA such that it detected only antisense cDNA during RT-PCR and could not recognise native *Pichia* sequences. Specificity of RT-PCR was therefore high as indicated by the mere-negative results found for the WT strain GS115 (Table 8).

Results from RT-PCR showed expression of antisense RNA when compared to wild-type strain (Tables 8 and 9). Among all clones analysed only two clones ASI CI and ASII C2 showed higher levels of antisense RNA expression from AOXI promoter when compared to wild-type GS115 stain. Samples isolated during 0 h of induction already showed antisense RNA expression due to depletion of dextrose in the medium there by derepressing *AOX1* locus but the level of expression was not over expressed during induction phase similarly during recombinant production. Antisense RNA expressing clones are Mut^+ clones which metabolize methanol fastly, therefore clones ASI CI, ASII C2 and wild-type cells were induced with methanol at regular time intervals such that they have a continuous flow of methanol to replenish methanol utilised. Surprisingly, on Western blot it was observed that ASI clones showed elevated levels of CPY protein when compared to GS115 and ASII clones (Figure 15) speculating over expression of *PRC1* gene to reimburse low level of CPY due to

down regulation. Transcriptome analysis revealed that change in carbon source from dextrose to methanol cells undergoes stress response elevating CPY. Elevated levels of CPY may be required to degrade enzymes involved in the metabolism of dextrose but also for the autophagy of vacoules.

In the present context selected clones described above their *AOX1* locus has been derepressed with sorbitol for antisense RNA expression. Result of derepression on Western blot showed identical result (Figure 16) similar to figure 15. High levels of CPY expression during 0 h of induction may be attributed either to the ideal levels of downregulation there by upregulating *PRC1* locus or integration of the antisense plasmid in reverse orientation into the *AOXI* locus ending in CPY overexpressing strain.

From this information it became clear that in the presence of methanol there is up regulation of the CPY, in order to know the situation during the derepression analysis of AOXI promoter with sorbitol was done. There is no deviation in results in comparison where cells are induced with methanol. These experiments will further confirm microarray data. Down regulation experiments confirm that CPY could be one of the most important protease during the transient phase, shift in carbon source from dextrose to methanol.

CPY protein expression in presence of dextrose /methanol it is better to express antisense RNA under constitutive GAP-promoter such that there is a continuous supply of antisense RNA depleted during inhibitory step. Length of antisense RNA expressed against mRNA could be crucial; with increase in size of antisense RNA its stability in the cell cytoplasm should be verified. A successful attempt in altering cell wall composition of *P. pastoris* to enhance recombinant protein expression by applying PTGS ended up in the expected results further indicating applicability of the principle in *P. pastoris* (24).

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Ref Type: Thesis/Dissertation

Translational Coupling **can be used in high-throughput screening to optimize protein expression in** ***Pichia pastoris***.

Abstract:

A set of *Pichia pastoris* transformants bearing identical cassettes for recombinant protein expression does not express heterologous protein to the same level. There are numerous reasons for the divergent protein expression levels such as variable number of the expression cassette within the cells, variable pattern of integration at the target loci, variable stringency conditions while expressing proteins, and variable physiology of the cells during protein expression due to undefined side affects of the transformation event. To select yeast transformants expressing recombinant proteins to high level is a challenging task and requires screening a large number of clones. To cut down screening time and effort, a novel stringent high-throughput screening system was established to quickly select the clones expressing highest amounts of recombinant protein. Bicistronic mRNAs were generated from constructs linking the gene of intrest to the coding sequence of an antibiotic resistance marker and placing the whole construct under the control of the *AOX1* promoter. Gene of intrest and antibiotic resistance marker gene were not directly fused but separated by a stop codon. Thus, translational coupling of protein expression from the resulting bicistronic mRNA will yield 2 independent proteins which are not covalently linked. Thereby, resistance to high levels of antibiotic should be conferred by the same clones that express protein of intrest to high amounts.

Key words: *Pichia pastoris*, high-throughput screening system, *translational coupling*, and recombinant protein expression

Introduction:

Translational coupling is a process during which several genes are translated from a single mRNA strand (polycistronic mRNA). Threonine operon is a role model for translational coupling. This operon contains 3 genes *thrA*, *thrB* and *thrC*. In *E. coli* K-12 in an attempt to express only *thrB* and *thrC* genes constructs were designed eliminating most of the *thrA* gene. On expressing this construct under the control of a strong promoter (*trp* or *tac*) transcription passed through *thrB* gene, but resulted in efficient translation of *thrC* gene only. To overcome this, a new translational coupling construct was developed using the cat gene in phase to the last 800 bp of *thrA* followed by *thrB* and *thrC*. Cells containing this translational coupling construct express high levels of both *thrB* and *thrC* gene products. In addition it was found that DNA sequence between 220 and 57 bp before the start of *thrB* was necessary to allow translational coupling to occur (1).

Prokaryotes are a very simple organism which is reflected in the simplicity of their translation machinery. A single 70S ribosome can translate a polycistronic mRNA producing multiple proteins.

Transferring the concept of translational coupling from bacterial cells to yeast, was supposed to allow screening for the few clones among many that would produce highest levels of recombinant protein. Should *translational coupling* work in *P. pastoris* this would, literally, enable us to search for the needle in the hay stack successfully.

Pichia pastoris is an excellent platform for expressing a diverse range of proteins, i.e. enzymes to biopharmaceuticals, which have broad commercial applications. Moreover, application of this organism in basic/applied research is increasing constantly, but to this day there is no widely applicable screening system available which could be helpful in screening for (single) clones showing elevated expression.

Typically *high-throughput screening* is used in the areas of recombinant protein expression and in protein engineering by searching for (single) clones which show higher levels of heterologous protein expression or altered activity.

Translational bypass of ribosomes over stop-codons has been discovered in yeast cells. Studies on *S. cerevisiae* showed that both genetic and epigenetic mechanisms affect

translation termination. Leakiness in translation termination has positive impact on the cell i.e. ability of cells to respond, and perhaps adapt to different environmental conditions on the longer term (2). Ribosome's containing any one of the three stop codons in the A site can trigger one of two reactions, (i) translation termination by release factors or (ii) alternatively, incorporation of some amino acid by tRNA which is coincidentally in the close vicinity of ribosome thereby extending translation. The ratio at which any one mechanism occurs in a living cell depends upon balanced activities of translation termination factors and the translation elongation machinery.(2)

Interestingly, the genome of rat line (L1Rn) DNA contains two overlapping open reading frames (ORFs) and apparently codes for DNA/RNA-binding protein in ORF1 and a reverse transcriptase in ORF2(3). To test whether ORF1 and ORF2 are expressed as a fusion protein or independently, a series of capped RNAs with progressive truncations containing one or both ORFs were prepared and translated *in vitro* in the rabbit reticulocyte lysate. Experimental analysis indicated that expression of reverse transcriptase from ORF2 is regulated by reinitation or internal initation of translation *in vitro* thereby expressing two proteins independently. To obtain highly productive mammalian cell lines that express a therapeutic protein at high level a selection marker with a start codon that confers attenuated translation initation frequency was placed upstream of the gene of interest. From the resulting bicistronic mRNA, the selection marker is translated at low frequency, and the protein of interest at high frequency (4).

To investigate the feasibility of translational coupling in yeast cells, two genes were selected. One was coding for *Hb*-Hnl mutant W128A and another encoded zeocin resistance protein.

Hydroxynitrile lyases/oxynitrilases (EC 4.1.2.10), *Hb-Hnl* catalyse the synthesis of cyano-hydrins from aldehydes or activated ketones. Optically pure cyanohydrins are important precursors in constructing a wide variety of chiral building blocks such as amino alcohols and hydroxy acids. These structural moieties are used as adducts in pharmaceuticals, food industries, veterinary products and crop protecting agents (5). (S)-selective Hnl obtained from *Hevea brasiliensis* is a 29 kDa single chain protein. Structurally, it shows an α/β hydrolase fold catalysing stereospecific, reversible addition of HCN to aldehydes or ketones yielding α- or β – cyanohydrins respectively. Alternatively, the enzyme may reverse the reaction and liberate HCN and aldehyde/ketone. The gaseous hydrocyanic acid can be detected by a visible

chromogenic reaction using a simple filter detection assay (Feigl-Anger test) (5). Additionally, another colorimetric assay is available which can be set in *high-throughput format* facilitating enzyme detection. Hnl acts on mandelonitrile (Benzaldehyde-cyanohydrin) producing benzaldehyde and liberating HCN. Benzaldehyde produced during the reaction is measured at 280 nm

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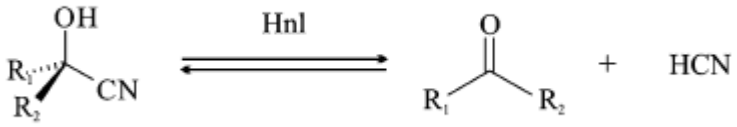


Fig 1: Hnl activity on cyanohydrins releases gaseous HCN

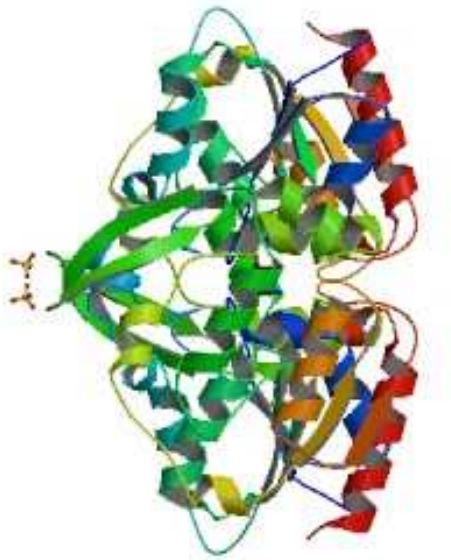


Fig 2: Protein structure of *Hb* Hnl when containing mandelonitrile (6).

Zeocin resistance gene, *Sh ble*, is a 375 bp long resistance marker gene isolated from *Streptoalloteichus hindustanus* conferring resistance to bleomycin related antibiotics, e.g. zeocin. The protein confers resistance to zeocin by selectively binding to the compound and thereby inhibiting its binding to DNA. Expression from the *Sh ble* gene can be visualised by the ability of yeast cells to resist zeocin by growing on nutrient agar plates containing antibiotic(7,8). Zeocin is a copper chelating glycopeptide antibiotic produced by *Streptomyces CL990*. It intercalates into DNA and triggers DNA cleavage. This antibiotic is active on all aerobic cells and, therefore is useful for selection in bacteria, eukaryotic micro- organisms, plants and animal cells (9,9,10).

In the study we try to develop a new *high-throughput* screening system based on *translational coupling* in *P. pastoris*. For this purpose 3 constructs were developed individually by fusing *HNL* genes followed one, two and three stop codons with *sh ble* gene. Bicistronic mRNA generated from this constructs is functional on transcribing produce Hnl enzyme and antibiotic resistance protein independently. Verifying efficiency of *translational coupling* construct and screening of the clones expressing elevated levels of Hnl was performed simultaneously by growing yeast transformants to high levels of zeocin there by ending in high amounts of Hnl protein.

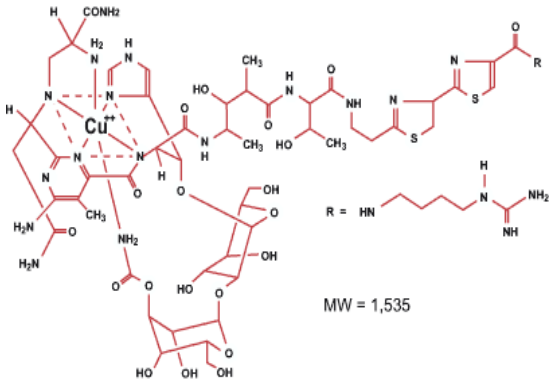


Fig 3: Structure of zeocin molecule (11)

Materials and Methods:

Strains and media:

Wild type strain *P. pastoris* GS115 (Invitrogen, Carlsbad, USA) was used throughout the study. It is auxotrophic for histidine which will be abrogated upon transforming recombinant plasmid into the *his4* locus of the wild type strain. Yeast cells were grown in YPD, 4/5 BYPD, MD, BMMS, and BMMS zeocin plates. *E. coli* TOP10 was grown on standard LB medium and used for plasmid propagation.

PCR amplification:

Hnl coding sequence was amplified from an *E. coli* expression plasmid pSE420_HNLW128A (kindly provided by Romana Wiedner). The *HNL* gene followed by 1, 2, or 3 stop codons was amplified separately in a total volume of 50 µl with 1 U of Phusion DNA polymerase, 1xHF buffer, 100 ng of template, 0.2 mM of dNTPs and 0.4 µM of each primer according to the Phusion High-Fidelity DNA polymerase Manual (Finzymes, Espoo, Finland). PCR conditions were 30 s 98°C, 30 cycles of (30 s 98°C, 30 s 54°C, 1 min 70°C) and 7 min 72°C. Zeocin

resistance gene was amplified from plasmid pPICZ designed for *Pichia pastoris*. This PCR product was used for all further constructions. PCR amplification was done as described before by 30 s 98°C, 30 cycles of (30 s 98°C, 30 s 46°C, 30 s 70°C) and 10 min 70°C. After 10 cycles, primer annealing temperature was increased to 50°C. *HNL* coding sequences were fused with the *Sh ble* gene by overlap extension PCR. Aiming for equimolar ratios of *HNL* and *Sh ble* PCR products as templates for overlap-extension PCR, reactions were performed in a total volume of 50 µl with 1 U of Phusion DNA polymerase, 1X HF buffer, 6.6 ng of *HNL*, 3.4 ng of *Sh ble*, 0.2 mM of dNTPs and 0.4 µM of each primer *Eco*RI *HNL* and Zeo *Eco*RI. PCR conditions were 30 s 98°C 30 cycles of (10 s 98°C, 20 s 50°C, 30 s 72°C) and 10 min 70°C.

Cloning of expression constructs:

PCR products and pHIL-D2 were cut with FastDigest™ *Eco*RI restriction enzyme (Fermentas, Burlington, Canada). After restriction inserts were purified over PCR purification columns to get rid of the buffer and enzyme. Linearized plasmid samples were purified by gel extraction kit (QIAGEN, Hilden, Germany). Dephosphorylation of the linearized plasmid DNA was performed using calf intestinal alkaline phosphatase (Roche, Basel, Switzerland) using 1 U of the enzyme per µg of the plasmid DNA. After 2 h of incubation at 37°C, alkaline phosphatase was inactivated by heating the sample to 80°C for 10 min. Concentrations of the plasmid and PCR products after purification have been analysed by agarose gel electrophoresis.

Calculating a 1:2 molar ratio of the plasmid backbone to insert, ligation reactions were set up in a final volume of 10 µl with 1.5 U T4 DNA ligase (Promega, Madison, USA), 1X Ligation buffer, 100 ng of vector DNA and 17 ng of PCR inserts. Ligations were conducted overnight at 16°C. Desalting of ligation reactions were carried out by placing the reaction mixtures on a floating millipore filter discs (0.25 µm) (Millipore, Tullagreen, Ireland) for 30 min.

Table 1: Restriction reactions with *EcoRI*

S.no	Contents	pHIL-D2	PCR products
1	DNA concentration	2 µl (1 µg)	10 µl (0.2 µg)
2	Fast digest enzyme	1 µl	1 µl
3	10 x fast buffer	2 µl	2 µl
4	Water	15 µl	17 µl
Total volume		20 µl	30 µl

Upon desalting ligated vectors were transferred into ice-cold electroporation cuvettes (Biozyme Scientific GmbH, Oldendorf, Germany) followed by 40 µl of electro-competent *E. coli* TOP10 cells. Electroporation cuvettes containing bacterial cells and DNA were chilled on ice for 5 min before transformation of the recombinant plasmids occurred by an electroporator (Bio-Rad, California, USA) with preset pulse program EC2 (2.49 kV, 6.10 ms). 200 µl aliquots of the regenerated transformants were been plated on LB- ampicillin plates.

Table 2: Primer used for amplifying *HNL* genes followed one, two and three stop codons and *Sh ble* gene

S.no	Primer sequences for isolating HNL gene	Primer name
1	ATGCGAATTCATGGCATTCGCTCAT	*Eco*RI *HNL*
2	TGGTCAACTTGGCCAT***TCA***ATTATAGGTATCA	Stop1 *HNL*
3	TGGTCAACTTGGCCAT***TCATCA***ATTATAGGTA	Stop2 *HNL*
4	TGGTCAACTTGGCCAT***TCATCATCA***ATTATAGGT	Stop3 *HNL*
5	ATGGCCAAGTTGACCAGTG	Zeo gene F
6	TTGGAATTCTCAGTCCTGCTCCTCGGC	Zeo *Eco*RI

Primers used in this study are given in 5' to 3' orientation with *Eco*RI site in red colour, Stop codons in green colour italicized, bold, and underlined. Primer sequence annealing to *HNL* sequence is highlighted in blue colour, Primer sequence annealing to *Sh ble* gene is highlighted in pink colour.

Eight transformants per construct were selected for plasmid isolation. Correct orientations of the inserts were verified by *Nde*I digestion then plasmids were sequenced to confirm the absence of any potential (point) mutations.

Transformation of *Pichia pastoris*:

Recombinant plasmids containing HNLZEO constructs with 1, 2, and 3 stop codons respectively, were linearized with *Sal*I for site specific recombination into *his4* locus. One microgram of plasmid DNA was linearized by 10 U of *Sal*I. After linearization, plasmid DNA was purified by ethanol precipitation. Cultivation of yeast cells, harvesting and transformation of the electrocompetent cells was done as described in chapter 2. Regeneration of transformants was carried out in 1 ml of 1 M YPS for 1 h at 28°C. After regeneration, 200 µl aliquots were plated onto the selective, regeneration dextrose sorbitol plates and incubated at

28°C for 2 days. Mut^+ and Mut^S screening was conducted by streaking individual transformants and Mut^+/ Mut^S controls on MM and MD plates.

Screening:

Cultivation and induction of yeast transformants with potential translational coupling construct was done in 96 deep-well plates as described in the previous chapters. After 48 h of induction, liquid phase in situ Hnl activity assay was conducted in microtiter plates to asses the levels of expression. To make substrate solution, 40 µl mandelonitrile was dissolved in the 5 ml buffer (Citrate-phosphate buffer pH 3.5). Forty microliter of yeast cell suspension from the deep well plates was equilibrated with 100 µl buffer (Citrate-phosphate buffer pH 3.5) for 10 min. Then 10 µl of the substrate solution was added to the cell suspension. Detection filters were placed directly on top of the microtiter plates to detect the released hydrogen cyanide gas. Clones which showed Hnl expression in the fluid phase assay were selected for the next round.150 µl of liquid cultures of these clones were transferred into new deep well plates and were induced with 350 µl of BMM sorbitol broth containing 100 µg/ml zeocin. After 8 h, yeast cells were induced with 1.5% v/v of absolute methanol. 16 h latter, liquid cultures were diluted 500 fold and 10 µl thereof were platted onto the BMM sorbitol plates with and without zeocin and incubated at 28°C. Colonies obtained under these conditions were tested for translational coupling by plating onto MD, BMM sorbitol plates with and with out zeocin.

Colony PCR and sequencing:

Clones which most probably did not carry any ectopically integrated expression cassettes were selected for colony PCR. A small colony was resuspended in 100 µl of distilled water, and heated for 10 min at 95°C. Immediately after heating samples were cooled on ice for 5 min and centrifuged at maximum speed and 4°C for 10 min. 3 µl of the supernatant were taken for colony PCR with the primer pair *EcoR*I *HNL* FP and Zeo *EcoR*I RP. 5 µl of PCR products were loaded onto an agarose gel. PCR products obtained were sequenced for checking the integrity of the expression constructs.

Reverse screening:

Since all the three categories of transformants showed the expected results in the primary screening, for further screening only a single construct containing *HNL* and zeocin resistance genes separated by the single stop codon was selected for the further screening rounds. Electrocompetent cells of *P. pastoris* GS115 were transformed with linearized recombinant

plasmids harbouring *HNL* and zeocin resistance genes separated by the single stop codon. Transformants were regenerated for 1 h in 2 ml of 1 M YPS. Then, 500 µl of regeneration culture were platted onto regeneration dextrose agar plates for assessing the efficiency of the transformation. The remaining volume of the transformants was further grown in 125 µl of YPD media and induced as described for expressing recombinant proteins. After induction, cells were counted using the haemocytometer i.e. neubauer chamber. Original cell culture was diluted $1:10^2$, $1:10^4$ and $1:10^5$ and 200 µl aliquots of each dilution were plated onto MD, BMMS and BMMS zeocin plates with 50 µg/ml, 100 µg/ml and 150 µg/ml of zeocin. Upon incubation transformants were obtained on all plates.

Eight clones from each plate, MD, BMMS and different BMMS zeocin plates were picked randomly from different dilutions plated. Along with these clones, three control strains were included in the assays. First *P. pastoris* GS115 pHIL-D2 1.17-Hnl, which is a Mut^S, $HIS^{+,}$ zeo^- strain (IMBT, culture collection number 2386) expressing high levels of cytosolic Hnl, i.e. up to 22 g/l which is the equivalent of 50% of the cytoplasmic protein (12). Secondly, *P. pastoris* GS115 pHIL-D2 Hnl tunnel mutant W128A, which is a Mut^+, $His^{+,}$ zeo^- single copy strain expressing average levels of Hnl tunnel mutant (IMBT, culture collection number 2661). A third strain *P. pastoris* X-33 pPICZ GFP, which is $Mut^+Hnl^-zeo^+$ will express GFP serving as internal reference for the induction conditions.

All test and control strains were inoculated for the deep well fermentation according to the protocol described in previous chapters. After 48 h of induction cultures were diluted 40 fold in sterile BMM in deep well plates. Diluted cell cultures were pinned onto YPD, BMMS, and BMMS zeocin plates containing 50, 100 and 150 µg/ml of zeocin respectively. After pinning the cells, OD_{600} of the dilutions was measured using a plate reader. In parallel 100 µl of the original cell cultures were collected in a sterile microtiter plates in triplicate and cells were harvested by centrifuging the samples at 4000 rpm for 10 min at 4°C. Supernatants were discarded and cell pellets were preserved at -20°C for Hnl assay and western blotting (13) (Fig 4).

In vitro Hnl assay:

50 µl of Y-PER (Invitrogen, Carlsbad, USA) were added to 10-20 mg of yeast cells in a microtiter plate to release the active enzyme. Samples were incubated at room temperature for half an hour on Titramax shaker 1000 (Heidolph instruments GmbH, Schwabach, Germany)

at maximum speed. After Y-PER extraction, samples were spun for 10 min at 4°C, and 3220 x g. 50 µl each of the supernatants were collected for the assay. 20 µl of the metaphenoxy benzaldehyde cyanohydrin were dissolved in 3 ml of dimethylformamide followed by the addition of 7 ml citrate-phosphate-buffer 0.1 M pH3.5 yielding substrate solution. 50 µl of enzyme preparations were diluted with 100 µl of 0.05 M citrate-phosphate-buffer, pH 5 and 10 µl of substrate solution were added. To detect the Hnl activity i.e. release of gaseous HCN, in the microtitre plate, a net and a detection filter were placed one above each other. Reactions were incubated until visible chromogenic reactions had developed. Detection papers were prepared by soaking filter disks (Whatman No. I) with a chloroform solution, containing 0.5% (w/v) copper-(II) ethyl-acetoacetate (Sigma–Aldrich Co., UK) and 0.5% (w/v) 4,4′-methylenebis (*N*,*N*-dimethyl-aniline) ($CH_2[C_6H_4N(CH_3)_2]_2$) (98%; Sigma–Aldrich Co., UK). When dried, pale green but almost colourless test-paper is ready to use

Western blotting for Hnl:

Randomly, test clones were selected along with a positive control and negative a control expressing GFP for Western blotting. 10 µl of the cell cultures were resuspended in 50 µl of SDS-loading dye and heated for 10 min at 95°C. Whole cell lysates generated thereby were centrifuged at a low speed for 5 sec. 20 µl thereof were loaded for SDS-gel electrophoresis. Electrophoresis, blot transfer and development of the blot were performed as described in the previous chapters. Western blots were decorated primarily with rabbit anti *Hb-Hnl* antibody (1:5,000 dilutions) followed by secondary goat-anti-rabbit antibody covalently attached alkaline phosphatase (1:30,000 dilutions). Upon addition of substrate BCIP / NBT (Calbiochem, Darmstadt, Germany) a violet colour band appeared between the 25 and 35 kDa bands of the standard corresponding to the molecular weight of Hnl, which is 29.2 kDa.

Results:

Generation of *P. pastoris* strains expressing Hnl and zeocin resistance from a translational coupling construct:

The *HNL* coding sequence one, two and three stop codons, respectively was amplified from the plasmid pSE420_HNLW128A using the primer pairs *Eco*RI *HNL* and stop1 *HNL*, stop2 *HNL* or stop3 *HNL*. Analysis of the PCR products by agarose gel electrophoresis showed ~780 bp long fragments (Fig 8a). 375 bp long *Sh ble* gene conferring resistance to zeocin was obtained from pPICZ plasmid using the primer pairs Zeo gene F and Zeo *Eco*RI (Fig 5a).

Equimolar concentrations of the *HNL* and *Sh ble* PCR products were mixed for overlap PCR reaction in order to generate full-length HNLZEO translational coupling inserts using primer pairs *Eco*RI *HNL* and Zeo *Eco*RI. Agarose gel electrophoresis showed 1150 bp long DNA band coinciding with the expected size of the HNLZEO construct (Fig 5b).

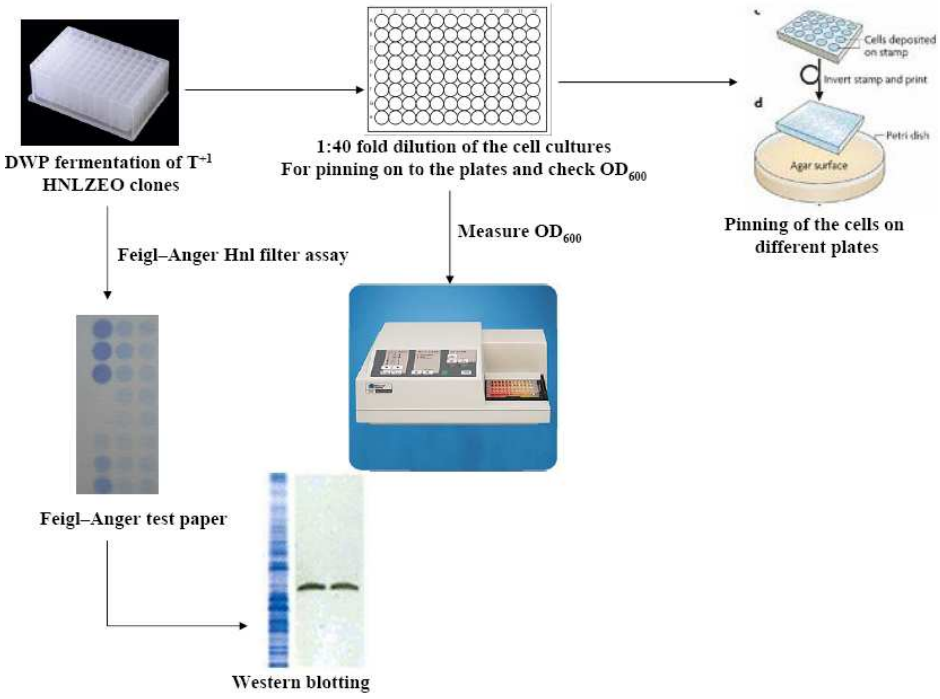


Fig 4: Work flow for correlating Hnl activity and zeocin resistance. (T^{+1} → Yeast transformants bearing translational coupling construct with one stop codon between *HNL* and zeocin resistance gene). Hnl expressing clones detected by Feigl-Anger assay was taken for Western blotting.

pHIL-D2 was selected for intracellular expression. It contains an *Eco*RI site for cloning inserts. Recombinant plasmids can be transformed into the *his4* locus upon linearizing the plasmid by restriction enzyme *Sal*I or into *AOX1* locus by restriction with *Not*I (Fig 7).

The three different constructs differing in the number of stop codons and plasmid pHIL-D2 were digested with fast-digest *Eco*RI enzyme. After digestion, PCR products were purified by ethanol precipitation, where as linearized Plasmid DNA was purified by gel extraction and dephosporylated with the CIP enzyme. Ligations were performed at 1:2 molar ratios of the vector to insert and a total concentration of 50 ng/20 µl ligation reaction.

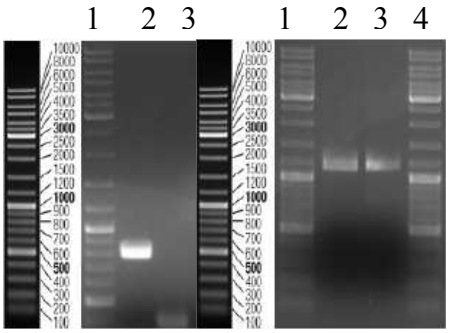


Fig 5: Results from the PCR amplification of *HNL* coding sequence with a single stop codon, *Sh ble* gene (a) and HNLZEO translational coupling construct (b). DNA Ladder mix from Fermentas (1, 4), *HNL* tunnel mutant W 128 A coding sequence (774 bp) (2), *Sh ble* /zeocin resistance gene (375 bp) (3) (Fig a) HNLZEO overlap PCR products (1149 bp) (Fig b) (2, 3).

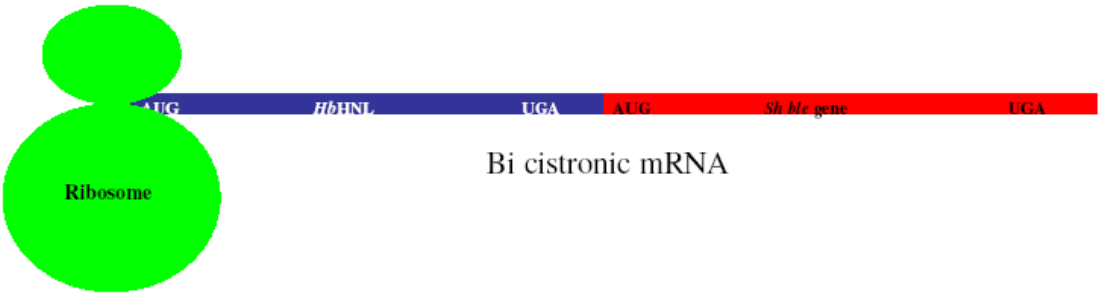


Fig 6: Proposed structure of bicistronic mRNA to be expressed from *AOX1* promoter. mRNA is to translated by a single ribosome expressing both Hnl and zeocin resistance proteins. Totally, 3 constructs were developed with 1, 2 and 3 stop codons separating *Hb HNL* and *Sh ble* genes respectively.

Upon desalting, ligation reactions were transformed into electrocompetent *E. coli*TOP10 cells and plated onto LB ampicillin plates. Randomly, 8 transformants per construct were selected for plasmid isolation, analysis of insert orientation and sequencing. Correct plasmids were selected for yeast transformation. *Sal*I linearized plasmid DNA was concentrated and purified by ethanol precipitation. DNA pellets were dissolved in 10 µl water and transformed into electrocompetent yeast cells as described in the chapter1.Transformants were regenerated and plated onto regeneration dextrose sorbitol plates.

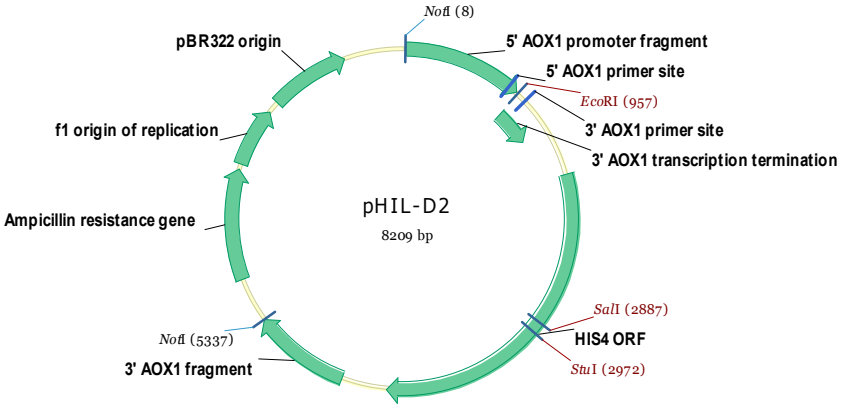


Fig 7: Schematic representation of plasmid pHIL-D2.

Transformants of GS115 with *Sal*I linearized pHIL-D2 constructs favour recombination at *his4* locus and, theoretically, all the transformants should be Mut^+. However, because of the *AOX1* promoter and termination sequences present in the plasmid there is a chance for recombination to occur at the *AOX1* locus, disrupting the wild-type *AOX1* gene and creating HIS^+Mut^S transformants.

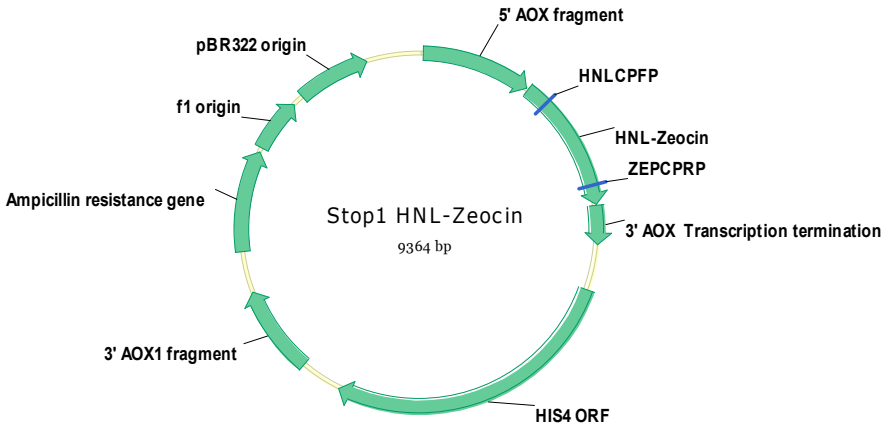


Fig 8: Schematic representation of one of the translational coupling construct with single stop codon used for yeast transformation.

Transformants transcribing the potential translational coupling constructs on bicistronic mRNA were screened in two ways. Primarily, clones were selected for Hnl expression and later tested for resistance to zeocin .In a latter stage, primary screening was done for increased zeocin resistance followed by Hnl activity assays.

Forward screening-Correlating zeocin resistance to Hnl expression:

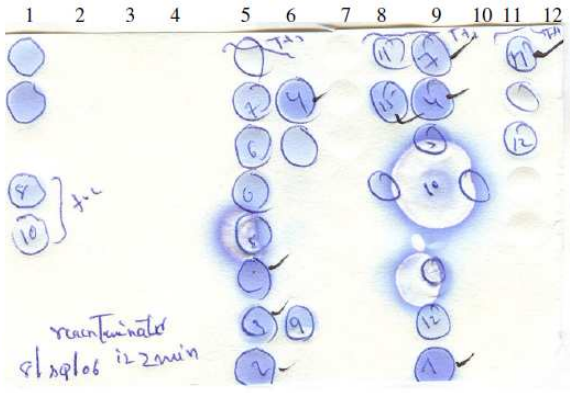
16 clones obtained per transformation event along with positive controls (X-33 strain pPICZB Hnl tunnel mutant W128A, which is Mut^S, His^+, zeo^+) and negative control (*P. pastoris*

GS115) were grown and induced in deep- well plate. Hnl assays were performed in liquid phase to select for clones expressing functional Hnl. Of 16 clones per each construct i.e. T^{+1} clones with single stop codon, T^{+2} clones with two stop codons and T^{+3} clones with three stop codons, screening T^{+1} yielded 3 strains, T^{+2} clones yielded 7 strains and T^{+3} yielded 6 strains (Fig 9).

Strains depicted in Fig 9 were grown and induced in BMM zeocin 100 µg/ml media to test for resistance of the strains to the antibiotic. After 12 h of induction the cultures were diluted 500 fold and 10 µl of the dilutions were plated onto BMM sorbitol plates with (100 µg/ml) and without zeocin. After 2 days of incubation there were minute colonies on the BMM sorbitol plates with zeocin and lawn of culture on the BMM sorbitol plates (Fig 10).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Rec HNL				T^{+3}	T^{+3}		T^{+2}	T^{+2}		T^{+1}	T^{+1}
B	Rec HNL				T^{+3}	T^{+3}		T^{+2}	T^{+2}		T^{+1}	T^{+1}
C					T^{+3}	T^{+3}		T^{+2}	T^{+2}		T^{+1}	T^{+1}
D	+C				T^{+3}	T^{+3}		T^{+2}	T^{+2}		T^{+1}	T^{+1}
E	+C				T^{+3}	T^{+3}		T^{+2}	T^{+2}		T^{+1}	T^{+1}
F					T^{+3}	T^{+3}		T^{+2}	T^{+2}		T^{+1}	T^{+1}
G	- C				T^{+3}	T^{+3}		T^{+2}	T^{+2}		T^{+1}	T^{+1}
H	- C				T^{+3}	T^{+3}		T^{+2}	T^{+2}		T^{+1}	T^{+1}

a



b

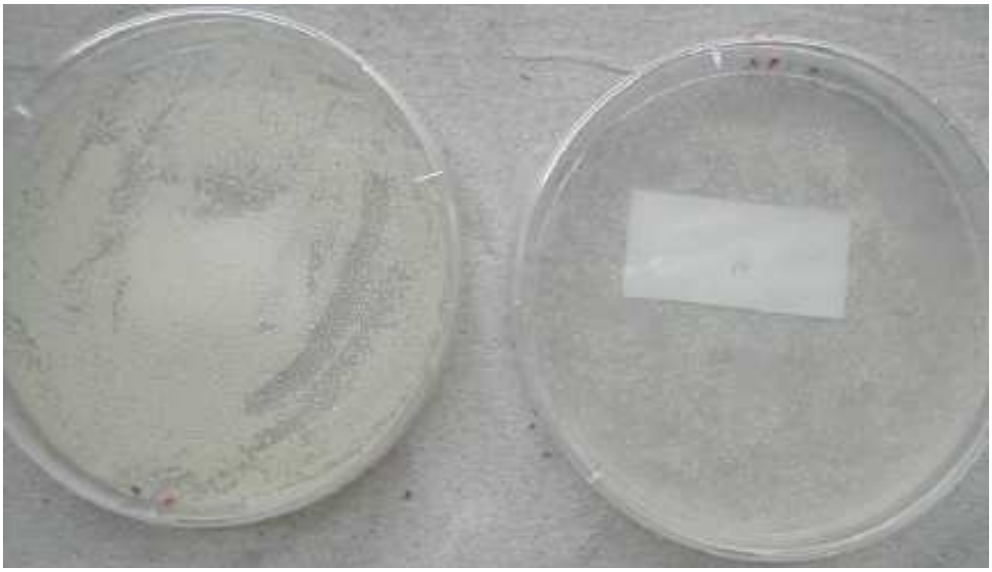
Fig 9) a) Arrangment of the clones and control strains for the Hnl assay b) Image of Hnl assay detection filter. RecHNL → Recombinant Hnl enzyme, +C → Positive control strain X-33 strain pPICZB HNL tunnel mutant W128A, -C → Negative control *P. pastoris* GS115.

Small zeocin resistant clones were re-checked for their viability onto MD and antibiotic plates. After 3 days of incubation, positive control strain grew on all plates, negative control strains grew only on MD and BMM sorbitol plates. Test strains grew on all plates although growth was slower because these strains were found to be Mut^S. After confirming antibiotic resistance, Hnl expression was confirmed by Feigl-Anger filter assay (Fig 11).

Pichia pastoris transformants which showed both Hnl expression and antibiotic resistance were tested for ectopic integration of the zeocin resistance marker by plating the uninduced clones onto the MD plates with and without zeocin (Fig 12). Without induction none of the clones grew on MD zeocin plates arguing against ectopic integration of the zeocin resistance

cassette. Upon methanol induction the same clones grown on BMM sorbitol plates with and without zeocin (Fig 13).

In order to confirm the integrity of the expression cassette insert, one clone per construct was selected for colony PCR with primer pairs *EcoR*I *HNL* FP and Zeo *EcoR*I RP giving a 1155 bp PCR products as expected. On sequencing colony PCR products showed intact translational coupling constructs (Fig 14).



a b

Fig 10: Five hundred-fold dilution of cell cultures plated onto BMMS (a) and BMMS 100 µg/ml zeocin plates (b). On incubation they show a lawn of cell culture on methanol plates but also many isolated colonies on the antibiotic plates as shown above.

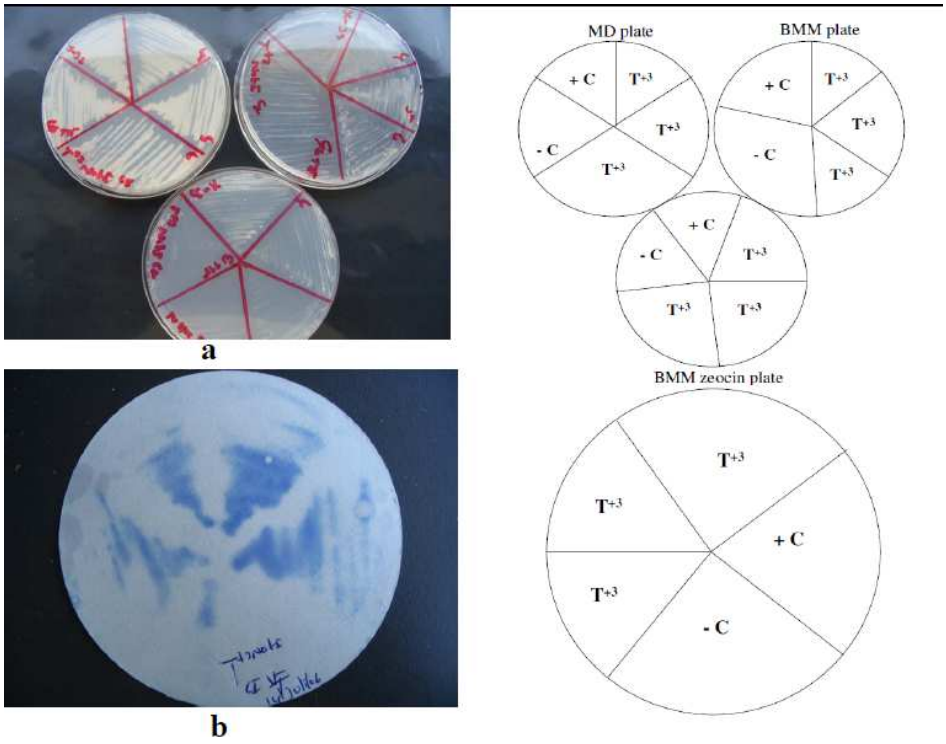


Fig 11: Clones recovered from antibiotic plates were restreaked on MD, BMM and BMM zeocin plates to check for viability and zeocin resistance of the clones (a). Colonies grown on methanol plates were assayed for Hnl activity as shown above (b).

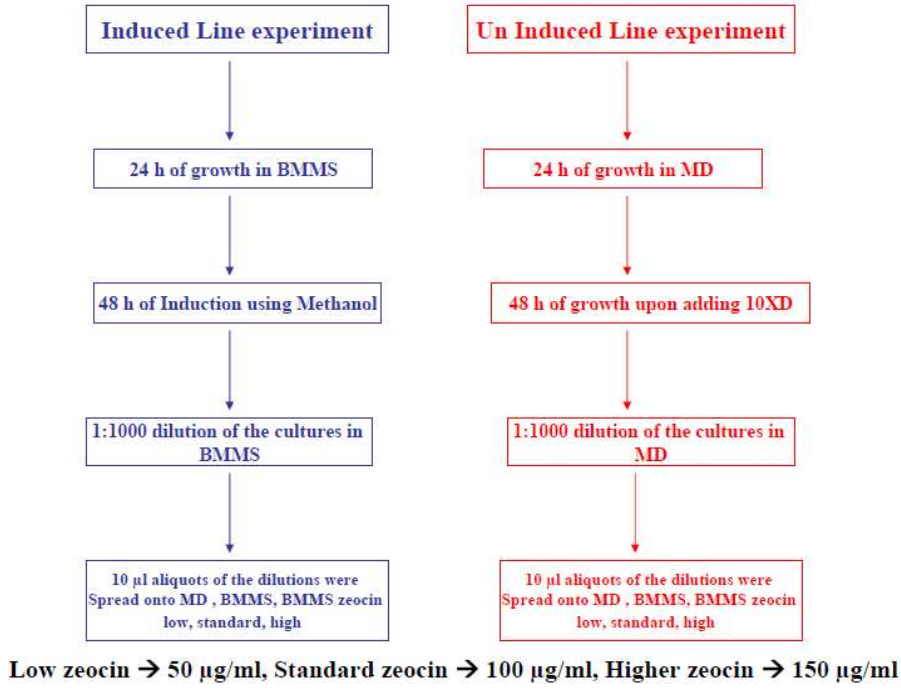


Fig 12: Growth and induction protocol for deep-well fermentation to check for ectopic integrations

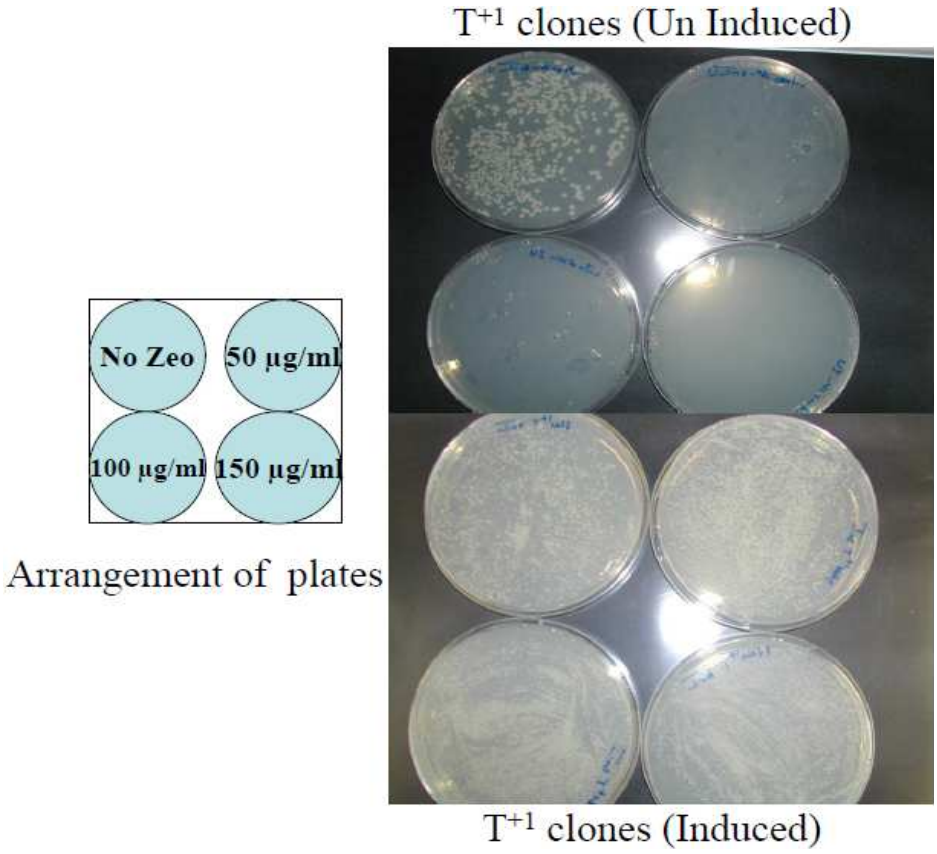


Fig 13: Upper figure shows the T^{+1} clones grown on dextrose medium and plated onto MD plates with and with out zeocin. Growth is confined to the antibiotic-free plates. Lower figure shows the induced clones growing on all the plates.

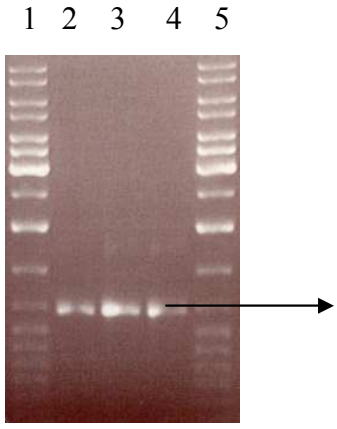


Fig 14: Results from colony PCR showing a band at 1155 bp atmost coinciding with the band standard at 1200 bp. Gene ruler DNA ladder from Fermentas (1,5), PCR product from T^{+1} clone (2), PCR product from T^{+2} clone (3) , PCR product from T^{+3} clone (4).

Backward screening- Correlating Hnl expression to zeocin resistance

As the initial experiments to correlate zeocin resistance and Hnl expression did not show any differences between the constructs containing one, two or three stop codons between Hnl and zeocin resistance coding regions further work was continued on the translational coupling construct with a single stop codon. Following a new round of transformation an induced culture was diluted 1:10^2, 1: 10^4 and 1:10^5 respectively. 200 µl aliquots of each dilution were plated onto plates MD, BMMS and BMMS zeocin plates containing 50 µg/ml, 100 µg/ml, and 150 µg/ml of antibiotic respectively. Yeast colonies were obtained on all the plates (Fig 15). There was perfect correlation between the number of colonies on zeocin containing plates and the respective dilutions.

After obtaining minute yeast colonies on all plates, randomly eight clones each from MD, BMMS, and BMMS 150 µg/ml zeocin plates were selected, grown and induced with methanol. After 48 h of induction, cell cultures were diluted 40 fold in BMM medium and pinned onto YPD, BMMS, and BMMS zeocin plates (Fig 17). OD_{600} of the dilutions were determined by a plate reader (Fig 16).

In order to check for the absence of the ectopic integrations paralelly dilutions have also been pinned on to the MD zeocin plates. All the clones including control grew on the YPD and BMMS plates. Clones originating from BMMS plates could resist up to 50 µg/ml zeocin, where as clones originating from plates with higher zeocin concentrations grew up to 150 µg/ml zeocin. On MD 100 µg/ml zeocin plates only positive control strain for antibiotic resistance grew indicating the absence of ectopic zeocin resistance integrations.

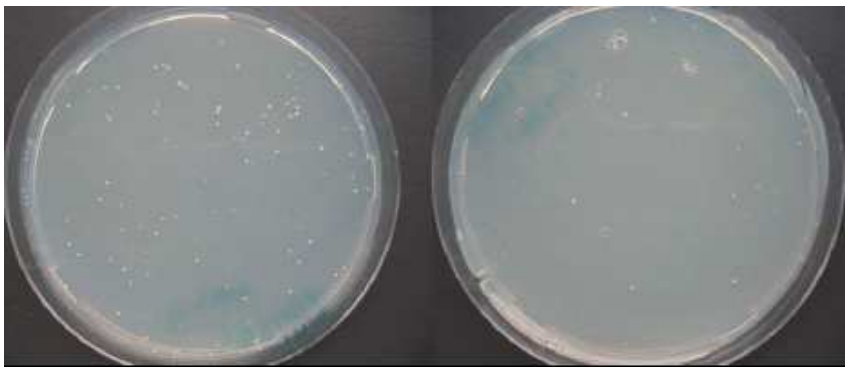
Yeast cells were harvested and lysed with Y-PER (Yeast Protein Extraction Reagent) for Hnl activity assay. After Y-PER treatment, samples were spun to remove debris. The resulting supernatants i.e. concentrated enzyme preparations, were diluted with citrate phosphate buffer pH 5. On adding the substrate metaphenoxy benzaldehyde cyanohydrin Hnl activity released gaseous HCN which passed through the nylon mesh to react with copper acetate and tetra base on the detection filter producing distinct blue spot (Feigl-Anger test) (5). Clones which had resisted 150 µg/ml zeocin in the screening showed Hnl activity (Fig 18).

Randomly few clones which had shown Hnl activity were selected for Western blotting. On developing the blot, a clear band was observed between the 25 and 35 kDa bands of the

standard indicating the expression of Hnl protein (Fig 19). The molecular weight of the Hnl is 29.2 kDa and zeocin resistance protein is 125 aa long is can be concluded that Hnl and zeocin resistance protein are not covalently linked.

Translational coupling construct was amplified from genomic DNA by PCR and analysed. by gel electrophoresis showing a DNA band in the range of 1345 bp (Fig 20).

A B C

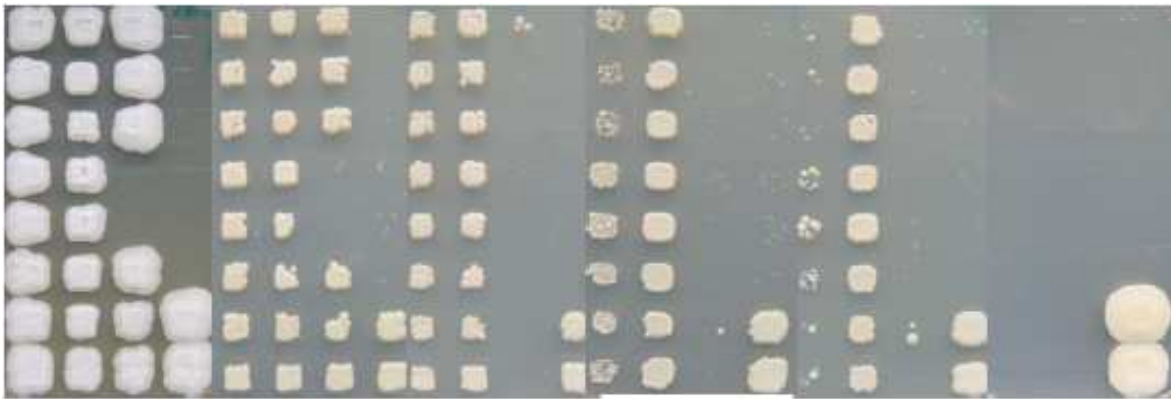


D E

Fig 15: The total number of yeast cells in an induced culture was determined to be 4.9 x 10^8/ml. This culture was diluted in three steps primarily hundred fold dilution of the original culture (4.9 x 10^8/ml → 4.9 x 10^6/ml), then a further hundred fold dilution (4.9 x 10^6/ml → 4.9 x 10^4/ml) and final ten fold dilution (4.9 x 10^4/ml → 4.9 x 10^3/ml). 200 µl aliquots of each dilution were plated onto the MD, BMMS and BMMS with different concentrations of the antibiotic zeocin. Roughly 1000 colonies were on MD (A), BMMS (B), BMMS (50 µg/ml) zeo (C), BMMS (100 µg/ml) zeo (D), BMMS (150 µg/ml) zeo (E).

0.111 T+1 HNLZEO	0.091 T+1 HNLZEO	0.054 HNL W128A SCS	
0.049 T+1 HNLZEO	0.074 T+1 HNLZEO	0.032 HNL W128A SCS	
0.087 T+1 HNLZEO	0.067 T+1 HNLZEO	0.069 HNL W128A SCS	
0.086 T+1 HNLZEO	0.050 T+1 HNLZEO		
0.116 T+1 HNLZEO	0.064 T+1 HNLZEO		
0.089 T+1 HNLZEO	0.059 T+1 HNLZEO	0.048 HNL MCS	
0.087 T+1 HNLZEO	0.052 T+1 HNLZEO	0.044 HNL MCS	0.038 -C GFP
0.066 T+1 HNLZEO	0.087 T+1 HNLZEO	0.038 HNL MCS	0.046 -C GFP

Fig 16: OD_{600} values from of diluted cultures used for pinning of selected clones and control cells onto different media. T^{+1} HNLZEO → translational coupling construct with single stop codon, HNL W128A → *P. pastoris* GS115 pHIL-D2 Hnl tunnel mutant W128 A is a single copy strain, HNL MCS → *P. pastoris* GS115 pHIL-D2 1.17-Hnl is a *HNL* multi copy strain



A B C D E F

Fig 17: Transformants and control strains were pinned onto YPD (A), BMMS (B), BMMS (50 µg/ml) zeo (C), BMMS (100 µg/ml) zeo (D), BMMS (150 µg/ml) zeo (E), and MD (100 µg/ml) zeo (F).

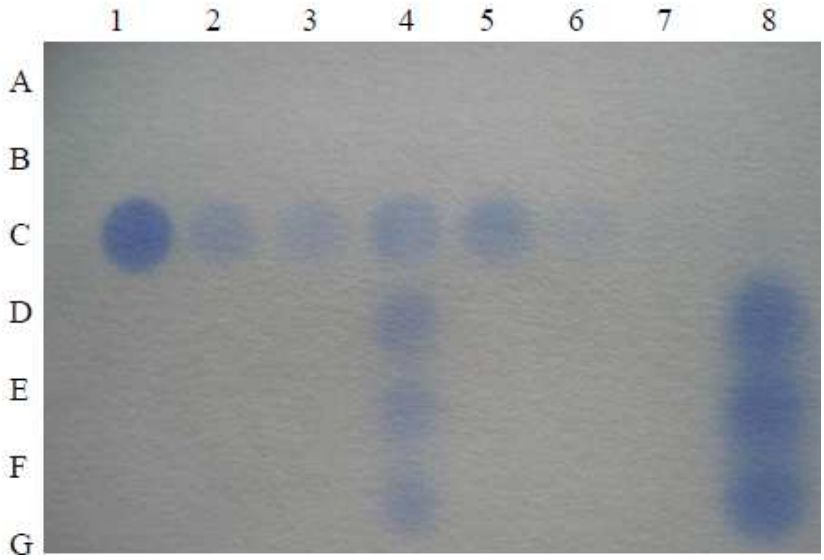


Fig 18: Detection filter after Hnl assay Row (A) shows clones taken from BMMS plates, row (C) shows the clones taken from BMMS (150 µg/ml) zeocin plates. Lower part of lane 1 are the X-33 GFP expressing strains (Negative control), Lane 4 is *P. pastoris* GS115 pHIL-D2 Hnl tunnel mutant W128 A is a single copy strain, Lane 6 is *P. pastoris* GS115 pHIL-D2 1.17-Hnl is a *HNL* multi copy strain.

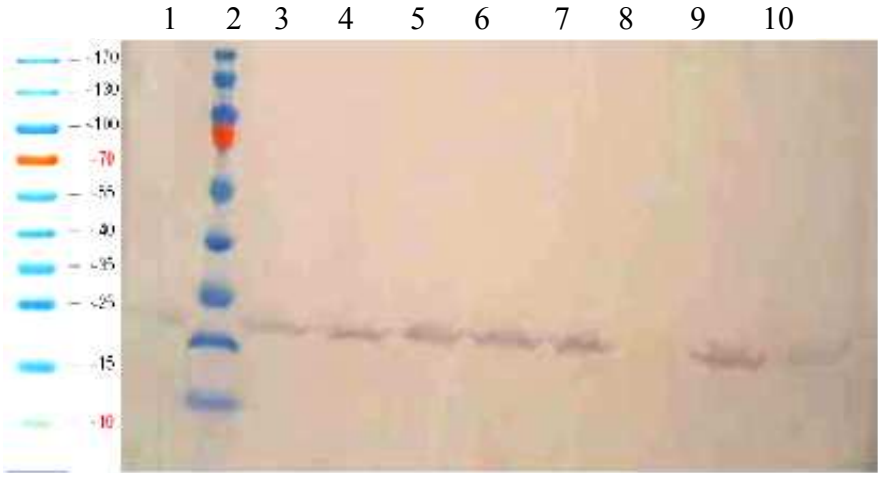


Fig 19: Western blotting of yeast whole cell lysates of clones which resisted high doses of zeocin. Hnl expression was verified by the western blot to asses the size of the expressed protein. Molecular mass of Hnl is 29.2 kDa. Whole cell lysates from the *P. pastoris* GS115 pHIL-D2 Hnl tunnel mutant W128A (1), Page ruler Pre stained from Fermentas (2), Whole cell lysates from the test samples (3, 4, 5, 6, 7, 9, 10), and whole cell lysates from the X-33 GFP expressing strain (8) as negative control were applied.

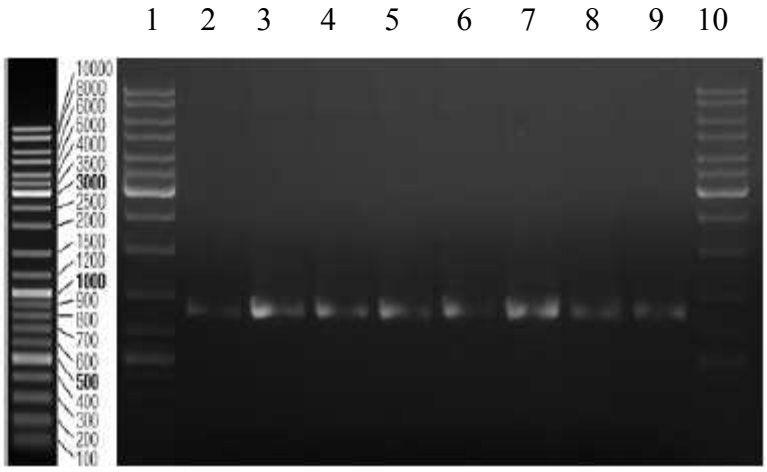


Fig 20: Agarose gel electrophoresis of PCR products. Products were assessed to be (1345 bp). DNA Ladder mix from Fermentas (1, 10), PCR product from a recombinant plasmid as positive control (2), and PCR products from the yeast transformants containing translational coupling construct (3-9) were applied.

Discussion:

High through-put screening is a most relative issue in the fields of pharmaceuticals, recombinant protein expression and protein engineering. Primarily in the field of pharmacology high through-put screening (HTS) is employed in drug discovery to screen a large number of potential compounds against a biological target (14). Secondarily, in the area of recombinant protein expression and protein engineering, screening for clones which express high levels of recombinant protein or proteins with improved properties from a large pool of protein variants is challenging and it is only possible with HTS. If the expressed recombinant protein is an enzyme, setting-up the screening system based on the enzymatic activity is much more convenient straightforward and last but not least user friendly. In some cases, it is not possible to set up a screening system in high through-put format due to non-availability of appropriate substrates, as the protein to be expressed does not show enzymatic activities etc. In such situations setting up the screening system is laborious, it leading to trial and error methods such as performing Western blots for a limited number of clones. Additionally it may be possible to select for clones with increased antibiotic resistance reasoning that higher antibiotic resistance may imply higher plasmid copy number and thus higher expression level.

To overcome this type of problem we report a new screening system based on translational coupling in *Pichia* which is applicable for expressing any recombinant protein. In the proposed screening system a gene of interest is linked by translational coupling to the expression of antibiotic resistance in a bicistronic manner. Bicistronic mRNA transcribed from this construct is translated by a single ribosome expressing two proteins individually and separately. This phenomenon is seen in bacteria where polycistronic mRNA is translated by a single ribosome. To execute the proposed project we choose *Hevea brasiliensis HNL* as a gene of choice for recombinant protein expression. *Hb-HNL* is a 29 kDa single chain protein with a typical α/β hydrolase fold catalysing stereo-specific reversible addition of HCN to aldehydes or ketones forming the corresponding α – hydroxynitriles. *Hb-HNL* was expressed in *P. pastoris* intracellularly at a high cell density of > 100 g cell dry weight /liter yielding 22 g/liter of the heterologous protein in the cytoplasm(12). Expressed *Hb-HNL* is detected by performing enzymatic assays in vivo and in vitro using the substrates mandelonitrile or metaphenoxy benzaldehyde. The gaseous HCN which is liberated by the hydroxynitrile lyase reaction can be detected by a visible, blue chromogenic reaction on a detection filter (5).

Antibiotic marker *Sh ble* gene has been applied in constructing the translational coupling construct. The advantage of taking this marker is that the coding sequence of the corresponding gene is short i.e. 375 bp, and expression thereof can be visualized by resistance against zeocin (7,8-10,15).

After transforming the translational coupling constructs, gene expression was verified by performing *HNL* assays and determining yeast cell resistance to zeocin. To detect protein expression from the translational coupling construct Western blots of whole cell lysate samples was decorated with a specific anti-*Hb-Hnl* antibody (13). On developing the blots with the BCIP/NBT a blue colour band at ~29 kDa coinciding with the Hnl molecular weight was observed indicating the expression of two independent proteins from the bicistronic mRNA.

In order to detect the efficiency of translational coupling construct and to correlate zeocin resistance with Hnl expression clones which exhibited resistance to 200µg/ml of zeocin were analysed for Hnl activity. In order to exclude that high zeocin resistance was derived from ectopic integrations of zeocin resistance marker, uninduced clones which had been pinned onto the BMMS zeocin plates has also been paralelly stamped onto MD zeocin plates. Transcribing zeocin resistance from a constitutive promoter only zeocin resistant control strains pinned grew on MD zeocin plates, whereas negative controls and the test clones did not grow, antibiotic resistance most likely arose from intact translational coupling constructs. Secondarily this growth pattern also gives a clue for the absence of the ectopic integrations in the test clones. In order to detect the presence of the intact construct at the genomic level, PCR on genomic DNA of the selected clones was performed using standard 5' *AOX1* and 3' *AOX1* sequencing primers. On sequencing the resulting PCR products intact DNA of the translational coupling construct without any mutations was found thus confirming translational coupling for *P. pastoris*.

We propose that we have established a new screening system based on the principle of translational coupling in *Pichia pastoris* and we have a proof of principle using the *Hb-HNL*-zeocin resistance system. The screening system should be tested with a further reporter systems and finally, be broad to application in a genuine HTS approach.

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Abbrevations

1) *ACT1* = Gene encoding actin protein
2) *AOXI* = Alcohol oxidase I gene
3) AS I , II = Antisense DNA/cDNA I and II
4) BMM = Buffered minimal methanol
5) BMMS = Buffered minimal methanol sorbitol
6) BSA = Bovine serum albumin
7) BYPD = Buffered Yeast extract, peptone, dextrose
8) CB staining = Coomassie blue staining
9) cDNA = Complementary DNA
10) CGI-58 = Comparative Gene Identification-58
11) CIP = Calf intestinal alkaline phosphatase
12) CO_2 = Carbon dioxide
13) CPY = Carboxy peptidase Y
14) DEPC = Diethylpyrocarbonate
15) dsRNAs = Double stranded RNAs
16) DTT = Dithiothreitol
17) ER lumen = Endoplasmic reticulum lumen
18) FAM = amine-reactive succinimidyl esters of carboxyfluorescein (fluorescent dye)
19) GFP = Green fluorescent protein
20) GST-tag = glutathione S-transferase tag
21) *Hb HNL* = *Hevea brasiliensis* hydroxynitrile lyases
22) HCN = Hydrogen cyanide
23) *HIS4* = Gene encoding for ~2.4 kb histidinol dehydrogenase
24) LB medium = Luria-Bertani medium
25) MD = Minimal dextrose
26) MM = Minimal methanol
27) MOPS = 3-(N-morpholino)propanesulfonic acid
28) N2 = Liquid nitrogen
29) NBIT-BCIP = Nitro blue tetrazolium chloride- 5-Bromo-4-chloro-3-indolyl phosphate
30) NTC = No template control
31) PBS = Phosphate buffered saline
32) PBS^{++} = Phosphate buffered saline, protease inhibitor ,1 M DTT
33) PCI solution = Phenol: Chloroform: Isoamylalcohol (25: 24: 1 v/v)

34) Protease inhibitor = 20 µg/ml leupeptin, 2 µg/ml antipain, 1 µg/ml pepstatin

35) PTGS = Post transcriptional gene silencing

36) qPCR = Quantitative PCR

37) RISC = RNA-induced silencing complex

38) RNasE = Ribonuclease enzyme

39) ROX dye = Glycine conjugate of 5-carboxy-X-rhodamine

40) RT-PCR = Reverse transcription polymerase chain reaction / real-time polymerase chain reaction

41) SDS = Sodium dodecyl sulfate

42) SDS-PAGE = Sodium dodecyl sulfate polyacrylamide gel electrophoresis

43) si RNA = Short interfering RNA

44) T^{+1}, T^{+2}, T^{+3} = Translational coupling constructs with one, two and three stop codons

45) TAMRA = Tetramethyl-6-Carboxyrhodamine (fluorescent dye)

46) Tgl3p and Tgl4p = TriacylGlycerol Lipase 3 and 4 from *S. cerevisiae*

47) UDG = uracil DNA glycosylase

48) *VPS10* = Type I transmembrane sorting receptor for multiple vacuolar hydrolases

49) YPD = Yeast extract peptone dextrose medium

50) Y-PER = Yeast protein extraction reagent

51) YPS = yeast extract, peptone and sorbitol

Media Recipes

LB medium composition (1 l)

Bacto-Tryptone 10 g

Bacto-yeast extract 5 g

NaCl 10 g

YPD medium (1 l)

Yeast extract 1%

Peptone 2%

Dextrose 2%

BYPD (1 l)

Potassium phosphate, pH6.0 100 mM

Yeast extract 1%

Peptone 2%

Dextrose 2%

4/5BYPD

Potassium phosphate, pH6.0 200 mM

Yeast extract 0.8%

Peptone 1.6%

Dextrose 1.6%

MD (1 l)

Yeast nitrogen base 1.34%

Biotin $4x\ 10^{-5}$

Dextrose 2%

BMD (1 l)

Potassium phosphate, pH6.0 200 mM

Yeast nitrogen base 1.34%

Biotin $4x\ 10^{-5}$

Dextrose 2%

MM (1 l)

Yeast nitrogen base	1.34%
Biotin	$4x\ 10^{-5}$
Methanol	0.5%

BMM (1 l)

Potassium phosphate, pH6.0	100 mM
Yeast nitrogen base	1.34%
Biotin	$4x\ 10^{-5}$
Methanol	0.5%

BMMS (1 l)

Potassium phosphate, pH6.0	100 mM
Yeast nitrogen base	1.34%
Biotin	$4x\ 10^{-5}$
Sorbitol	2%
Methanol	0.5%

YPS (1 l)

Sorbitol	1 M
Yeast extract	1%
Peptone	2%

RD (1 l)

Sorbitol	1M
Dextrose	2%
Yeast nitrogen base	1.34%
Biotin	$4x\ 10^{-5}$

Note: Additionally 2% of agar is added to respective media to make agar plates

Buffer compositions

1xTBST buffer:
50 mM Tris. HCl, 150 mM NaCl, 0.1% Tween 20

1 M potassium phosphate buffer, pH6.0:
132 ml of 1M K_2HPO_4, 868 ml of 1M KH_2PO_4, pH is adjusted to 6.0

Citrate-Phosphate-Buffer (0.05 mol/l; pH 5.0):
a) 10.51 g Citric acid-Monohydrate in 1000 ml Distilled water
b) 11.41 g $K_2HPO_4.3H_2O$ in 1000 ml Distilled water
1000 ml solution (b) in to 500 ml solution (a) set final pH of the solution to 5.0

Citrate-Phosphate-Buffer (0.1 mol/l; pH 3.5):
a) 21.02g Citric acid-Monohydrate in 1000 ml Distilled water
b) 22.82g $K_2HPO_4.3H_2O$ in 1000 ml Distilled water
Solution (b) in to solution (a) set final pH of the solution to 3.5

H-buffer:
250 µl 8M LiCl, 800 µl 0.25M EDTA, 200 µl 1M Tris (pH 7.5), 500 µl 20% SDS, 18.25 ml frisenius water

TE-8 buffer:
10mM Tris-HCl (pH 8.0), 1 mM EDTA

Elution Buffer:
50 mM Tris(pH 8.0), 15 mM reduced glutathione (aliquot and store at -20°C), 1M NaCl, 10% Glycerol, 0.1% NP-40 or Triton x-100, 1 mM EDTA, + 1M DTT, 1 mM benzamidine, 1 mM PMSF

Protoplast solution:
6.5 mg/ml Zymolyase, 1 M Sorbitol, 67 mM potassium phosphate buffer pH 7.4, 0.1% mercapthoethanolamine

PBS buffer:

150 mM KCl, 10 mM potassium phosphate buffer pH 7.4

PBS^{++}:

50 ml 1x PBS, 50 µl protease inhibitor, 50 µl 1M DTT