Expression of the *Rhizopus oryzae* lipase in *Pichia pastoris* under the control of the *FLD1* promoter

---

PhD Thesis
2006

David Resina Rodriguez

Departament d'Enginyeria Química
Universitat Autònoma de Barcelona

FRANCESC VALERO BARRANCO, professor titular del Departament d'Enginyeria Química i PAU FERRER ALEGRE, investigador de la Universitat Autònoma de Barcelona,

CERTIFIQUEM:

Que el Llicenciat en Biologia David Resina Rodríguez ha dut a terme sota la nostra direcció el treball amb el títol "Expression of the *Rhizopus oryzae* lipase in *Pichia pastoris* under the control of the *FLD1* promoter" que es presenta en aquesta memòria i que constitueix la seva Tesi per optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

I perquè es prengui coneixement i consti als efectes oportuns, presentem a l'Escola Tècnica Superior d'Enginyeries de la Universitat Autònoma de Barcelona l'esmentada Tesi, signant el present certificat a

Bellaterra, Juny 2006

Dr. Francesc Valero Barranco Dr. Pau Ferrer Alegre

Haig de donar les gràcies per haver-me ajudat a dur a terme aquest treball a la meva família i a la Yolanda que han tingut i tenen tanta paciència amb mi.

Als meus directors, en Paco i en Pau els hi vull agrair l'oportunitat que m'han donat per estar aquí, i evidentment per la seva ajuda y el seu recolzament durant tot el temps que he passat amb ells.

Als companys i amics de grup amb els que m'he divertit tant fent la tesi, la Surri, en Ramon, l'Oriol, l'Anna Ubach, en Manuel, i els nouvinguts Kristin, Carol i Albert.

Vull donar les gràcies també als companys i companyes de despatxos on hem passat molt bones estones, en Juli, en Martí, en Soley, l'Enric, l'Ernest, en Marcos, el Juan Pedro, la Mar, en Luis i molt d'altres que em deixo....

També a la Montse i la Nati per la seva ajuda i a la Vanessa, la Lorena i la Noelia pel seu sentit del humor.

Com no als amics que he fet durant aquest trajecte, a la Maite, en Jaume, la Paqui, la Sandra, l'Albert Guisasola, la Trinitat, la Irene, la Neus, la Núria, la Cristina Sendra, l'Anna Montràs i en Bartro

Thanks to the people from Dr Mattanovich's laboratory, specially Gitti, Hansi, Michael and Didi for their hospitality and friendship.

També als amic d'Oulu i finalment també al Ministerio de Ciencia y Tecnología que em va concedir la beca per a realitzar aquest treball.

Table of contents

Summary..... 1

Objectives..... 5

Chapter 1. Introduction..... 7

1.1 The methylotrophic yeasts..... 7

1.2 *P. pastoris* as a protein expression system. 7

1.2.1 Promoters..... 8

1.2.2 Strains and phenotypes..... 9

1.2.3 Vectors..... 10

1.2.4. Integration into the genome..... 11

1.2.5. Secretion signal..... 12

1.3 Cultivation techniques..... 13

1.4 The *Rhizopus oryzae* lipase..... 14

1.5 Previous works with ROL..... 14

1.6. References..... 16

Chapter 2. Materials and methods..... 19

2.1 Strains..... 19

2.2 Culture maintenance..... 19

2.3 Inoculum preparation..... 19

2.4 Glycerol, methanol, sorbitol, methylamine and ammonium determination.. 20

2.5 Lipase activity determination..... 20

2.6 Expression of ROL in *E. coli* for polyclonal antibody production..... 21

2.7 SDS-PAGE and western blotting analyses..... 22

2.8 Protease activity determination..... 23

2.9 Total protein determination..... 23

2.10 Biomass analysis..... 23

2.11 Biomass elemental composition..... 23

2.12 Molecular biology methods..... 23

2.12.1 Reagents and enzymes..... 23

2.12.2 DNA extraction..... 23

2.12.3 Agarose gel electrophoresis..... 24

2.13 Transformation..... 24

2.13.1. *E. coli* transformation..... 24
2.12.2 *P. pastoris* transformation..... 24
2.14 References..... 26
Chapter 3. Cloning and expression of the *Rhizopus oryzae* lipase in *P. pastoris* under the control of the nitrogen source-regulated *FLD1* promoter..... 27
3.1 Introduction..... 27
3.1.1 Methanol metabolism..... 28
3.1.2 Methylamine metabolism..... 29
3.1.3 Previous studies and aims of the work..... 30
3.2 Materials and methods..... 31
3.2.1 Strains..... 31
3.2.2 Media composition..... 31
3.2.3 Shake flask and bioreactor cultivation conditions…..... 32
3.2.4 Plasmid and strain construction…..... 33
3.3 Results..... 35
3.3.1 Construction of the *P. pastoris* production strain……..... 35
3.3.2 ROL expression under the control of *PFLD1* and comparison to *PAOX1* in shake flask cultures..... 35
3.3.3 Bioreactor batch cultivations..... 38
3.4 Conclusions..... 42
3.5 References..... 44
Chapter 4. Developing fed-batch cultivation strategies for heterologous production of *R. oryzae* lipase in *P. pastoris* using the *FLD1* promoter..... 47
4.1 Introduction..... 47
4.2 Materials and methods..... 48
4.2.1 Strains..... 48
4.2.2 Fed-batch cultivation set up and operational conditions..... 48
4.2.3 Western blot analyses..... 49
4.2.4 Pre-programmed exponential feeding rate..... 49
4.2.5 Calculation of specific rates..... 50
4.3 Results..... 51
4.3.1 Fed-batch cultures with *FLD1*-based system..... 51
4.3.2 Comparision of *FLD1*-based fed-batch cultures with the classic *AOX1*

system..... 58
4.4 Conclusions..... 61
4.5 References..... 62
Chapter 5. Analysis and engineering of bottlenecks in *R. oryzae* lipase production in *P. pastoris* using the nitrogen source-regulated *FLD1* promoter.. 65
5.1 Introduction..... 65
5.2 Materials and methods..... 68
5.2.1 Strains..... 68
5.2.2 Flow cytometry..... 69
5.2.2.1 Immunofluorescence staining..... 69
5.2.2.2 Viability staining..... 71
5.3 Results..... 72
5.3.1 Monitoring cell's viability in fed-batch cultivations of *P. pastoris* expressing ROL under the control of the *PFLD1*..... 72
5.3.2 Monitoring of intracellular BiP and intra/extracellular ROL levels in fed-batch cultivations of *P. pastoris* expressing ROL under the control of the *PFLD1*..... 74
5.3.3 Monitoring of intracellular BiP and intra/extracellular ROL levels in fed-batch cultivations of *P. pastoris* co-expressing *HAC1* constitutively and ROL under the control of the *PFLD1*..... 79
5.3.4 Comparison of X-33 versus GS115H/HAC1 strains producing ROL.... 83
5.4 Conclusions..... 87
5.5 References..... 89
Chapter 6. Quantitative analysis of specific mRNAs of P. pastoris cells growing in fed-batch cultivations using a sandwich hybridization assay..... 93
6.1 Introduction..... 93
6.2 Materials and methods..... 95
6.2.1 Strains..... 95
6.2.2 Shake flask cultures..... 95
6.2.3 Sample preparation..... 96
6.2.4 Yeasts lysis and RNA extraction..... 96
6.2.5 Oligonucleotides probes design and synthesis..... 96
6.2.6 Synthesis of in vitro transcripts for standard values..... 98

6.2.7 Sandwich hybridization assay..... 98
6.3 Results..... 100
6.3.1 Shake flask experiment..... 100
6.3.2 Fed-batch fermentations..... 102
6.3.2.1 Fed-batch of X-33 strain at a low specific growth rate..... 103
6.3.2.2 Fed-batch of X-33 strain at specific growth rate near to maximum..... 104
6.3.2.3 Fed-batch of the GS115H strain at specific growth rate near to maximum..... 106
6.3.2.4 *26S* rRNA analyses..... 108
6.3.3 Discussion..... 108
6.4 Conclusions..... 110
6.5 References..... 111
Chapter 7. Deletion of the P. pastoris *GAS1* gene and its application in ROL secretion..... 115
7.1 Introduction..... 115
7.2 Materials and methods..... 116
7.2.1 Strains..... 116
7.2.2 Cloning and disruption of the *GAS1* homologue in *P. pastoris*..... 116
7.3 Results..... 117
7.3.1 Shake flask experiments..... 117
7.3.2 Bioreactor fed-batch cultivations..... 117
7.4 Conclusions..... 122
7.5 References..... 123
Chapter 8. Cloning and expression of a GFP-ROL fusion protein in *P. pastoris*..... 125
8.1 Introduction..... 125
8.2 Materials and methods..... 126
8.2.1 Strains..... 126
8.2.2 Media composition..... 126
8.2.3 Shake flask and bioreactor cultivation conditions..... 127
8.2.4 Plasmid and strain construction..... 127
8.2.5 Fluorescence measurements..... 131

8.2.6 FITC straining for total protein..... 131
8.2.7 Flow cytometry measurements..... 131
8.2.8 Confocal microscopy..... 132
8.2.9 Western blot analyses..... 132
8.3 Results..... 132
8.3.1 Constructions and isolation of transformants expressing GFP/ROL fusions..... 132
8.3.2 GFP-ROL expression analysis in shake flask cultivations..... 133
8.3.3 Bioreactor batch culture..... 135
8.3.3.1 Fluorimetric analyses from batch culture samples..... 136
8.3.3.2 Intracellular fusion protein content analyses..... 140
8.3.3.3 Flow cytometry analyses..... 142
8.3.3.4 In situ analysis of product subcellular localization by confocal microscopy..... 143
8.4 Conclusions..... 145
8.5 References..... 147
Conclusions..... 151
List of publications..... 155
Notes..... 157

SUMMARY

During the course of this work, the expression of a *Rhizopus oryze* lipase (ROL) under the control of the *FLD1* promoter in the methylotrophic yeast *Pichia pastoris*, as well as the development of methanol-free cultivation processes and metabolic engineering strategies for process improvement has been carried out.

First, an expression vector containing the *ROL* gene and the *FLD1* promoter from *P. pastoris* was constructed and subsequently transformed in *P. pastoris*. Subsequent studies in shake flask and bioreactor batch cultivations revealed the synergetic effect of methanol and methylamine on the *FLD1* promoter induction. Moreover, the use of sorbitol as carbon source, combined with methylamine as nitrogen source, was found to be an attractive alternative to the use of methanol for high cell density cultivations.

Second, a new fed-batch fermentation strategy was developed using sorbitol and methylamine as carbon and nitrogen source, respectively. Three fed-batches were performed at different specific growth rates, 0.005 h^{-1}, 0.01 h^{-1}, and 0.02 h^{-1}. The specific growth rate was controlled during induction phase in the 0.005 h^{-1}, 0.01 h^{-1} fermentations by implementing an exponential feeding rate at limiting substrate concentration. In the near-to-maximum specific growth rate fermentation performed at about 0.02 h^{-1}, sorbitol concentration was keep into the medium at about 8 $g \cdot L^{-1}$ throughout the induction phase. The highest lipase productivity was obtained in the fed-batch performed at the 0.02 h^{-1} specific growth rate. However, product production rate of all three fermentations dropped soon after reaching a maximum at about 30 hours of culture.

Third, in order to analyze potential bottlenecks in the protein folding or secretion pathway and, in particular, the potential activation of the unfolded protein response (UPR) during ROL overexpression and intracellular accumulation, the levels of a reticle endoplasmatic-resident chaperone (BiP), its induction has been related to the activation of the UPR, as well as the intracellular levels of ROL were analyzed by immunofluorescence staining combined with flow cytometry. The increase of BiP and

ROL levels during the induction phase of fed-batch cultivations indicated the possible activation of the UPR.

Fourth, based on these analytical studies, a metabolic engineering strategy was proposed to alleviate the supposed bottleneck in the folding and secretion pathway. The *Saccharomyces cerevisiae HAC1* gene was cloned and constitutively expressed in *P. pastoris* co-expressing ROL. The *HAC1* gene codifies for a transcriptional factor which activates the UPR related genes expression. Thus, the constitutively activation of the UPR was aimed at sustaining a better protein folding and secretion efficiency. Results from fed-batch cultivations with the HAC1-engineered strain showed an increase in the heterologous product expression, demonstrating the positive effect of such modification on protein folding and secretion.

Besides, a transcriptional analysis of some bioprocess-relevant genes of the ROL-expressing *P. pastoris* strains was carried out. In particular, the alcohol oxidase 1 (*AOX1*), formaldehyde dehydrogenase (*FLD1*), protein disulfide isomerase (*PDI*), binding protein (*KAR2*), 26S rRNA and the recombinant product (*ROL*) mRNA levels were monitored in both shake flask and fed-batch cultivations. The mRNA levels of these genes were quantified by using a sandwich hybridization assay based on immobilization of the desired mRNA on magnetic beads and subsequent detection by a fluorescence-based technique. The specific growth rate showed to be a key factor in the transcriptional level of the analyzed genes; moreover, the results gave additional evidence to the hypothesis that ROL triggers the UPR.

Fifth, a second genetic modification was carried out in order to improve the secretion efficiency of ROL. The *P. pastoris GAS1* gene, which codifies for a glycoprotein anchored to the outer layer of the plasma membrane performing cross links between the cell wall β-glucans, was disrupted. The deletion of this gene provoked a change in the cell wall conformation, causing an increase of the cell wall porosity and permeability, i.e. allowing for a better release of the recombine product. Although the mutant strain experienced a reduction of the maximum specific growth rate, the specific productivity, compared to the control strain, was doubled.

Finally, in order to gain further insights on the potential (subcellular) accumulation of the recombinant product, the ROL gene was fused with the *Aequorea victoria* green

fluorescence protein (GFP) gene and expressed in *P. pastoris*. The GFP was fused in N-terminus and C-terminus with ROL. However only the GFP-ROL fusion transformants showed detectable lipase activity. A batch culture was performed with the GFP-ROL fusion expressing strain, and compared with the ROL expressing control strain. The expression of the ROL as a fusion protein caused an important decrease in the extracellular levels of the recombinant lipase activity when compared with the results obtained with the control strain. Moreover, the quantification of the extracellular GFP fluorescence levels by direct fluorimetry was not possible due to the presence in the culture broth of a fluorescent by-product produced by *P. pastoris* during cultivation. The removal of this product (probably riboflavin) from culture supernatants allowed the detection of GFP fluorescent signal. On the other hand, the intracellular accumulation of the fusion product was followed by confocal microscopy and flow cytometry. The GFP-fusion protein appeared to accumulate in the cell wall periplasm as well as intracellularly into large, vacuole-likely, compartments.

Overall, this study has allowed the development and improvement of the *P. pastoris* expression system based on the formaldehyde deshydrogenase promoter as an alternative to the classic methanol inducible alcohol oxidase promoter.

OBJECTIVES

The main objective of this thesis is to express the *Rhizopus oryzae* lipase in *Pichia pastoris* under the control of the nitrogen source-regulated promoter *FLD1* and to characterize the system using different methodologies and approaches.

Secondary objectives of the thesis are:

- To test different carbon and nitrogen sources to evaluate the possibility of expressing ROL without the necessity of using methanol as carbon source.
- To develop a fed-batch strategy, based in an alternative to methanol carbon source, to produce ROL in high cell density fermentations.
- To identify and characterize the possible bottlenecks in the synthesis, folding and protein secretion pathways that might produce the reduction of process productivity.
- To develop new strategies to overcome these possible bottlenecks based in the genetic modification of the host microorganism.

CHAPTER 1

Introduction

1.1. The methylotrophic yeasts

By the year 1970, the interest of the company Phillips Petroleum in the production of biomass (single-cell protein) from methanol led to the isolation of methylotrophic yeasts from the nature. Only six genera from the 39 yeast genera were identified as methylotrophic yeast, these are: *Hansenula*, *Pichia*, *Candida, Turolopsis*, *Kloeckera* and *Rhodotorula* (Ogata et al., 1969)(cited in Higgins et al., 1998). These six genera were sub-divided in two groups: the asporogenous *Candida*, *Turolopsis*, *Kloeckera* and *Rhodotolula* genera, and the ascomycetous yeasts *Hansenula* and *Pichia.* Nowadays, the principal interest in the methylotrophic yeast is focused in their use as cellular factories for recombinant proteins production and in the study of the specific compartments which are very abundant in methanol-grown cell, namely peroxisomes (Romanos et al., 1992).

1.2. *P. pastoris* as a protein expression system

During the decade of the 80's, efficient classical and molecular tools were developed for *Hansenula polymorpha* and *P. pastoris* genetic manipulation, the results from this new advance combined with the previous knowledge in high density cultivation became in an expression system that led to high levels of heterologous protein expression.

P. pastoris as protein expression system combines the unique eukaryotic capacity of performing post-translational modifications with the easy genetic manipulation and the ability to growth on minimal media. Next, a list of the characteristics that define *P. pastoris* as a suitable expression system is detailed:

- A high similitude with *Saccharomyces cerevisiae* regarding genetic manipulation techniques.

- The aptitude to growth to high cell densities, up to 100 $g \cdot L^{-1}$ of dry cell weight, due to its preference to the respiratory metabolism than the fermentative (Cereghino et al., 2002).
- The capacity to growth on minimal synthetic media, unlike other eukaryotic systems as mammalian or insect cells.
- As an haploid organism, any genetic modification is phenotipically revealed in further generations (Cregg et al., 2000).
- As an eukaryotic organism has the ability to perform many of the post-translational modifications usually performed in higher eukaryotes, e.g. correct folding, disulphide bond formation, O and N-linked glycosylation and processing of signal sequences.
- A very low secretion of endogenous proteins, which makes easy the recovery and purification of the secreted product from the culture medium.
- The presence of a highly induced and tightly regulated promoter that controls the expression of the AOX1 gene. This promoter has been widely used to control the expression of foreign genes; this will be further discussed.

1.2.1. Promoters

The AOX1 promoter has been the most widely utilized of all available promoters for *P. pastoris*. The alcohol oxidase is the key enzyme in the methanol oxidation pathway. It oxidize methanol to formaldehyde and hydrogen peroxide. Studies about this enzyme revealed the existence of two genes AOX1 and AOX2 (Cregg and Madden, 1987) that codify for two enzymes that are 95% homologues in their amino acid sequences. The AOX1 enzyme has a poor affinity for oxygen that is compensated by the up-regulation of the AOX1 promoter to drive expression of the AOX1 gene and produce large amounts of AOX1 enzyme. Actually, the abundance of the AOX1 enzyme can achieve the 30% of the total cellular protein in methanol growth conditions. In contrast to the methylotrophic yeast *Hansenula polymorpha* where the AOX gene can be induced by limiting concentrations of glycerol or glucose, or without the addition of methanol (Mayer et al., 1999; Gellisen et al., 2000), other authors argue that in *P. pastoris* the induction of the AOX1 is restricted to the use of methanol (Tschopp et al., 1987).

The AOX2 gene yields a protein that represents only the 10% of the total AOX enzyme within the cell. Nevertheless, some authors have reported successful expression of recombinant proteins using the AOX2 promoter (Kobayashi et al., 2000). However, the expression levels of foreign proteins obtained with the AOX1 are often much higher than those achieved with the AOX2.

An alternative to the AOX1 promoter was described by Waterham et al. (1997), the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (GAP) was cloned and used to control the expression of foreign proteins. This promoter allows the constitutively expression of the recombinant protein, and thus no use of methanol for induction is needed. However, the possibility that the constitutive expression of recombinant proteins could have a cytotoxic effect has avoided the extensive used of this promoter. In despite of this, recently, Hohenblum et al. (2004) and Menendez et al. (2004) have reported levels of foreign protein expressed under the control of the GAP promoter similar to those obtained with the AOX1.

On the other hand, Shen et al. (1998) proposed the used of the formaldehyde dehydrogenase (*FLD1*) promoter. This promoter controls the expression of the *FLD1* gene, which codifies for an enzyme involved in the formaldehyde degradation. This enzyme is key in the methanol metabolism and also in the methylamine metabolism when it is used as sole nitrogen source. The FLD enzyme is thought to play an important role protecting the cell from the toxic effect of formaldehyde accumulation. The advantage of using the *FLD1* promoter is that expression is either induced by methanol as carbon source or methylamine as nitrogen source. Hence, the use of methanol could be replaced by another carbon source, i.e. glucose or glycerol, according to Shen et al. (1998).

Recently, the cloning and use of the isocitrate liase (ICL1) promoter has been reported (Menendez et al., 2003). Limiting concentrations of glucose or ethanol were utilized as carbon sources to express the dextranase from *Penicillium minioluteum*. However, no bioreactor experiments have been carried out yet and further studies are necessary to implement the fermentation conditions.

1.2.2. Strains and phenotypes

Regarding its capacity to growth on methanol, *P. pastoris* strains are divided in three different phenotypes (Cregg et al., 2000). The Mut^+, or methanol utilization plus phenotype, has the two intact AOX genes, and grows on methanol as at the wild type rate. The Mut^S phenotype, or methanol utilization slow, has a disruption in the AOX1 gene, thus, its capacity to growth on methanol is affected, and the growth rate is slower. The Mut^-, or methanol utilization minus phenotype, lacks the two AOX genes, as a result cells are unable to growth on methanol (Chiruvolu et al., 1997).

There are many commercial available *P. pastoris* strains that can be used to express recombinant proteins. In table 1 the most used are summarized. The choice of a specific strain is determined by the production recruitments, i.e. intracellular or extracellular expression or culture condition.

The GS115 strain, as well as the KM71, MC 100-3 and SDM strains are auxotrophic for histidine (phenotype HIS-), which means that lacks the histidin dehydrogenase gene (*his4*), a gene that codifies for an enzyme of the histidine synthesis pathway. The auxotrophic mutants allow the selection of transformants by their ability to growth on minimal medium without histidine. The SMD1163 and SMD1168 strains are defective in the vacuolar peptidase A (*pep4*). This enzyme is responsible for the activation of carboxipeptidase Y and protease B1, thus, these strain are also defective in this enzymes. Additionally, the strain SMD1163 and the SMD1165 are defective for proteinase B.

Table 1. Genotype and phenotype of the *P. pastoris* strains.

Soca	**Genotip**	**Fenotip**
GS115	*his4*	Mut^+ His^-
X-33	Wild type	Mut^+ His^+
KM71	aox1Δ::SARG4 his4 arg4	Mut^s His^-
MC 100-3	aox1Δ::SARG4 aox2Δ:: his4 his4 arg4	Mut^- His^-
SMD 1168	pep4Δ his4	Mut^+ His^- protease-
SMD 1165	pbr1 his4	Mut^+ His^- protease-
SMD 1163	pep4 pbr1 his4	Mut^+ His^- protease-

1.2.3. Vectors

There are many commercial available expression vectors for *P. pastoris*, either for intracellular or extracellular expression. Vectors for extracellular expression usually contain a copy of a signal sequence as the alpha-mating factor pre-pro leader sequence (α-MF), before the cloning site. Vectors such as pHIL-D2 or pPIC9 contain a functional copy of the histidine dehydrogenase gene (HIS4), which works as a selection marker in transformation on *his4* (histidine dehydrogenase defective) *P. pastoris* strains. These

vectors contain as well gene that confers resistence to ampicilin as selectable marker for subcloning into *E. coli* . A gene conferring resistance to kanamycin is used to select tandem multi-copy integrations events of the expression cassette in the *P. pastoris* genome.

In the pPICZ derived vectors, resistance to ampicilin and kanamycin have been substituted for ressistance to the antibiotic zeocin, conferred by the gene *Sh ble*. This gene allows selection of transformants into *E. coli* and *P. pastoris*. Moreover multi-copy integration events of the expression cassette can be selected by resistance to increasing concentration of zeocin. The use of the *Sh ble* gene has reduced considerably the size of the expression vectors, resulting in an easier manipulation during cloning.

1.2.4. Integration into the genome

As in *S. cerevisiae*, integration into the genome by homologous recombination of linear plasmids can be achieved. Integrated plasmids are more stable than episomal vectors, i.e. loss of vector is less than 1% per generation in the absence of selectable marker. All *P. pastoris* vectors contain at least one *P. pastoris* DNA fragment such as the *HIS4* gene, the *AOX1*, *GAP* or *FLD* promoter, with a unique restriction sites that can be cleaved and used to direct the vector to integrate into the host genome by a single crossover type insertion event (figure 1a).

Moreover, vectors such as pPIC9 that include a 3' *AOX1* sequence can be integrated into the genome by gene replacement event at the AOX1 locus (figure 1b). This event takes place when crossover occurs at both *AOX1* promoter and 3'*AOX1* regions in the vector and the genome. The consequence of this event is the deletion of the *AOX1* gene, resulting in a Mut^S phenotype.

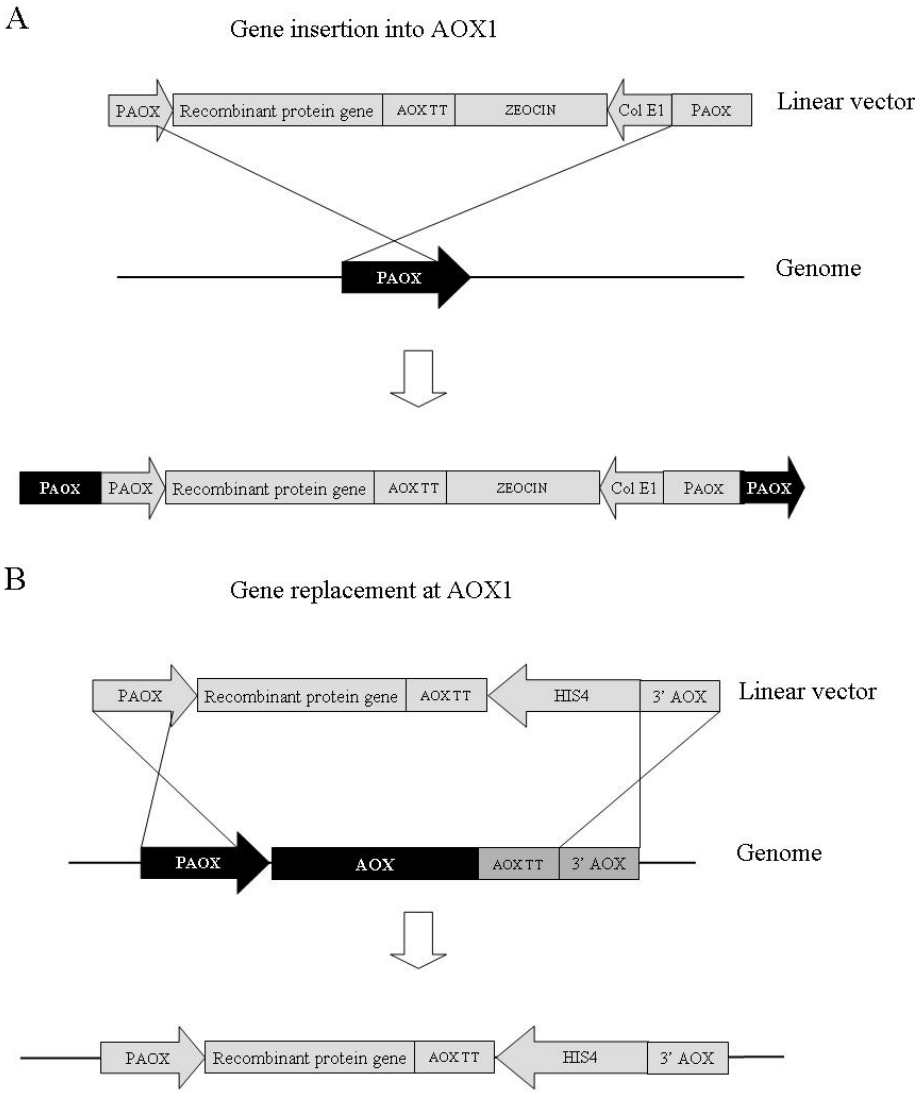


Figure 1. Integration events into the *P. pastoris* genome by insertion (A) and gene replacement (B).

1.2.5. Secretion signal

Frequently, the secretion of the recombinant protein is necessary in order to asses a correct folding and post-translational processing of this proteins, and to avoid toxicity of this proteins within the cell. Moreover, secretion facilitates the product recovery and purification. The secretion pathway in yeasts is similar to the higher eukaryotes pathway, secretion is lead by an amino-terminal signal sequence. This sequence drives the co-translational translocation of proteins into the endoplasmatic reticulum.

The most used secretion signal sequences in *P. pastoris* are: the *S. cerevisiae* alpha-mating factor pre-pro leader sequence (α-MF), the acid phosphatase signal sequence (PHO) and the invertase signal sequence (SUC2). The most commonly used is the *S. cerevisiae* alpha-mating factor pre-pro leader sequence (α-MF) which is a peptide with a 19 amino acid pre-sequence and a hydrophilic 60 amino acid pro-region (reviewed by Daly et al., 2005). The 19 amino acid pre-region is responsible for translocation of the pro-protein into the ER and is cleaved by an endopeptidase during the translocation process. The pro-protein is transported the Golgi, where a dibasic aminopeptidase, product from the gene *KEX2*, cleaves in the carboxyl terminal region of the Lys-Arg, between the α-MF and the protein.

1.3. Cultivation techniques

The bases of *P. pastoris* fed-batch fermentation were provided by Invitrogen Corporation in its operational manual. *P. pastoris* fermentation strategies in fed-batch have been recently reviewed by Cos et al (2006). Fed-batch fermentations protocols usually include three different phases:

First, a glycerol phase is carried out in order to achieve a high cell density within the reactor. The glycerol phase is independent of the *P. pastoris* phenotype, in general, the initial glycerol concentration is about 40 g per liter. After the glycerol consumption a sharp increase in the dissolved oxygen occurs, indicating the end of the batch phase.

After the glycerol batch phase, a transition phase is initiated. The transition phase might consist in a constant glycerol feeding rate at a limiting concentration to induce AOX1 derepression. Other strategies consist in a mixed substrate feeding, including glycerol and methanol at decreasing and increasing concentrations respectively. The objective of this phase is to derepress completely the AOX1 promoter and to prepare the cells for the following phase where induction takes place.

Third, the induction phase is the most important phase of the process, where methanol is added into the bioreactor using different strategies. The chosen strategy is one of the most important factors for maximizing the heterologous protein production and may depend of the phenotype, operational conditions or specific characteristics of the heterologous protein expressed.

1.4. The *Rhizopus oryzae* lipase

Rhizopus oryzae is a lipolytic fungus that grows on the palm tree fruit. Fungi from the genera *Rhizopus* are natural producers of lipolytic enzymes, more than 30 different lipases have been reported to be produced by this genus, and the differences between these lipases are basically in some amino acid, e.g. the *Rhizopus oryzae* lipase (ROL), the *Rhizopus delemar* lipase (RDL) and the *Rhizopus javanicus* lipase (RJL) have a substitution in the His134 and the Leu234 by a Asn and a Leu respectively (Minning et al., 1998). Beer et al. (1998) allege that the different lipases are product of different proteolytic processing of the same gene, producing diverse three-dimensional structures. The native *Rhizopus oryzae* lipase is a 392 amino acid protein. It is thought that the 26 first amino acids are a signal sequence, followed by a 97 amino acid pro-region and finally the 269 amino acid mature protein sequence (Beer et al., 1998).

The characterized mature ROL has a molecular weight of 32 kDa, according to SDS-PAGE analysis (Ben Salah et al., 1994), it has 4 potential sites for N-linked glycosition and three disulphide bonds, between the amino acids 152 and 391, 163 and 166, and the 358 and 367. The native enzyme is stable between pH 4.5 and 7.0 at 30º C and its optimal pH is 8.5.

1.5 Previous works with ROL

Heterologous expression of ROL was firstly carried out by Beer et al. (1998), using the *E. coli* expression system. Although the proteolytic processing of the protein was successfully achieved, it was not possible to detect any lipolytic activity and the protein was found as inclusion bodies. After purification and refolding of the inclusion bodies, between 10-15 mg of active ROL per liter were obtained.

On the other hand, Di Lorenzo et al. (2005) achieved, for the first time, the expression of *R. oryzae* lipase in *E. coli* in an active and correctly folded form using the host strain *E. coli* Origami (DE3). Similar results were achieved using *S. cerevisiae* as host organism by Takahashi et al. (1998). Here, the *S. cerevisiae* alpha-mating factor pre-sequence was fused to the pro-form of the *R. oryzae* lipase in order to obtain the extracellular expression. However, when the mature ROL encoding gene was directly fused to the alpha-mating factor pre-sequence and expressed in *S. cerevisiae*, the

activity neither was detected in the culture medium nor in the cells (Takahashi et al., 2001).

Minning et al., (1998) expressed the ROL in *P. pastoris* under the control of the AOX1 promoter. ROL was secreted into the culture medium using the *S. cerevisiae* alpha-mating factor pre-prosequence. Up to 60 mg per liter of active enzyme were obtained in a 5 L bioreactor. The properties of the obtained recombinant lipase were similar to those reported for the native enzyme and the expressed in *E. coli* .

Further, Minning et al. (2001) increased the productivity 2.5-fold by using a feeding strategy based on the monitoring of methanol concentration by gas chromatography. Moreover, the introduction of a transition phase, between the glycerol batch-phase and the methanol fed-batch-phase, consisting in a decreasing and increasing gradient of glycerol and methanol respectively, showed an improvement in ROL expression levels.

Recently, Cos et al. (2006) observed and increase of ROL expression using an on-line methanol analyzer, based in the methanol concentration of exhaust gas. This works has proved that keeping a constant value of methanol concentration into the culture medium is a key parameter to obtain high yields of recombinant proteins using *P. pastoris* and the AOX1 promoter using a Mut^S strain.

1.6. References

Beer HD, McCarthy JEG, Bornscheuer UT and Schmid RD (1998) Cloning, expression, characterization and role of the leader sequence of a lipase from *Rhizopus oryzae*. Biochimica et Biophysica Acta-Gene Structure and Expression 1399: 173-180.

Ben Salah A, Fendri K and Gargoury Y (1994) The enzyme of *Rhizopus oryzae*: production, purification and biochemical characteristics. Revue Francaise des Corps Gras 41: 133-137.

Cereghino GPL, Cereghino JL, Ilgen C and Cregg JM (2002) Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. Current Opinion in Biotechnology 13: 329-332.

Chiruvolu V, Cregg JM and Meagher MM (1997) Recombinant protein production in an alcohol oxidase-defective strain of *Pichia pastoris* in fed-batch fermentations. Enzyme and microbial technology 21: 277-283.

Cos O, Ramon R, Montesinos JL and Valero F (2006) A simple model-based control for *Pichia pastoris* allows a more efficient heterologous protein production bioprocess. Biotechnology and Bioengineering.

Cos O, Ramon R, Montesinos JL and Valero F (2006) Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: A review. Microbial cell factories 5: 17.

Cregg JM and Madden KR (1989) Use of site-specific recombination to regenerate selectable markers. Molecular and general genetics 219: 320-3.

Cregg JM, Cereghino JL, Shi JY and Higgins DR (2000) Recombinant protein expression in *Pichia pastoris*. Molecular Biotechnology 16: 23-52.

Daly R and Hearn MTW (2005) Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. Journal of Molecular Recognition 18: 119-138.

Higgins DR and Cregg JM (1998) *Pichia* protocols. Totowa, New Jersey.

Hohenblum H, Gasser B, Maurer M, Borth N and Mattanovich D (2004) Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris*. Biotechnology and Bioengineering 85: 367-375.

Kobayashi K, Kuwae S, Ohya T, Ohda T, Ohyama M and Tomomitsu K (2000) Addition of oleic acid increases expression of recombinant human serum albumin by the AOX2 promoter in *Pichia pastoris*. Journal of Bioscience and Bioengineering 89: 479-484.

Mayer AF, Hellmuth K, Schlieker H, Lopez-Ulibarri R, Oertel S, Dahlems U, Strasser AWM and van Loon APGM (1999) An expression system matures: A highly efficient and cost-effective process for phytase production by recombinant strains of *Hansenula polymorpha*. Biotechnology and Bioengineering 63: 373-381.

Menendez C, Hernandez L, Banguela A and Pais J (2004) Functional production and secretion of the *Gluconacetobacter diazotrophicus* fructose-releasing exo-levanase (LsdB) in *Pichia pastoris*. Enzyme and microbial technology 34: 446-452.

Menendez J, Valdes I and Cabrera N (2003) The ICL1 gene of *Pichia pastoris*, transcriptional regulation and use of its promoter. Yeast 20: 1097-1108.

Minning S, Schmidt-Dannert C and Schmid RD (1998) Functional expression of *Rhizopus oryzae* lipase in *Pichia pastoris*: high-level production and some properties. Journal of Biotechnology 66: 147-156.

Romanos MA, Scorer CA and Clare JJ (1992) Foreign gene expression in yeast : a review. Yeast 8: 423-488.

Shen SG, Sulter G, Jeffries TW and Cregg JM (1998) A strong nitrogen source-regulated promoter for controlled expression of foreign genes in the yeast *Pichia pastoris*. Gene 216: 93-102.

Takahashi S, Ueda M, Atomi H, Beer HD, Bornscheuer UT, Schmid RD and Tanaka A (1998) Extracellular production of active *Rhizopus oryzae* lipase by *Saccharomyces cerevisiae*. Journal of Fermentation and Bioengineering 86: 164-168.

Takahashi S, Ueda M and Tanaka A (2001) Function of the prosequence for in vivo folding and secretion of active *Rhizopus oryzae* lipase in *Saccharomyces cerevisiae*. Applied Microbiology and Biotechnology 55: 454-462.

Tschopp JF, Sverlow G, Kosson R, Craig W and Grinna L (1987) High level secretion of glycosylated invertase in the methylotrophic yeast, *Pichia pastoris*. Bio-Technology 5: 1305-1308.

Waterham HR, Digan ME, Koutz PJ, Lair SV and Cregg JM (1997) Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. Gene 186: 37-44.

CHAPTER 2

Materials and methods

This chapter details the most common material and methods used througout this work. Specific materials or methods are detailed at the correspondent chapters.

2.1. Strains

The wild type *P. pastoris* X-33 strain was used throughout this work. Other *P. pastoris* strains utilized in this work are detailed in the corresponding chapters.

For subcloning tasks *Escherichia coli* DH5α (Invitrogen), genotype F^- φ80d*lacZ*ΔM15 Δ (*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1, was employed.

2.2. Culture maintenance

P. pastoris strains were grown on YPD plates containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 2% (w/v) agar and stored at 4^o C. Long term stocks were prepared as recommended by Invitrogen and stored frozen at -80^o C.

2.3. Inoculum preparation

Pre-inoculums for bioreactor cultures were grown for 24 h in baffled shake flasks at 30°C, 250 rpm, in BMGY (buffered glycerol-complex medium) containing 1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 4×10^{-5} % (w/v) biotin, 1% (w/v) glycerol in 50 ml of final volume. The 50 ml culture was used to inoculate a 500 ml (final working volume) of BMGY in a 1-litre top-bench bioreactor (Braun Biotech). The culture was grown overnight at 30^oC and subsequently centrifuged at 5000 rpm. Harvested cells were resuspended in bioreactor culture medium and used to inoculate a 5-litre Biostat ED bioreactor (Braun Biotech).

2.4. Glycerol, methanol, sorbitol, methylamine and ammonium determination

Glycerol and sorbitol concentration was determined by a HP 1050 liquid chromatograph (Hewlett Packard) and an Aminex HPX- 87H ion-exchange column from Biorad. The mobile phase was 15 mM sulphuric acid. Data was quantified by the Millenium 2.15.10 software (Waters Corporation) being 3% the RSD obtained.

Methanol was analysed by a HP 5890 gas chromatograph (Hewlett Packard) equipped with an automatic injector (HP 7376), a FID detector and a capillary column Tracsil TR-FFAP 25 m x 0.53 mm x 1 μm (Tracer-Teknokroma). Operating conditions were 200°C and 280°C for injector and detector, respectively, oven-temperature profile 40°C (2 min), 20ºC/min to 200ºC, 200ºC (5 min) giving a total analysis time of about 15 min. Helium was used as carrier gas with a flow rate of 9 $mL \cdot min^{-1}$. The selected fuel gas was hydrogen. Internal standard was isopropanol (4 $g \cdot L^{-1}$) mixed at 50 % (v/v) with samples. Data was quantified by Millenium 32 software (Waters Corporation). The estimated RSD was about 2%.

Methylamine was analysed by HPLC (Hewlett Packard 1090) with UV-Vis diode array detector using a Hypersil AA-ODS column (Hewlett Packard, Palo Alto, CA, USA) with a Hypersil ODS Guard precolumn. Solvent A: 20 mM sodium acetate, 0.3% (v/v) tetrahydrofurane (THF), 0.018% (v/v) Triethilamine (TEA); Solvent B: 100 mM sodium acetate, 40% (v/v) methanol, 40% (v/v) acetonitrile.
Ammonium was determined with the ammonium-nitrogen assay kits LCK-302 and LCK-305 from DrLange, following the manufacturer instructions.

2.5. Lipase activity determination

Lipolytic activity determination was carried out using the Lipase colorimetric assay (kit 1821792 from Roche Diagnostics) as follows: 0.3 mL of substrate (1,2-*O*-dilauryl-rac-glycero-3-glutaric-(methylresorufin)-ester) was mixed with 0.5 mL 400mM Tris·HCl buffer, pH 7.25, and 0.5 mL of sample in a thermostatically controlled cuvette (30°C). The increase in absorbance at 580 nm was followed for 7 min with a UV-VIS Varian Cary 3 spectrophotometer. The absorbance increase per second was calculated from the slope of the curve and correlated to the lipolytic assay using pH-stat analysis as

described in Minning et al., (2001). Equation 1 was applied to convert slope into lipolityc activity units per millilitre, where Y is the lipolityc activity (Activity Units per milliliter) and X is the slope of the curve of measured absorbance. One unit of lipolytic activity was defined as the amount of lipase necessary to hydrolyse 1 μmol of ester bond per minute under assay conditions.

$$Y = 2.162X - 0.0162 \qquad (1)$$
$$r^2 = 0.995$$

Extracellular lipase activity was measured after removing cells from cultivation samples by centrifugation in a microcentrifuge at 12,000 rpm.

Intracellular lipase activity (defined as the soluble fraction of the cell bound lipase activity) was measured from cleared supernatants from cell lysates. Cells were mechanically disrupted as follows: Cells were harvested by centrifugation, washed once in PBS buffer and further resuspended in lysis buffer (PBS plus Protease Inhibitor Cocktail Tablets, Roche Diagnostics). Resuspended cells were mechanically disrupted using a Constant Systems cell disrupter, following manufacturer's instructions. Lipase activity in cleared cell lysates was related to the total protein content of such lysates, as measured using the Bradford assay (Pierce).

2.6. Expression of ROL in *E. coli* for polyclonal antibody production.

The *Rhizopus oryzae* lipase was expressed in *E. coli* and purified in order to immunize mice to obtain polyclonal antibodies anti-ROL. Expression and purification was carried folloing the QIAexpressionist handbook (Qiagen). The *ROL* gene was amplified by PCR adding a BamHI and PstI restriction target in its forward and reverse primers, respectively. A 6His-tag was expressed in N-terminus with ROL to facilitate further protein purification. Plasmid pQE40 and the *E. coli* M15 strain were used for cloning and expression.

E. coli M15 expressing ROL was growth in 1L LB medium containing 100 mg/mL ampicilin and 25 mg/mL kanamycin according to the QIAexpressionist handbook. Cells

were withdrawn and purification of ROL was performed under denaturing conditions using 8 M urea. The heterologous product was purified using a HisTrap column (Amersham) following the manufacturer instructions. The purified product was subsequently used to immunize mice at the Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences, Vienna, Austria.

2.7. SDS-PAGE and western blotting analyses

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 12%, was carried out in a Mini-PROTEAN II (BioRad) apparatus following the standard procedures recommended by the manufacturer and according to the protocol of Laemmli et al. (1970). Broad range protein markers (BioRad) were used to molecular weight determination. Gels were stained using silver stain procedure (BioRad) or Coomassie

Western blot were performed as follows: after running SDS-PAGE gels were incubated for 30 minutes in blotting buffer (Tris/HCl 3.03 $g \cdot L^{-1}$, glycine 14.41 $g \cdot L^{-1}$, methanol 175 $mL \cdot L^{-1}$), nitrocellulose membranes (Amersham) and fiber pads were also incubated as cited above. After incubation, transference was carried out using the Mini Trans-Blot apparatus (BioRad), transference was carried out during 90 minutes at 250 mA and 100 V. Once transference was completed, membranes were incubated in blocking buffer (Na_2HPO_4 1.42 $g \cdot L^{-1}$, KCl 0.20 $g \cdot L^{-1}$, KH_2PO_4 0.20 $g \cdot L^{-1}$, NaCl 8.00 $g \cdot L^{-1}$, Tween 20 1 $mL \cdot L^{-1}$, plus 5% skimmed milk) overnight at room temperature and gentle agitation. After blocking, the membranes were incubated for one hour and room temperature in blocking solution plus primary antibody at the suitable dilution (detailed at the corresponding chapters). Once incubated with primary antibody, the membranes were washed with wash buffer (Na_2HPO_4 1.42 $g \cdot L^{-1}$, KCl 0.20 $g \cdot L^{-1}$, KH_2PO_4 0.20 $g \cdot L^{-1}$, NaCl 8.00 $g \cdot L^{-1}$, Tween-20 1 $mL \cdot L^{-1}$) four times during 10 minutes. Incubation with secondary antibody was performed under the same conditions mentioned above and with the appropriate dilution detailed at the corresponding chapters. Finally, the membrane was washed four times for 10 minutes using wash buffer and incubated for five minutes with the chemiluminescent substrate SuperSignal West Pico (Pierce) and the signal was collected by a photographic film.

2.8. Protease activity determination

Protease activity in the culture supernatant was determined using the QuantiCleave Protease Assay Kit (Pierce) according to the manufacturer's instructions.

2.9. Total protein determination

Bradford assay (Pierce) was used for total protein quantification. Absorbance was measured in 96-wells plates using the spectrophotometer Microplate Reader 2001 (Whittaker Bioproducts).

2.10. Biomass analysis

Biomass was expressed as dry cell weight. This was measured by withdrawing 8-mL samples of the bioreactor; these were then filtered through pre-weighted glass microfibre filters (Whatman), which were subsequently washed with two volumes of distilled water and dried at 105^{o}C to a constant weight. Additionally, optical density was measured using an spectrophotometer Uvikon 941 Plus (Kontron) at a wavelength of 600 nm.

2.11. Biomass elemental composition

Cells were harvested by centrifugation (CentriKon H-401 ZK401, Kontron Hermle) for 5 min at 4ºC and 4500 ×g. Pellets were collected, washed with 10 mM Tris·HCl, pH 8.0, and centrifuged twice. Washed cells were freeze-dried (Virtis Sentry 5L, Virtis Company Gardiner) for 24 h, further dried at 105ºC to constant weight and analyzed for composition of C, H, N, O and S in an elemental analyzer (Carlo Erba EA 1108, Carlo Erba Instruments). Ash content was quantified about 9 %.

2.12. Molecular biology methods

2.12.1. Reagents and enzymes

DNA modifying enzymes utilized in molecular biology were purchased at Roche Molecular Biochemicals. For PCR reactions, the KOD HiFi DNA polymerase (Novagen) was employed.

2.12.2 DNA extraction

Plasmidic DNA extraction was performed using the Plasmid Extraction kit (Qiagen) according to the manufacturer instructions. DNA fragments were recovered from agarose gels applying the Gel Extraction kit (Qiagen). Genomic DNA from *P. pastoris* was extracted using the DNeasy Tissue kit (Qiagen) following the manufacturer's instructions.

2.12.3. Agarose gel electrophoresis

Agarose gel electrophoreses were carried out according to the protocol of Sambrook et al. (1989). Photographs of the gels were obtained by the Electrophoresis Documentation and Analysis System 120 (Kodak). To determine the DNA fragments size the1 kb DNA ladder was used (Invitrogen), agarose was purchased from Sigma.

2.13. Transformation

2.13.1. *E. coli* transformation

Competent cells were prepared according to Sambrook et al. (1989) as follows: A 10 mL LB (Luria-Bertani medium containing 10% yeast extract, 10% peptone and 5% NaCl) culture was inoculated from a criostock and incubated overnight at 37ºC. 1-5 mL of the overnight culture were used to inoculate a 1000 mL LB culture. This culture was incubated at 37ºC until reach an optical density of 0.5-1.0. Once achieving the desired optical density, cells were chilled in ice for 20 minutes and subsequently centrifuged at 4000 rpm and 4ºC for 20 minutes. Supernatant was discarded and the cells resuspendended in 500 mL of cold 10% glycerol solution. This washing step was then repeated resuspending in 250, 20, and finally 2 mL 10% glycerol. Cells were frozen at -80ºC in 40 μL aliquots or used for electroporation.

Electrocompetent *E. coli* DH5α cells were transformed with a Gene Pulser (BioRad) at 2000 V, 25μF and 200 Ω. Cells were thawed in ice and 40 μL were mix with 1 to 5 μL of DNA. The mixed solution was transferred to a 0.2 cm gap electroporation cuvette (BioRad), previously chilled on ice. Then, cells were pulsed and 500 μL of LB was added immediately. The solution was transferred to a sterile tube and incubated for one hour at 37ºC. After time was elapsed, aliquots were spread on corresponding agar plates.

2.12.2 *P. pastoris* transformation

Competent *P. pastoris* were prepared following the protocol by Higgins et al. (1998) as follows: A 10 mL YPD (20% glucose, 20% peptone, 10% yeast extract) were inoculated from a criostock and grown overnight at 30ºC. The overnight culture was transferred to a new 500 mL YPD culture and incubated at 30ºC until an optical density of about 1.5. Then, culture was chilled in ice for 15-20 minutes and centrifuged at 4000 rpm and 4ºC for 20 minutes. Supernatant was discarded and cells were resuspended in 100 mL of a new medium containing 100 mL of YPD plus 20 mL of 1M Hepes pH 8.0, and 2.5 mL DTT 1M. Cells were incubated for 30 minutes at 30ºC and subsequently centrifuged at 4000 rpm 15 minutes. The cell pellet was resuspended with 250 mL of cold water and centrifuged again under the same conditions. Supernatant was discarded and the pellet resuspended with 20 mL of 1M cold sorbitol, the centrifugation step was repeated once again and the pellet resuspended in an final approximately volume of 1.5 mL of 1M cold sorbitol. Aliquots of 80 µL were performed for subsequent electroporation or for storage at -80ºC.

Electrocompetent *P. pastoris* cells were transformed using a Gene Pulser (BioRad) at 1500 V, 25µF and 400 Ω. A volume of 80 µL of fresh cells and about 1 µg of DNA in no more than 10 µL total volume were transferred into a 0.2 cm gap electroporation cuvette (BioRad) previously chilled on ice. Cells were pulsed and immediately 1 mL of cold 1 M sorbitol was added. The cuvette content was transferred to a sterile tube and incubated for 1 hour at 30ºC without agitation. Selected aliquots were spread onto agar plates containing an appropriate selective medium. Plates were incubated at 30ºC for 2-4 days.

2.2. References

Higgins DR and Cregg JM (1998) *Pichia* protocols. Totowa, New Jersey.

Laemmli UK (1970) Cleavage of structural proteins during assembly of head of bacteriophage-T4. Nature 227: 680-&.

Minning S, Serrano A, Ferrer P, Sola C, Schmid RD and Valero F (2001) Optimization of the high-level production of *Rhizopus oryzae* lipase in *Pichia pastoris*. Journal of Biotechnology 86: 59-70.

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular cloning. A laboratory manual. Cold Spring NY.

CHAPTER 3

Cloning and expression of the *Rhizopus oryzae* lipase in *P. pastoris* under the control of the nitrogen source-regulated *FLD1* promoter.

3.1. Introduction

Although a wide variety of proteins have been produced successfully using the *PAOX1*, this promoter may not be appropriate or convenient in some situations (Shen et al., 1998). For instance, in small and micro scale cultures (e.g. shake flasks and micro plates), methanol rapidly evaporates and it is inconvenient to on-line monitor and control methanol concentrations within an optimal range. Also, methanol metabolism utilises oxygen at high rates, and expression of foreign proteins is negatively affected by oxygen limitation, which may take place under these cultivation conditions. The constitutive strong promoter from the glyceraldehyde-3-P dehydrogenase (*PGAP*) is readily available for heterologous expression of proteins in *Pichia pastoris* (Waterham et al., 1997). However, strong constitutive promoters are not a good choice for the over expression of proteins showing cell toxicity.

The cloning and characterisation of the *P. pastoris FLD1* gene and its promoter have been reported (Shen et al., 1998), showing an alternative to the *AOX1* promoter for regulated expression of foreign proteins in *P. pastoris*. The gene encodes a formaldehyde dehydrogenase (FLD), a key enzyme required for the catabolism of methanol as carbon source and certain primary amines, such as methylamine as nitrogen sources in methylotrophic yeasts. In order to understand the operation of the formaldehyde dehydrogenase, an overview of the methanol and the methylamine metabolism is needed.

3.1.1. Methanol metabolism

In yeasts, oxidation of methanol is carried out in the peroxisomes were the alcohol oxidase AOX catalyzes the oxidation of methanol into formaldehyde and hydrogen peroxide (figure 1). The formation of hydrogen peroxide is potentially toxic for the cell, thus a catalase is responsible for the elimination of the hydrogen peroxide in the peroxisomes (van Dijken et al., 1975).

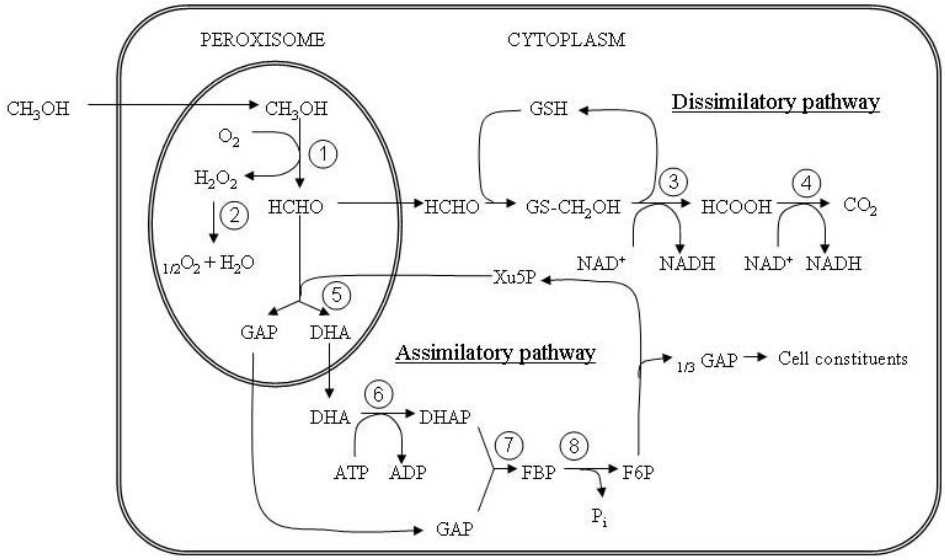


Figure 1. Schematic representation of methanol metabolism in methylotrophic yeasts. Peroxisome: 1, alcohol oxidase; 2, catalase, 5, dihydroxiacetone syntase. Cytoplasm: 3, formaldehyde dehydrogenase; 4, formate dehydrogenase; 6, dihydroacetone kinase; 7, fructose 1,6-biphosphate aldolase; 8, fructose 1,6-biphosphate phosphatase.

Formaldehyde, on the other hand, enters either the assimilatory pathway or the dissimilatory pathway. In the assimilatory pathway, formaldehyde enters the biosynthetic way to form cell constituents. In methylotrophic yeasts that growth on methanol the synthesis of cellular biomass is made via the xilulose 5-phosphate. In this pathway, two trioses are synthesized in the peroxisome, the dihydroxiacetone (DHA) and the glyceraldehydephosphate (GAP), by a dihydroxiacetone synthase (DHAS). This enzyme transfers a glycoaldehyde from xilulose 5-phosphate to formaldehyde (figure 1). Next, DHA is phosphorylated in the cytoplasm and condenses with GAP forming a fructose 6-phosphapte (F6P) via a fructose 1,6-biphosphate aldolase and a phosphatase.

In the dissimilatory pathway, the formaldehyde synthesized in the peroxisomes by AOX is oxidized by two dehydrogenases NAD dependent (figure 1). These two enzymes, which are located into the cytoplasm, are the formaldehyde dehydrogenase (FLD) and the formate dehydrogenase (FDH).

The FLD is an enzyme that strictly depends of glutathione to perform its catalytic activity. The substrate for FLD is the complex formed by the spontaneous union of formaldehyde and glutathione, the result is S-hydroximethylglutathione. The product of the FLD reaction is the S-formilglutathione (Harder and Veenhuis 1989). Studies performed by Kato et al., (1979) and Trotsenko et al., (1984) showed that NADH and ATP may strongly inhibit the FLD activity. Also, FLD has been proposed to play a primary role in the protection of the cell from toxic formaldehyde that accumulates with excess of methanol in the medium (Sibirny et al., 1990).

For the following steps of this pathway, two alternatives have been postulated: in the first one, an S-formilglutathione hydrolase might act on the S-formilglutathione obtaining formate and glutathione; then, a formate dehydrogenase would oxidize formate to carbon dioxide (Kato et al., 1974). In the second one, the low affinity of formate dehydrogenase for its substrate has suggested that FHD may oxidize directly S-formilglutathione to glutathione and carbon dioxide (van Dijken et al., 1976).

3.1.2. Methylamine metabolism

It is known as metazotrophy the capacity of certain microorganisms to growth on methylated amines a sole nitrogen source. Although metazotrophy is widely extended within yeasts (461 species tested by Djiken and Bos, 1981) it is not known yet any organism capable of growing on this substrates as sole carbon and nitrogen source at the same time.

The main metabolic pathway of methylamine assimilation is depicted in figure 2. First step takes places in the endoplasmatic reticulum, where trimethylamine and dimethylamine are oxidized by the action of a trimethylamine monoxygenase and a dimethylamine monoxygenase, respectively (Large and Green, 1984). Resulting from these reactions, two molecules of formaldehyde, one for each oxidation step, are formed as well as methylamine that is translocated to the cytoplasm.

Methylamine oxidation takes places in the yeast organelle called peroxisome, in the peroxisomes methylamine is oxidized by a methylamine oxidase (Haywood and Large, 1981). The products obtained from this oxidation are ammonia, hydrogen peroxide and

formaldehyde. Hydrogen peroxide is eliminated by a catalase located inside the peroxisomes. On the other hand, ammonia and formaldehyde leave the peroxisome to the cytoplasm.

Ammonia generated in the peroxisomes is assimilated by the cell via a NADPH-dependent glutamate dehydrogenase. The activity of this enzyme is increased drastically in cells grown under ammonium or amine limitation or, under carbon limitation, in the presence of amines as sole source of nitrogen (Zwart and Harder, 1983). The fate of formaldehyde produced by methylamine oxidation is not well known yet. According to Large and Green (1984) and Jones et al. (1991) *Hansenula polymorpha* is able to assimilate the formaldehyde coming from methylamine to cell constituents, however in *P. pastoris* this ability has not been reported.

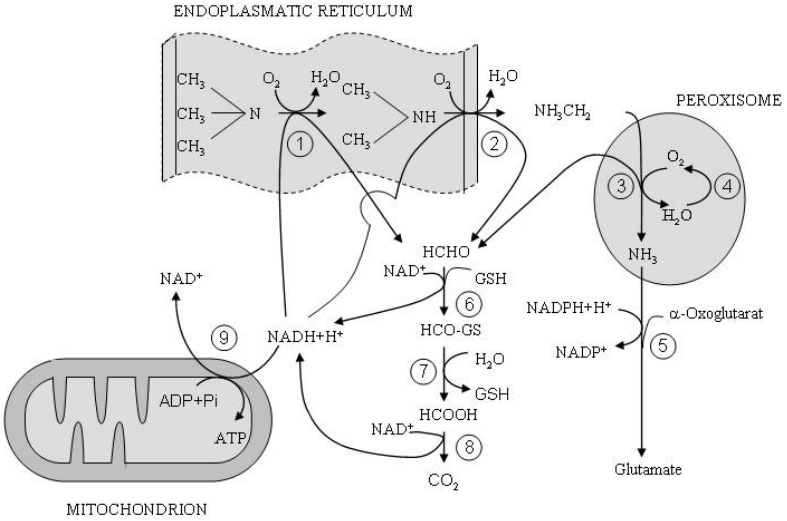


Figure 2. Intracellular compartments and reactions that takes place in the oxidation of methylated amines according to Largen and Green (1984). Endoplasmatic reticulum: 1, trimethylamine monooxygenase; 2, dimethylamine monooxygenase. Peroxisome: 3, methylamine oxidase; 4, catalase. Cytosol: 5, glutamate dehydrogenase; 6, formaldehyde dehydrogenase; 7, S-formylglytathione hydrolase; 8, formate dehydrogenase. Mitochondrion: 9, NADH dehygrogenase.

3.1.3. Previous studies and aims of the work

As mentioned before, Shen et al. (1998) cloned and characterized the *P. pastoris FLD1* promoter. Using β-lactamase as a reporter, they showed that the *FLD1* promoter (*PFLD1*) can be strongly and independently induced either by methanol or methylamine as carbon and nitrogen source respectively. In this work they analysed the intracellular FLD activity levels as well as the β-lactamase activity. The β-lactamase activity was

measured in cells grown in media containing either methanol or glucose as carbon source and ammonium sulphate or methylamine as nitrogen source. The highest β-lactamase activity was achieved in the methanol and methylamine culture. While in the methanol and ammonium sulfate culture the 88% of the maximum activity was obtained, in the glucose and methylamine culture, only the 48% of the maximum activity was achieved.

In the work described here, the recently characterized *FLD1* promoter from *P. pastoris* was used for the heterologous expression of the lipase gene (GeneBank accession No. AF229435) from *Rhizopus oryzae* (Ben Salah et al., 1994; Beer et al., 1998) in *P. pastoris*. In previous studies, this *R. oryzae* lipase (ROL) has been successfully overexpressed in *P. pastoris* under the control of the *AOX1* promoter (Minning et al., 2001). Despite this, ROL expressed under the control of the constitutive *GAP* promoter (Waterham et al., 1997) resulted in very low expression levels (Serrano, 2002). The study described here will be useful for designing methanol-free high cell density cultivation strategies for controlled heterologous protein production with based in the *FLD1* promoter system in *P. pastoris*.

3.2. Materials and methods

3.2.2. Strains

Escherichia coli DH5α was used for plasmid construction and amplification. The wild-type phenotype *Pichia pastoris* X-33 strain (Invitrogen Co.) was used as a host strain for expressing a *Rhizopus oryzae* lipase gene, *ROL*, under the transcriptional control of the *FLD1* promoter. The *P. pastoris* X-33/pPICZαA_*ROL* derivative strain expressing and secreting the *ROL* gene under the *AOX1* promoter (Minning et al., 2001) was used for comparative cultivation experiments.

3.2.2. Media composition

E. coli strains were cultured in Luria broth medium supplemented with 50 μg ml^{-1} zeocin (Invitrogen Co.) as required. *P. pastoris* strains were cultured in YPD medium (1% yeast extract, 2% peptone, 2% glucose) supplemented with 100 $\mu g \cdot mL^{-1}$ of zeocin when required. Shake flask cultures were performed using a minimal medium (Shen et al., 1998) composed of: 0.17% yeast nitrogen base (YNB) without ammonium sulphate and amino acids (Difco), a carbon source (0.4% w/v glucose, 0.4% w/v glycerol, 0.4%

w/v sorbitol, or 0.5% w/v) and a nitrogen source (0.5% w/v ammonium sulphate or 0.25% w/v methylamine chloride). This medium was supplemented with 1×10^{-4} % w/v biotin. The YNB, biotin, and methanol components were sterilised separately by micro filtration and then added to the shake flasks.

Bioreactor batch cultivations were carried out using a mineral medium (MM) (d'Anjou et al., 2001) having the following composition: KH_2PO_4 4.8 $g\cdot L^{-1}$, $MgSO_4\cdot 7H_2O$ 1.88 $g\cdot L^{-1}$, $CaCl_2\cdot 2H_2O$ 0.144 $g\cdot L^{-1}$, a carbon source (glycerol, sorbitol, or methanol) 20 $g\cdot L^{-1}$, a nitrogen source (ammonium sulphate 8 $g\cdot L^{-1}$ or methylamine chloride 8 $g\cdot L^{-1}$), 0.1 $mL\cdot L^{-1}$ of antifoam Mazu DF 7960 –a polyoxyalkylene glycol- (Mazer Chemicals, PPG Industries Inc., USA), 1 $mL\cdot L^{-1}$ of a biotin solution (400 $mg\cdot L^{-1}$), and 1 $mL\cdot L^{-1}$ of trace salts solution (0.2 mM $CuSO_4\cdot 5H_2O$, 1.25 mM KI, 4.5 mM $MnSO_4\cdot 4H_2O$, 2 mM $Na_2MoO_4\cdot 2H_2O$, 0.75 mM H_3BO_3, 17.5 mM $ZnSO_4\cdot 7H_2O$, 44.5 mM $FeCl_3\cdot 6H_2O$). The biotin and trace salts components were sterilised separately by micro filtration. The medium used for starter cultures was YPD medium containing 1%(w/v) yeast extract, 2%(w/v) peptone, 2% (w/v) dextrose.

For strain maintenance**,** *P. pastoris* strains were grown in YPD agar plates and maintained at 4°C for up to one month. Long term stocks were prepared as recommended by Invitrogen and stored frozen at -80°C.

3.2.3. Shake flask and bioreactor cultivation conditions

Small scale cultures were carried out at a working volume of 100 mL in 1 L baffled shake flasks, in an orbital shaker (Infors) at 30ºC and 200 rpm for 48 h. Cultures were carried out per duplicate. A starter culture of 100 ml grown in YPD medium was cultivated in 1 L baffled shake flasks at 200 rpm, 30ºC for 24h. Cells were harvested by centrifugation and resuspended in 50 mL of sterile water prior to the inoculation of the shake flasks to the desired initial optical density.

Bioreactor batch cultivations were carried out at a working volume of 1 L in a 1.5 L bench-top bioreactor (BiofloIII, NewBrunswick) at 30ºC, for 72 h. The pH of the culture was maintained at 5.5 by automatic addition of 5M KOH. The airflow was maintained at 2 L min^{-1}, assuring a minimum of 30% dissolved oxygen along the cultivation. The agitation speed was set to 500 rpm. The exhaust gas of the bioreactor was cooled in a condenser at 2-4°C (*Frigomix R*, B. Braun Biotech Int). Starter cultures (100 ml) were grown in 1 L baffled shake flasks at 200 rpm, 30ºC for 24h. Cells were

harvested by centrifugation and resuspended in fresh MM medium prior to the inoculation of the bioreactor.

3.2.5. Plasmid and strain construction

For expression of the *ROL* gene under the transcriptional control of *FLD1* promoter, a 1.5 kb *Bgl*II-*Not*I fragment composed of a 0.6 kb region beginning immediately 5' of the methionine initiator ATG of *FLD1* (Shen et al., 1998) was fused to a 1.2 kb region comprising sequences coding for the *S. cerevisiae* α-factor secretion signal fused to the mature *ROL* sequences (Minning et al., 1998). The 0.6 kb *FLD1* promoter region was generated by PCR using plasmid pSS040 (Shen et al., 1998) as template and oligonucleotides composed of the following sequences as primers: AS4: 5'-AGTCTACGAGATCTGCATGCAGGAATCTCTGGCACG-3' (5' primer), with a *Bgl*II site (underlined), and AS2: 5'-*TGAAGGAAATCTCAT*TGTGAATATCAAGAATTGTATGAAC-3' (3' primer), with a 5'-end 15-b region (italics) complementary to the 5' region coding for the α-factor secretion signal from *S. cerevisiae*. Plasmid pSS040 contains an expression cassette composed of *FLD1* promoter, the β-lactamase gene, and the *AOX1* transcriptional activator. It also contains the zeocin-resistance gene as selectable marker for both *E. coli* and *P. pastoris*.

The α*ROL* fragment was generated by PCR using plasmid pPICZαA_ROL (Minning et al., 1998) as template, and the oligonucleotides with the following sequences as primers: AS1: 5'-*CAATTCTTGATATTCACA*ATGAGATTTCCTTCAATTTTTACTG-3' (5' primer), which has a 5' 18-b region (italics) complementary to the 3'region of *PFLD1*, and AS3: 5'-GATCCACGTGCGGCCGCTTATTACAAACAAGC-3' (3' primer), introducing a *Not*I site (underlined). Plasmid pPICZαA_ROL is a derivative from pPICZαA (Invitrogen Co.) containing the expression cassette composed of the *PAOX1*, the coding region for the α-factor secretion signal fused to the mature ROL coding region, and the *AOX1* terminator. It also contains the zeocin-resistance gene as selectable marker for both *E. coli* and *P. pastoris*. The obtained PCR fragments were fused by SOE-PCR (Splicing by Overlap Extension) (Horton et al., 1989). The SOE-PCR conditions were: 94ºC for 10 minutes, and 25 cycles of 94ºC denaturation (1 minute), 55ºC annealing (1 minute), 72ºC elongation (1 minute), and finally 10 minutes at 72ºC. The resulting fused 1.8 kb fragment was cut with *Bgl*II and *Not*I and ligated

into *Bgl*II- and *Not*I-digested pPICZαA backbone (Figure 3). The DNA ligation reaction was then transformed into *E. coli*, and plasmids in the resulting colonies examined. A plasmid with the expected DNA construct and sequence, pPICZFLDα_ROL (Figure 3), was recovered and retransformed into *P. pastoris* X-33 cells via electroporation as described in (Cregg and Russell, 1998).

Recombinant DNA methods were performed essentially as described in Sambrook et al., (1989). Oligonucleotides were synthesised by Roche Molecular Biochemicals-Research. DNA sequencing was performed at the DNA Service Facility of the Universitat Autònoma de Barcelona, UAB, (Bellaterra, Spain) using specific primers in order to verify the complete nucleotide sequence of the *PFLD1* promoter region fused to the α-factor secretion signal and mature ROL coding regions.

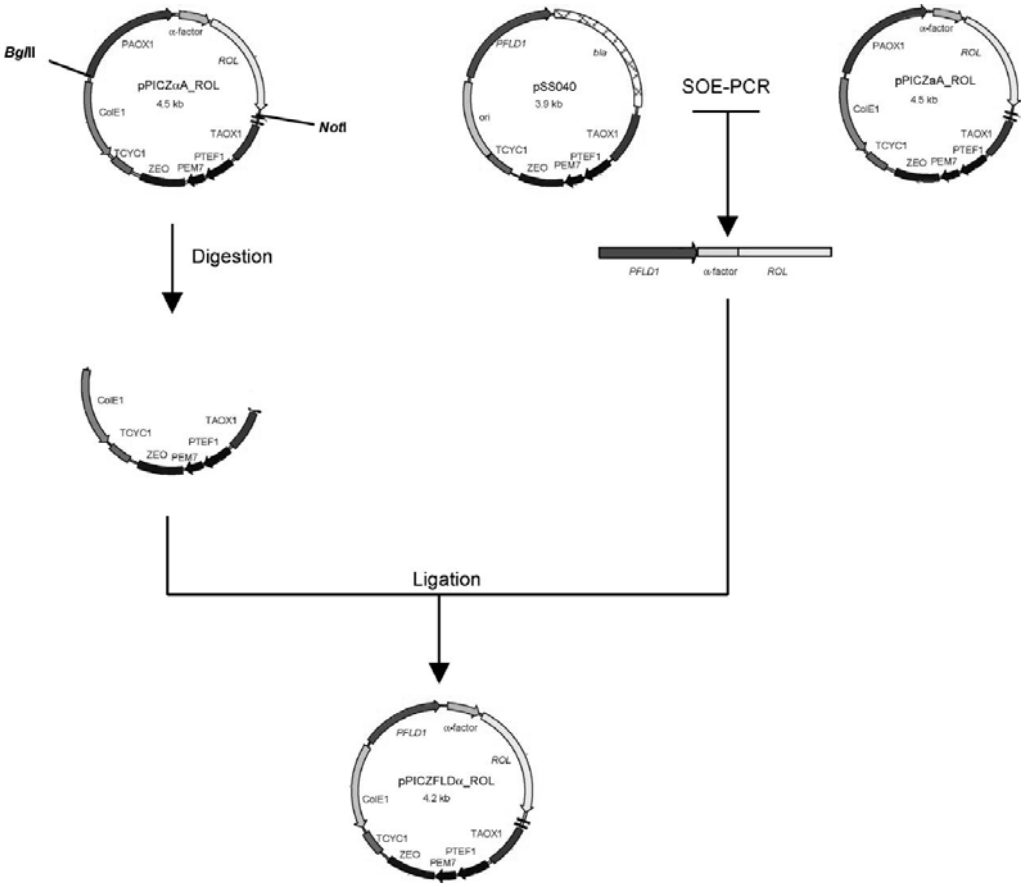


Figure 3. Physical map of the vectors used in this study and construction strategy of the pPICZFLDαROL expression vector.

3.3. Results

3.3.1. Construction of the *P. pastoris* production strain

To study heterologous gene expression under the transcriptional control of *PFLD1*, a pPICZ-derived expression vector incorporating the 0.6 Kb region comprising the sequences originating from just 5' of the methionine initiator ATG codon of *FLD1* fused to the 1.2 Kb region containing a fusion of the α-factor secretion signal and the mature ROL encoding sequences was constructed. The resulting vector, pPICZFLDα_ROL, was linearised at its *Nsi*I unique restriction site within the *PFLD1* region and subsequently transformed into *P. pastoris*. This resulted in the integration of the vector at the *PFLD1* locus. Transformants selected and checked for growth on methylamine, indicating integrity of *FLD1*. Four transformants were isolated and tested for lipase production in shake flask cultivations under fully inducing conditions (methanol as carbon source and methylamine as nitrogen source) (figure 4). The best lipase producer transformant, *P. pastoris* X-33/pPICZFLDα_ROL-2, was selected for further cultivation studies.

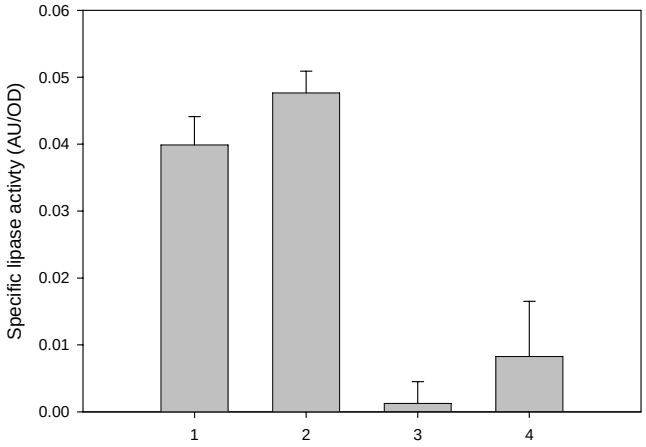


Figure 4. Specific lipase activity at 24h of culture of the four different clones obtained.

3.3.2. ROL expression under the control of *PFLD1* and comparison to *PAOX1* in shake flask cultures

The *P. pastoris* X-33/pPICZFLDα_ROL-2 strain was grown in six expression test media in shake flasks. These six media contained as carbon and nitrogen sources,

respectively: (1) glucose and ammonium sulphate (G/NH_4^+), (2) glucose and methylamine (G/MA), (3) methanol and ammonium sulphate (M/NH_4^+), (4) methanol and methylamine (M/MA), (5) glycerol and methylamine (GL/MA), and (6) sorbitol and methylamine (S/MA). Also, the *P. pastoris* X-33/pPICZα_ROL strain (Minning et al., 2001) expressing the *ROL* gene under the transcriptional control of *AOX1* promoter was cultivated in the test medium containing methanol and ammonium (M/NH_4^+) in order to compare levels of ROL activity under the control of *FLD1* promoter with those of a strain under the transcriptional control of *AOX1*. Both strains were derived from the X-33 strain and contained identical expression vectors except for their corresponding promoter regions. Figure 5 shows the biomass and extra cellular lipolytic activity levels observed in the shake flask cultures after 48 hours of inoculation. Four major observations could be made:

First, lipolytic activity levels produced under carbon and nitrogen sources combinations 1 to 4, i.e. G/NH_4^+, G/MA, M/NH_4^+ and M/MA, followed a partially different pattern of expression levels when compared to the results previously reported for the intracellular expression of β-lactamase in *P. pastoris* using the *FLD1* promoter and analogous culture conditions and carbon and nitrogen sources combinations (Shen et al., 1998). As expected, cells grown in glucose and ammonium sulfate did not produce detectable active lipase levels, whereas cultures grown with the inducer substrates methanol and/or methylamine did. However, unexpectantly, we could not detect lipolytic activity in glucose with methylamine (G/MA) cultures, whereas Shen et al., (1998) reported that β-lactamase levels in cells grown in this culture medium were 48% of those detected in M/MA cultures. In the methylotrophic yeast *H. polymorpha*, enzymes involved in the methanol metabolism such as the alcohol oxidase and formaldehyde- and formate-dehydrogenases are virtually absent during exponential growth on glucose, but their synthesis occurs after depletion of the substrate in the stationary phase of growth (Eggeling and Sahm, 1978). Thus, formaldehyde produced in methylamine oxidation might have entered the assimilatory pathway. However, neither the derepression phenomenon nor the assimilation of formaldehyde from methylamine has been described for *P. pastoris* yet.

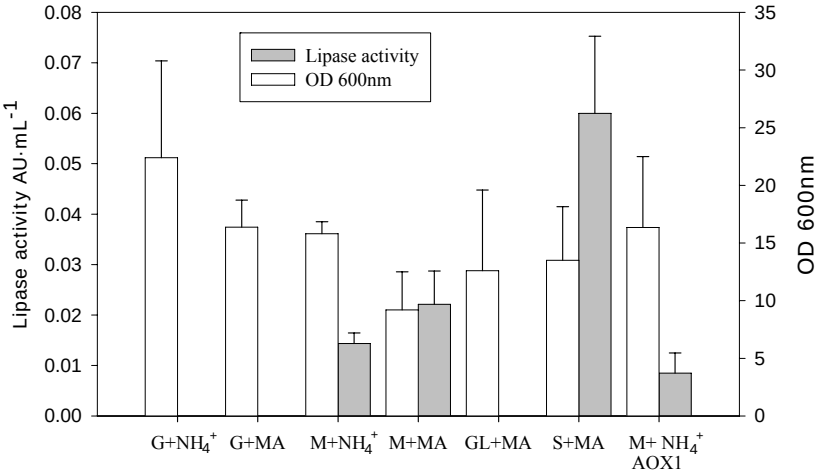


Figure 5. Comparison of cell growth and extracellular lipase activity in shake flask cultures of *P. pastoris* expressing ROL under the control of the *FLD1* and *AOX1* promoters using different carbon and nitrogen sources. Culture using the AOX1 promoter is denoted with AOX1.

Second, no lipolytic activity was detected in the glycerol and methylamine (GL/MA) culture, indicating that glycerol exerts a similar degree of repression upon the *FLD1* promoter as glucose. In contrast, when sorbitol was used as alternative carbon source in the presence of methylamine, the highest lipolytic activity levels were detected using the *FLD1* promoter. Interestingly, sorbitol has been described as a non-repressive carbon source of the methanol metabolism in *P. pastoris* (Thorpe et al., 1999; Inan et al., 2001). These results show that induction of the *FLD1* promoter by methylamine is not totally independent from the carbon source used for cell growth. Nevertheless, since intracellular and cell wall-bound lipase activity levels were not analysed, we can not rule out the possibility that the undetectable levels of extra cellular lipase in G/MA and GL/MA shake flask cultures may also reflect different kinetics of lipase transport to the extra cellular medium resulting from cell growth on different carbon/nitrogen sources combinations (i.e. different growth kinetics).

Third, we compared levels of secreted lipase activity produced under the control of *FLD1* promoter with those of a strain in which *ROL* expression was under the transcriptional control of *AOX1* promoter growing on methanol. Comparable levels of lipase activity were present in culture broths of the *FLD1* and *AOX1* promoter strains grown in methanol and ammonium sulphate (M/NH_4^+), whereas cultures using the *FLD1* promoter grown in M/MA produced significantly higher lipase activities than

both strains grown in M/NH_4^+. These results are in accordance with those reported for intracellular expression of β-lactamase in *P. pastoris* strains using both the *AOX1* and *FLD1* promoters under analogous culture conditions. This indicated that heterologous expression from *FLD1* promoter is at least as efficient as expression from the *AOX1,* both for intracellularly and extracellularly-expressed proteins.

Fourth, the use of methylamine as nitrogen source clearly resulted in lower final biomass levels when compared with corresponding cell cultures grown on ammonium sulphate. This phenomenon is probably related with a physiological condition of nitrogen limitation that cells experience when growing in the presence of methylamine as sole nitrogen source (Zwart and Harder, 1983).

3.3.3. Bioreactor batch cultivations

The shake flask culture experiments provided preliminary information on the pattern of ROL expression under the control of *FLD1* promoter by cells grown on different carbon and nitrogen sources combinations. However, a major objective of this study was to explore the potentially of this system for bioreactor culture processes, particularly in the future design of high-cell density cultivation strategies using the *FLD1*-based expression system. Therefore, we performed a number of batch bioreactor cultivations, where higher reproducibility and higher cell densities can be achieved because of controlled environmental conditions (dissolved oxygen, pH). We used a selection of the media used in previous shake flask cultures. In particular, four carbon-nitrogen source combinations were tested: methanol plus ammonium sulphate (M/NH_4^+), methanol plus methylamine (M/MA), glycerol plus methylamine (GL/MA) and sorbitol plus methylamine (S/MA). The initial carbon source concentration in these cultures was 20 $g \cdot L^{-1}$, except for the methanol-grown cultures where this substrate was added in two steps of 10 g each in order to minimise a possible toxic effect on the growth of the micro organism due to an excess of methanol.

The main fermentation parameters are showed in figure 6 to 9 and table 1. In terms of final lipase activities and biomass attained, the bioreactor cultures behaved in the same pattern as that observed in shake flask cultures.

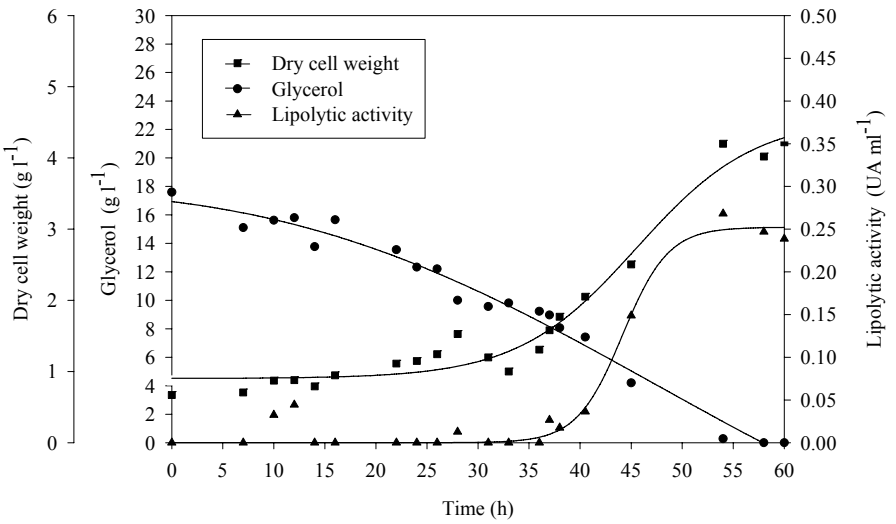


Figure 6. Bioreactor batch cultivation of *P. pastoris* expression ROL using glycerol and methylamine as carbon and nitrogen source respectively.

In all tested conditions, the substitution of ammonium for methylamine provoked a clear decrease in the specific growth rate and yield of biomass per gram of carbon source, $Y_{x/s}$. As already mentioned, this is probably due to the physiological situation of nitrogen limitation that cells experience when growing on methylamine as single carbon source (Zwart and Harder, 1983), in spite the fact that significant concentrations of methylamine were still detected at the end of all cultures.

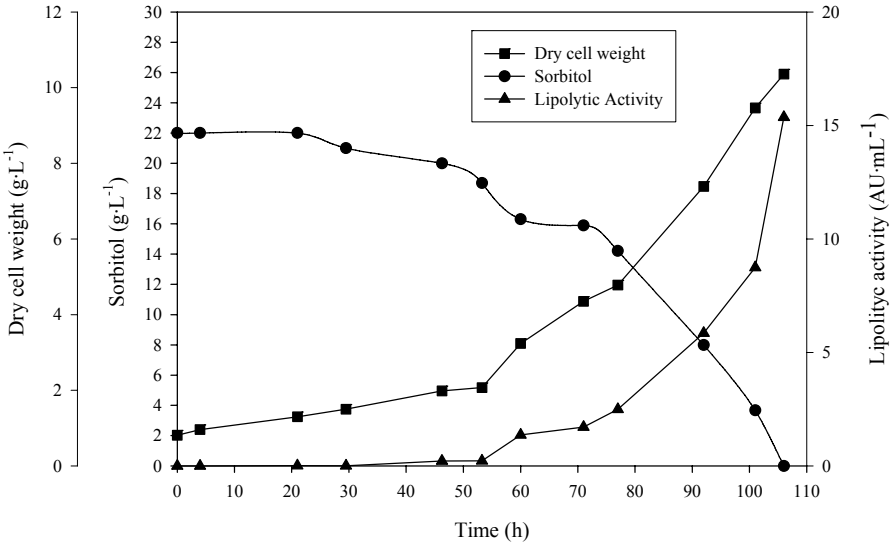


Figure 7. Bioreactor batch cultivation of *P. pastoris* expression ROL using sorbitol and methylamine as carbon and nitrogen source respectively.

	Glycerol methylamine FLD	Sorbitol methylamine FLD	Methanol ammonium FLD	Methanol ammonium AOX	Methanol methylamine FLD
Lipolytic activity (UA ml^{-1})	0.3	15	12	8	19
$Y_{X/S}$ ($gX\ gS^{-1}$)	0.25	0.45	0.14	0.12	0.06
$Y_{lipase/X}$ ($UA\ gX^{-1}$)	120	1666	4987	1875	16727
μ (h^{-1})	0.06	0.025	0.04	0.04	0.012
Productivity ($UA\ h^{-1}$)	5.17	144	300	266	292

Table 1. Comparison of *P. pastoris* bioreactor cultures expressing ROL under control of *FLD1* or *AOX1* promoter. X: dry cell weight ($g{\cdot}L^{-1}$), S: substrate ($g{\cdot}L^{-1}$), μ: specific growth rate (h^{-1}), UA: units of activity.

When glycerol was used as the carbon source and methylamine as the inducer substrate (figure 6), lipase levels were practically null. Nevertheless, low levels of lipolytic activity were detected in the culture supernatant at the end of the cultivation (ca. 0.3 U ml^{-1}), i.e. when glycerol concentration became limiting. These results confirm that excess of glycerol in the medium results in strong catabolite repression of the *FLD1* promoter; only when glycerol concentration decreases substantially and becomes limiting the *FLD1* promoter becomes gradually (partially) derepressed and a maximum of lipolytic activity is achieved. This pattern has also been observed for ROL expression under the control of *AOX1* promoter in *P. pastoris* glycerol-limited chemostat cultures (unpublished results). In contrast, as observed in the sorbitol and methylamine culture, catabolite repression on the *FLD1* promoter by sorbitol seems to be much lower, as significantly higher levels of lipolytic activity were detected along the cultivation course, in the presence of an excess of sorbitol. The maximum specific growth rate on sorbitol was significantly lower than on glycerol. In fact, sorbitol in the medium was not exhausted after 105 hours.

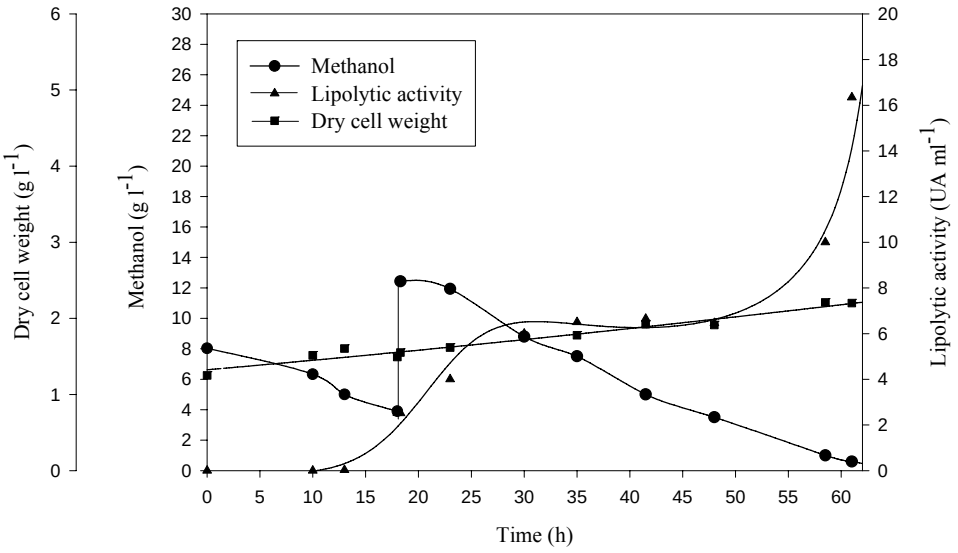


Figure 8. Bioreactor batch cultivation of *P. pastoris* expression ROL using methanol and methylamine as carbon and nitrogen source respectively.

In both methanol-grown cultures, high concentrations of methanol seem to reduce the transcription rates from *FLD1* promoter, as observed after the second addition of methanol. This suggests that methanol limiting concentrations might increase transcription efficiency from *FLD1* promoter, as reported for *AOX1* (Lin Cereghino and Cregg, 2000). This would be in agreement with the hypothesis that energy limitation increases the amount of formaldehyde available for dissimilation to CO_2 and NADH generation via FLD and FDH, being formaldehyde the inducer of *FLD1* gene expression (Harder and Veenhuis, 1989). Overall, there is evidence that induction of *FLD1* promoter by methylamine is not totally independent on the carbon source (and its concentration) used for cell growth.

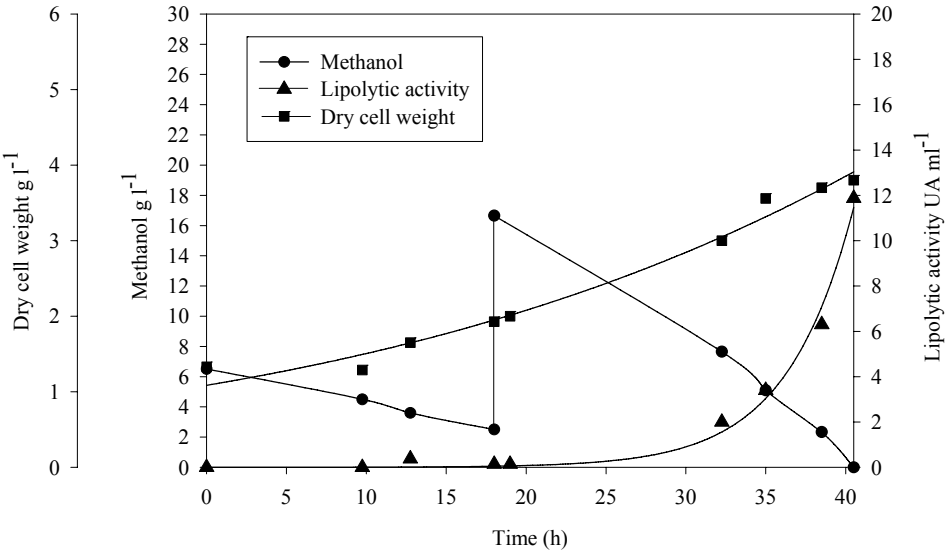


Figure 9. Bioreactor batch cultivation of *P. pastoris* expression ROL using methanol and ammonium as carbon and nitrogen source respectively.

In terms of yield of active lipase per gram of biomass ($Y_{lip/X}$), the combination of methanol and methylamine showed the highest value. This is, co-induction with methanol and methylamine resulted in a synergetic effect on ROL expression levels, which were higher than the sum of expression levels observed in cultures with either methanol or methylamine being present as unique inducers (i.e. M/NH_4^+ and GL/MA). Importantly, high $Y_{lip/X}$ values were obtained in S/MA cultures, indicating that sorbitol could be a valid alternative carbon source to the use of methanol for heterologous protein production in *P. pastoris* high-cell density fed-batch cultivations using the *FLD1* promoter.

3.4. Conclusions

In this chapter, the cloning, expression, and the preliminary study of the conditions for the production of the *Rhizopus oryzae* lipase in *P. pastoris* have been reported. The *Rhizopus oryzae* lipase (ROL) and the promoter from the *P. pastoris FLD1* gene were cloned into the plasmid pPICZα and successfully expressed in *P. pastoris*. Moreover, a set of experiments were performed in bioreactor to study the effect of different carbon sources on the *PFLD1* induction. The main conclusions obtained from this work are:

- *R. oryzae* lipase was successfully cloned into the plasmid pPICZα in *P. pastoris* under the control of the nitrogen source-regulated promoter *FLD1*.
- Extracellular ROL expression was achieved in shake flask cultures grown on different carbon and nitrogen sources. While the glucose and glycerol containing cultures did not produce quantifiable amounts of lipase, the methanol containing cultures reached high expression levels, either with ammonium or methylamine as carbon source. Moreover the sorbitol and methylamine culture obtained also high expression levels of ROL, using methylamine as inducer.
- Controlled batch experiments in bioreactor were performed. Glycerol and methylamine culture presented very low expression levels of ROL. On the other hand, the sorbitol and methylamine batch culture obtained 10-fold higher expression levels ($AU \cdot mL^{-1}$) than the glycerol culture. However, the highest lipase activity was achieved in the methanol and methylamine culture, where both inducers were present in the medium, producing a synergetic effect on ROL expression.
- The viability of the *FLD1* promoter to express ROL in *P. pastoris* was proved. The *FLD1* promoter has demonstrated to be at least as efficient as the *AOX1*. Moreover, the use of sorbitol has proved to be an interesting and feasible alternative carbon source for methanol-free high cell density cultures of *P. pastoris* using the *FLD1* promoter.

3.5. References

Beer HD, McCarthy JEG, Bornscheuer UT and Schmid RD (1998) Cloning, expression, characterization and role of the leader sequence of a lipase from *Rhizopus oryzae*. Biochimica et Biophysica Acta-Gene Structure and Expression 1399: 173-180.

Bensalah A, Fendri K and Gargoury Y (1994) The Enzyme of *Rhizopus-Oryzae* - Production, Purification and Biochemical Characteristics. Revue Francaise des Corps Gras 41: 133-137.

Cregg JM and Russell KA (1989) Transformation. In: Higgins DR and Cregg JM (eds) *Pichia* protocols.

d'Anjou MC and Daugulis AJ (2001) A rational approach to improving productivity in recombinant *Pichia pastoris* fermentation. Biotechnology and Bioengineering 72: 1-11.

Eggeling L and Sahm H (1978) Derepression and partial insensitivity to carbon catabolite repression of methanol dissimilating enzymes in *Hansenula polymorpha*. European Journal of Applied Microbiology and Biotechnology 5: 197-202.

Harder W and Veenhuis M (1989) Metabolism of one-carbon compounds. In: Rose AH and Harrison JS (eds) The yeast, pp 289-316. Academic press, London.

Haywood GW and Large PJ (1981) Microbial oxidation of amines - Distribution, purification and properties of 2 primary amine oxidases from the yeast *Candida boidinii* grown on amines as sole nitrogen source. Biochemical Journal 199: 187-201.

Horton RM, Hunt HD, Ho SN, Pullen JK and Pease LR (1989) Engineering hybrid genes without the use of restriction enzymes - Gene-splicing by overlap extension. Gene 77: 61-68.

Inan M and Meagher MM (2001) Non-repressing carbon sources for alcohol oxidase (AOX1) promoter of *Pichia pastoris*. Journal of Bioscience and Bioengineering 92: 585-589.

Jones JG and Bellion E (1991) Methylamine metabolism in *Hansenula polymorpha* - An In vivo C-13 and P-31 nuclear magnetic resonance study. Journal of Bacteriology 173: 4959-4969.

Kato N, Sahm H and Wagner F (1979) Steady-State Kinetics of Formaldehyde dehydrogenase and formate dehydrogenase from a methanol-utilizing yeast, *Candida boidinii*. Biochimica et Biophysica Acta 566: 12-20.

Large PJ and Green J (1984) Oxidation of mono, di, and trimethylamine by methazotrophic yeast: properties of the microsomal and peroxisomal enzymes involved and comparision with bacterial enzyme systems. In: Crawford RL and Hanson RS (eds) Microbial growth on C_1 compounds, American Society for Microbiology, Washington, D.C.

Minning S, Schmidt-Dannert C and Schmid RD (1998) Functional expression of Rhizopus oryzae lipase in Pichia pastoris: high-level production and some properties. Journal of Biotechnology 66: 147-156.

Minning S, Serrano A, Ferrer P, Sola C, Schmid RD and Valero F (2001) Optimization of the high-level production of *Rhizopus oryzae* lipase in *Pichia pastoris*. Journal of Biotechnology 86: 59-70.

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular cloning. A laboratory manual. Cold Spring NY.

Serrano A. Estudio de la producción the una lipasa del hongo *Rhizopus oryzae* en la levadura metilotrófica *Pichia pastoris*. 2002. Bellaterra, Catalonia, Spain, Universitat Autonoma de Barcelona. PhD thesis.

Shen SG, Sulter G, Jeffries TW and Cregg JM (1998) A strong nitrogen source-regulated promoter for controlled expression of foreign genes in the yeast *Pichia pastoris*. Gene 216: 93-102.

Sibirny AA, Ubiyvovk VM, Gonchar MV, Titorenko VI, Voronovsky AY, Kapultsevich YG and Bliznik KM (1990) Reactions of direct formaldehyde oxidation to CO_2 are nonessential for energy supply of yeast methylotrophic growth. Archives of Microbiology 154: 566-575.

Thorpe ED, d'Anjou MC and Daugulis AJ (1999) Sorbitol as a non-repressing carbon source for fed-batch fermentation of recombinant *Pichia pastoris*. Biotechnology Letters 21: 669-672.

Trotsenko YA, Bystrykh LV and Ubiyvovk VM (1984) Regulatory aspects of methanol metabolism in yeast. In: Crawford RL and Hanson RS (eds) Microbial growth on C_1 compounds, American Society for Microbiology, Washington, D.C.

van Dijken J.P and Bos P (1981) Utilization of amines by yeast. Archives of Microbiology 3: 320-4.

van Dijken JP, Ootra-Demkes GJ, Otto R and Harder W (2006) S-fomilglutathione: the substrate for format dehydrogenase in methanol utilizing yeast. Archives of Microbiology 2-3: 77-83.

Waterham HR, Digan ME, Koutz PJ, Lair SV and Cregg JM (1997) Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. Gene 186: 37-44.

Zwart KB and Harder W (1983) Regulation of the metabolism of some alkylated amines in the yeasts *Candida utilis* and *Hansenula polymorpha*. Journal of General Microbiology 129: 3157-3169.

CHAPTER 4

Developing fed-batch cultivation strategies for heterologous production of *Rhizopus oryzae* lipase in *P. pastoris* using the *FLD1* promoter.

4.1. Introduction

As seen in chapter 3, the *P. pastoris FLD1* promoter can be a good alternative to the classic *AOX1* promoter for controlled expression of heterologous genes in *P. pastoris*. Initial characterization of the *FLD1* promoter showed that it was strongly and independently induced either by methanol as carbon source or methylamine as nitrogen source (Shen et al. 1998).

However, the studies presented in the previous chapter indicate that this is not the case, suggesting that the use of sorbitol combined with methylamine as nitrogen source could be the basis for the development of methanol-free fed-batch fermentation processes for heterologous protein production in *P. pastoris* based in the *FLD1* promoter. Sorbitol showed to be a non repressive carbon source for the methanol metabolism-related enzymes (Thorpe et al., 1999; Inan et al., 2001)

In this chapter, the basis for a new methanol-free fed-batch strategy is explored, focused on a pre-programmed exponential feeding of sorbitol and methylamine solution at a stoichiometrically fixed ratio to maintain a constant specific growth rate during the induction phase. By using this strategy we have studied the behaviour of the main fermentation parameters under three different specific growth rates. Moreover, this strategy, based on the use of the *FLD1* promoter has been compared to the conventional one used in *AOX1* promoter-based systems.

4.2. Materials and methods

4.2.1. Strains

A *P. pastoris* X-33-derived strain containing the expression vector pPICZFLDαROL integrated in its genome *FLD1* locus was used throughout this study.

4.2.2. Fed-batch cultivation set up and operational conditions

Bioreactor fed batch cultivations were carried out using a mineral media with the following basal composition per liter: for the batch phase, KH_2PO_4 12.0 g, $MgSO_4 \cdot 7H_2O$ 4.70 g, $CaCl_2 \cdot 2H_2O$ 0.36 g, 0.1 mL of antifoam, alkoxylated ester JG73 (Strucktol, Hamburg, Germany), 1 mL of a biotin solution (400 mg L^{-1}), and 1 mL of trace salts solution (0.2 mM $CuSO_4 \cdot 5H_2O$, 1.25 mM KI, 4.5 mM $MnSO_4 \cdot 4H_2O$, 2 mM $Na_2MoO_4 \cdot 2H_2O$, 0.75 mM H_3BO_3, 17.5 mM $ZnSO_4 \cdot 7H_2O$, 44.5 mM $FeCl_3 \cdot 6H_2O$). The biotin and trace salts components were sterilized separately by microfiltration (Millex GS 0.22 μm, Millipore, Malsheim, France).

Fed-batch cultures were performed in a 5-litre Braun Biostat ED bioreactor (Braun Biotech, Melsungen, Germany), with an initial working volume of 3.5 L. The cultivation process comprised three phases:

Firstly, a batch growth phase was performed using glycerol (40 g L^{-1}) as sole carbon source and ammonium sulfate at the corresponding stoichiometric quantity (9.2 g L^{-1}) as sole nitrogen source. The ammonium sulfate concentration was estimated to achieve the exhaustion of both at the same time at the end of the batch phase. Such amount was calculated on the basis of the elementary composition of *P. pastoris* cells grown in batch cultivation on glycerol and ammonium as sole carbon and nitrogen source, respectively, $C\ H_{1.87}\ O_{0.56}\ N_{0.18}\ S_{0.008}$, and the $Y_{X/S}$ of 0.5 g of biomass per g of glycerol. The calculated stoichiometric ratio was 0.23 g $(NH_4)_2SO_4$/g glycerol.

Secondly, after glycerol depletion, a batch of 10 g L^{-1} of sorbitol and 3 g L^{-1} of methylamine ($CH_3NH_2 \cdot HCl$) was added into the bioreactor to induce the *FLD1* promoter.

This transition phase lead to the third phase of the cultivation, i.e. the induction phase, during this phase, a pre-programmed exponential feeding rate strategy ensured a constant specific growth rate along the fed-batch induction phase. In the induction phase, the sorbitol:methylamine ratio in the feeding stock solution reflected the stoichiometric ratio between these substrates, 0.118 g methylamine g $sorbitol^{-1}$, as calculated on the

basis of the elementary composition of *P. pastoris* cells growing in batch cultivations on sorbitol and methylamine, $C\ H_{1.74}\ O_{0.51}\ N_{0.14}\ S_{0.008}$, and a yield biomass/substrate, $Y_{X/S}$, of about 0.43 g X g sorbitol. The feeding stock solution contained 300 g L^{-1} of sorbitol and 35 g L^{-1} of methylamine, and was added to the reactor by an automatic micro burette MicroBU-2031 from Crison Instruments (Alella, Barcelona, Spain). The cultivation conditions were: stirring rate 800 rpm, temperature 30 ºC, pH controlled at 5.5 by adding 5M KOH, dissolved oxygen controlled above 30 % with an airflow rate between 1.5 – 20 L min^{-1}.

4.2.3 Western blot analyses

Western blots were carried out according to the protocol detailed in chapter 2 (page 22). For the *Rhizopus oryzae* lipase (ROL) detection a mouse anti-ROL antiserum was used (raised at the Institute of Applied Microbiology, BOKU – University of Natural Resources and Applied Life Sciences, Vienna, Austria) with a dilution 1:100. Antimouse IgG horseradish peroxidase conjugate (Sigma) was used as a secondary antibody diluted 1:1000.

4.3.4. Pre-programmed exponential feeding rate

A pre-programmed exponential feeding rate strategy was designed according to D'Anjou and Daugulis (2001) with the objective to control the specific growth rate at a constant value along the fed-batch induction phase. If a quasi-steady state is assumed for the residual substrate concentration, the specific growth rate of the culture at time (t) can be obtained from fed-batch substrate balance (eq.1).

$$\mu(t) = \frac{Y_{X/S}\, F(t)\, S_0}{V(t)\, X(t)} \qquad (1)$$

By integrating the fed-batch cell mass balance (eq.2) and combining it with equation 1, the feeding rate F(t) for a fixed specific growth rate can be expressed by equation 3.

$$X(t)V(t) = X(t_0)V(t_0)\exp(\mu(t-t_0)) \qquad (2)$$

$$F(t) = \frac{\mu\left[X(t_0)V(t_0)\right]}{Y_{X/S}\, S_0}\exp\left[\mu(t-t_0)\right] \qquad (3)$$

This feeding rate equation can be applied if V, X and $Y_{X/S}$ are known at t_0 and the yield can be assumed as a constant along the fermentation. At start of fed-batch phase, t_0, the biomass concentration was around 30 g L^{-1}, the culture volume was 3.6 L, and the sorbitol feed concentration was fixed at 300 g L^{-1}. The biomass/sorbitol yield was considered to be constant at 0.43 g X g sorbitol^{-1} under these conditions.

For programming reasons, the feeding rate (eq. 3) was expressed as a function of the previously added feed rate in equation 4. In this way, the feed rate (F) does not depend on the absolute time because the new addition rate can be expressed as the product of the previous feed rate and the exponential factor. The equation only requires the initial value of $F(t_o)$ when the induction phase starts and the time between two additions (Δt: 1min).

$$F(t+\Delta t)=F(t)\exp(\mu\,\Delta t) \quad (4)$$

4.2.5. Calculation of specific rates

Estimated reaction rates were specific growth-rate (μ) [h^{-1}], specific substrate consumption rate (q_S) [g methanol g biomass^{-1} h^{-1}] and specific lipase production rate (q_P) [AU g biomass^{-1} h^{-1}]. The estimation procedures were performed by Oriol Cos Busquets using suitable smoothing routines (Matlab 6.1 Curvefit Toolbox, The Mathworks Inc., Natick, USA) and mass balances that permitted to supply complete data sets with coincident off-line biomass, substrate and lipase activity data.

Firstly, biomass, substrate and lipase activity data were smoothed. After that, the first derivatives of the smoothed curves were obtained. Feed-flow rate was time-averaged between consecutive off-line biomass samples. Total volume was estimated taking into account feed-flow rate including carbon sources (methanol and/or glycerol), acid and base solutions, as well as volume samples.

Finally, mass balances inside the bioreactor for fed-batch operation were conducted to obtain the reaction rates as follows:

$$\mu = \frac{1}{(X \cdot V)} \frac{d(X \cdot V)}{dt} \quad (5)$$

$$q_S = -\left(\frac{dS}{dt} + \frac{F \cdot (S - S_0)}{V}\right) \cdot \frac{1}{X} \quad (6)$$

$$q_P = \left(\frac{dP}{dt} + \frac{F \cdot P}{V}\right) \cdot \frac{1}{X} \quad (7)$$

4.3. Results

4.3.1. Fed-batch cultures with *FLD1*-based system

Using the growth parameters obtained from the previously performed batch experiments a high cell density fed-batch cultivation strategy was implemented. The specific growth rate has been proved to have an important effect on secreted recombinant protein productivities in *P. pastoris* (Zhang et al., 2000; Sinha et al., 2003). Hence, two fed-batch cultivations were initially performed using such strategy, with their fed-batch phases pre-set at a 0.005 h^{-1} (i.e. about 20% of the μ_{max}) and 0.01 h^{-1} (i.e. about 40% of the μmax), respectively. The evolution of the key parameters during the transition and induction phases of these cultivations is shown in figure 1A and figure 1B.

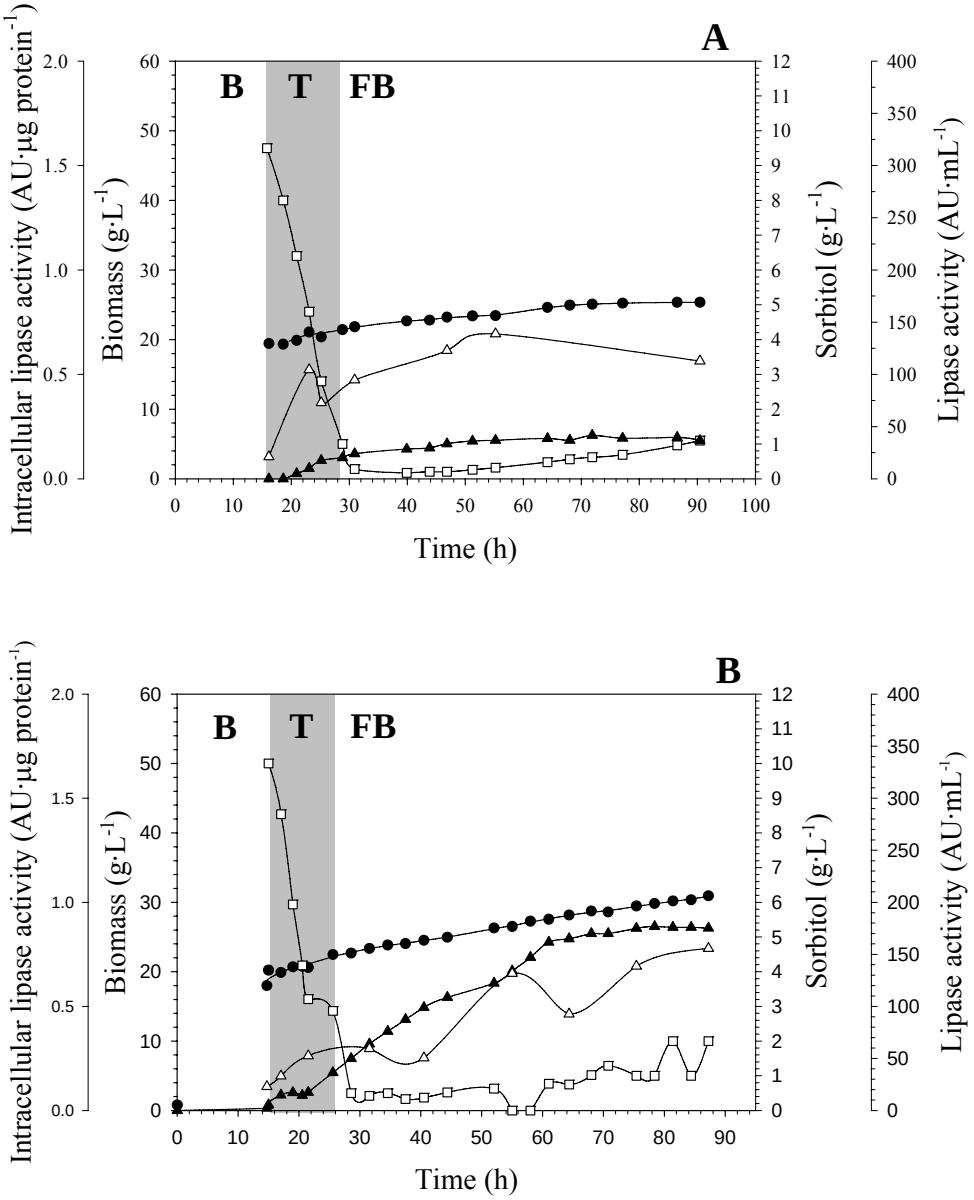


Figure 1. A: Fed-batch cultivation of *P. pastoris* expressing ROL at a controlled specific growth rate of 0.005 h^{-1}. B: Fed-batch cultivation of *P. pastoris* expressing ROL at a controlled specific growth rate of 0.01 h^{-1}. Biomass (dry cell weight) (●), sorbitol concentration (□), extracellular lipase activity (▲) and intracellular lipase activity (△) are indicated.

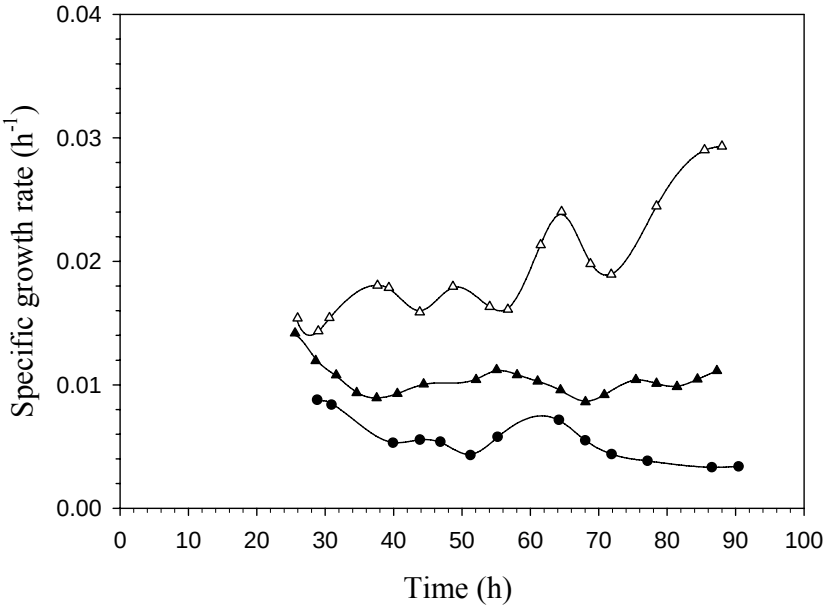


Figure 2. Specific growth rate profiles of three different fed-batch cultures of *P. pastoris* expressing ROL. Mean specific growth rate of 0.005 (h^{-1}) (●), mean specific growth rate of 0.01 (h^{-1}) (▲) and at constant residual sorbitol concentration of 8 g L^{-1} (near μ_{max})(△).

As can be seen in figures 1A and 1B, induction of the *FLD1* promoter is quite fast; both intracellular and extracellular lipase activities could be detected early after the sorbitol and methylamine pulse. Moreover, the levels of extracellular lipase at the end of the transition phase were similar in both cultivations. This fact indicates the reproducibility of the cultivation strategy used for product induction.

The experimentally estimated μ values during the fed-batch phase (figure 2) closely matched the pre-set values within a ± 0.001~0.0015 variation/range. Only in later stages of the lower growth rate cultivation there is a slight decrease in the experimental μ values. As expected, during this phase, residual sorbitol levels were below 0.5 g L^{-1} and 1.0 g L^{-1} in the cultivations at a μ of 0.005 h^{-1} and 0.01 h^{-1}, respectively, and only in the later stages of the cultivations these values showed a slight increase. Also, the evolution of the q_s values are well in agreement with the pre-programmed exponential feeding parameters and, consistently, q_s values stayed constant along the fed-batch phase in both cultivations (figure 3).

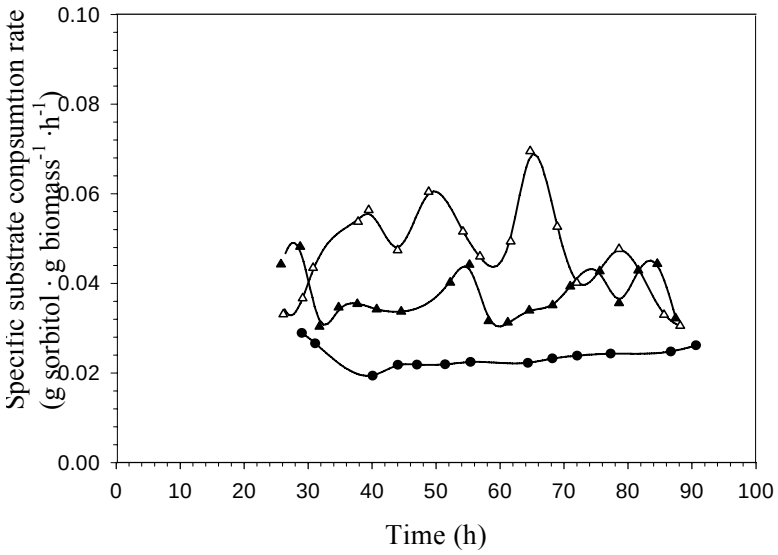


Figure 3. Specific substrate compsumtion rate profiles of three different fed-batch cultures of *P. pastoris* expressing ROL. Mean specific growth rate of 0.005 (h^{-1}) (●), mean specific growth rate of 0.01 (h^{-1}) (▲) and at constant residual sorbitol concentration of 8 g L^{-1} (near μ_{max})(△).

Remarkably, the extracellular lipase activity profiles in these two cultivations are very different between the lower and the higher growth rate fed-batch phases. In the 0.005 h^{-1} fed-batch cultivation, extracellular lipase exponential accumulation ceased soon after the transition phase, reaching only a maximum of 42 AU mL^{-1}. In contrast, in the fed-batch at 0.01 h^{-1} extracellular lipase levels increased steadily up to 60 h of cultivation. These extracellular lipase accumulation profiles are reflected in the evolution of q_p values throughout the cultivation (figure 4). In both cases, q_p values in the early stages of the transition phase are similar (about 70 ~ 110 AU g X^{-1} h^{-1}). However, in the lower growth rate cultivation, a maximum q_p of 88 AU g X^{-1} h^{-1} is obtained at the end of the transition phase, whereas in the 0.01 h^{-1} specific growth rate a maximum q_p of 206 AU g X^{-1} h^{-1} is reached soon after the start of the induction. After these maxima, an exponential decrease in q_p was observed in both cases, except for a transient increase in the 0.01 h^{-1} fed_batch cultivation at 55~60 hours after the start of the fermentation.

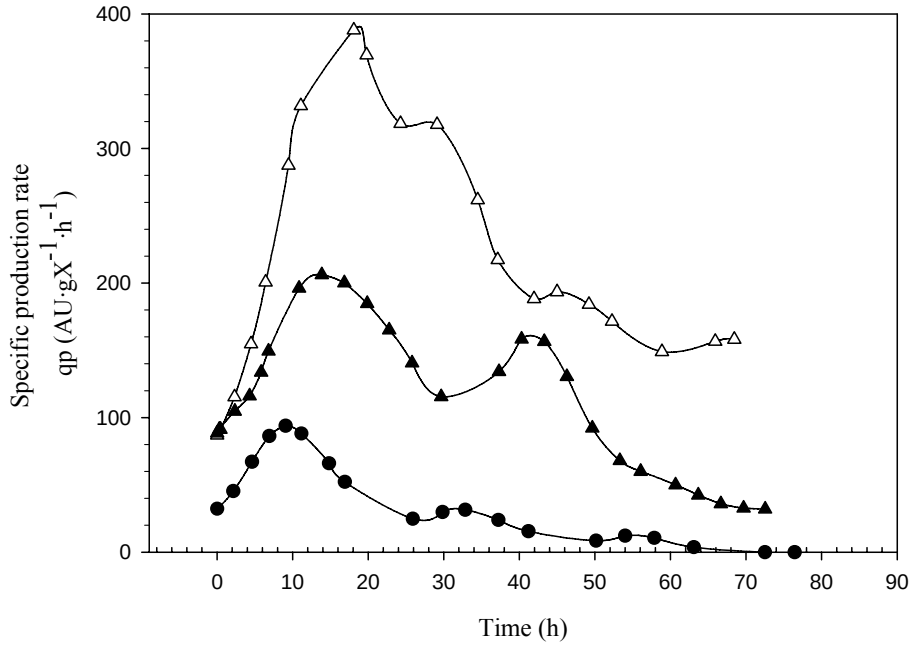


Figure 4. Specific product production rate (q_p) profiles of three different fed-batch cultures of *Pichia pastoris* expressing ROL at different specific growth rate. Mean specific growth rate of 0.005 (h^{-1}) (●), mean specific growth rate of 0.01 (h^{-1}) (▲) and constant residual sorbitol concentration of 8 g L^{-1} (near μ_{max}) (△).

Hence, it appears that ROL secretion can not be sustained after induction under growth limiting conditions. Further, the decrease in q_p (i.e. in the ROL secretion rate) does not appear to be concomitant with an increasing intracellular product accumulation: After an initial exponential increase of intracellular lipase levels upon ROL expression induction, these values stayed rather constant along the induction phase.

The exponential decrease in q_p could also be attributed to the presence of endogenous proteases released to the extracellular medium e.g. as a result of cell lysis phenomena. Nevertheless, extracellular protease levels in the lower growth rate fed-batch cultivation were very low/basal, i.e. indicating low cell lysis and product degradation levels. Western blotting analyses of cultivation broth samples using anti-ROL antibodies confirmed that ROL degradation was minimal (figure 5).

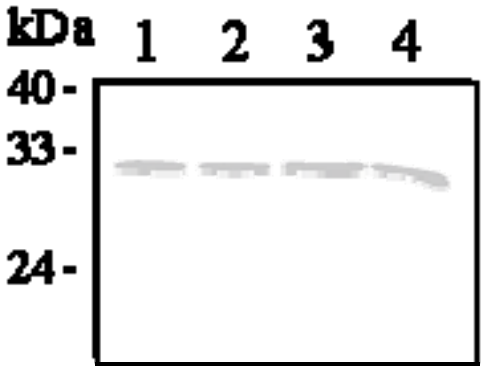


Figure 5. Western blot analyses of culture supernatants from fed-batch cultivations of *P. pastoris* expressing ROL. Lane 1: Culture supernatant at 86 h of fed-batch cultivation performed at a low specific growth rate (0.005 h^{-1}). Lane 2: Culture supernatant at 88 h of fed-batch cultivation performed at a medium specific growth rate (0.01 h^{-1}). Lane 3 and 4: Culture supernatants at 75 and 88 h of fed-batch cultivation, respectively, performed at constant residual sorbitol concentration of 8 g L^{-1} (near μ_{max}).

Therefore, these results suggest that extracellular ROL production is favored when the microorganism grows at higher growth rates and, therefore, in a situation of carbon excess.

Thus, a third fermentation was performed where the feeding rate was manually programmed to maintain sorbitol in excess of 8 g L^{-1}. This set concentration value allowed to maintain the cultivation under carbon and nitrogen excess conditions, as well as keeping sorbitol residual concentrations well below growth-inhibitory levels (>40 g L^{-1}, Thorpe et al., 1999). For such cultivation, the feeding rate was manually programmed according to the consumption rate estimated from the off-line measured residual sorbitol concentration in the cultivation broth. In this way, sorbitol was maintained in excess.

The results of this cultivation are shown in figure 6. As observed, the simple off-line control strategy allowed maintaining sorbitol residual levels within ± 2 g L^{-1} range of the set point, i.e. allowing cells to grow at a near-μ_{max} growth rate. Under these conditions, extracellular lipase levels steadily increased until the end of the cultivation time (90 h), reaching a maximum of 385 AU mL^{-1}, i.e. 2.2-fold higher than in cultivation at μ 0.01 h^{-1} and 9.2-fold higher than in cultivation at μ 0.005 h^{-1} (table 1).

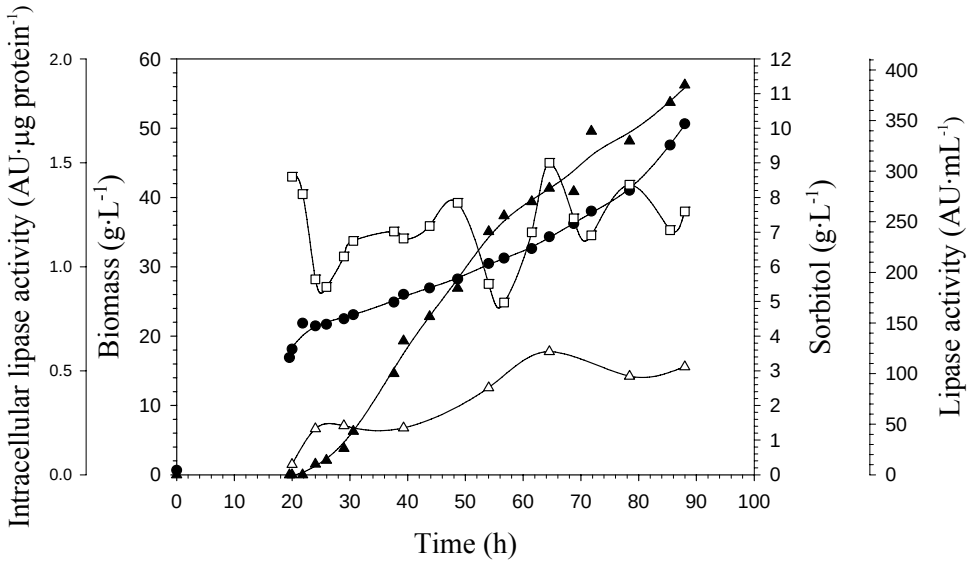


Figure 6. Fed-batch cultivation of *P. pastoris* expressing ROL at constant residual sorbitol concentration of 8 g L^{-1} (near μ_{max}). Biomass (dry cell weight) (●), sorbitol concentration (□), extracellular lipase activity (▲) and intracellular lipase activity (△) are indicated.

The average specific growth rate during the fed-batch phase was 0.02 ± 0.004 h^{-1}, i.e. about 80% of the μ_{max} observed in batch cultivations (0.025 h^{-1}). As expected, q_s values were higher than in fed-batch cultivations at lower specific growth rates. Most interestingly, q_p profiles during the fed-batch phase at near-μ_{max} were remarkably different from those observed in growth-limiting conditions (figure 4): The maximum q_p value (388 AU g X^{-1} h^{-1}) was obtained after about 20 h of the induction phase start, that is, 1.9-fold and 4.4-fold higher than in cultivations at µ 0.01 and 0.005 h^{-1}, respectively. Moreover, this maximum was reached in a later stage compared with the cultivations under growth-limiting conditions. After reaching this maximum, q_p values decreased exponentially down to a value of about 150 AU g X^{-1} h^{-1}. After this point, reached 50 h after the start of the induction phase, q_p values remained stable. Interestingly, the intracellular lipase activity levels followed similar profiles and maximum values to those observed in the cultivations at lower growth rates. Notably, the fact that intracellular lipase activity is detected in all three cultivations reflects that some active (i.e. properly folded and processed) ROL is retained within the cell's periplasm, and/or in the secretion vacuoles.

A comparative analysis of the fed-batch cultivations in terms of $Y_{P/X}$, productivity and specific productivity (table 1) revealed that the overall efficiency of the system increased together with the specific growth rate of the corresponding fed-batch cultivation. That is, at the highest specific growth rate cultivations, $Y_{P/X}$ values were 1.3-fold and 4.6-fold higher, the productivity was 1.9-fold and 7.6-fold higher, and the specific productivity was 1.1-fold and 3.8-fold higher than in cultivations at μ 0.01 and 0.005 h^{-1}, respectively (table 1). These results clearly indicate that the best strategy for ROL extracellular production using the P*FLD1*-based system is a fed-batch induction phase with a constant sorbitol excess residual concentration in the cultivation broth.

	AU max (AU mL^{-1})	$Y_{P/X}$ (AU g X^{-1})	μ mean (h^{-1})	Productivity (AU L^{-1} h^{-1})	Specific productivity (AU g X^{-1} h^{-1})
P*AOX*[a] Mut[s]	205	5775	0.005	2246	63
P*AOX*[a] Mut[+]	150	2470	0.036	3000	49
P*FLD1* μ = 0.005 h^{-1}	42	1658	0.005	579	23
P*FLD1* μ = 0.01 h^{-1}	177	5932	0.010	2254	76
P*FLD1* μ = 0.02 h^{-1}	385	7634	0.020	4379	87

[a] data taken from Cos et al. (2005)

Table 1. Maximal lipase activity, lipase yield, mean specific growth rate and productivities obtained in fed-batch cultivations of P*FLD1* and P*AOX*-based *P. pastoris* strains expressing ROL. X: dry cell weight ($g \cdot L^{-1}$), S: substrate ($g \cdot L^{-1}$), μ: specific growth rate (h^{-1}), UA: units of activity.

4.3.2. Comparision of *FLD1*-based fed-batch cultures with the classic *AOX1* system.

In table 1 and 2 are compared data taken from *AOX1* promoter-based high cell density fed-batch cultivations (Cos et al., 2005) with the newly performed *FLD1* based fermentations carried out at equivalent constant growth rates. Specifically, the cultivation of the *P. pastoris* Mut[s] strain expressing *ROL* was carried out at about the same growth rate than for the present *P. pastoris* cultivation at the lower growth rate (about 0.005 h^{-1}).

Remarkably, we find that, *P. pastoris* growing on methanol and ammonium sulfate as sole carbon and nitrogen sources, respectively, allow for sustained ROL production, in clear contrast to the cultivation with the combination sorbitol plus methylamine, clearly showing the impact of cells' metabolism on process productivity. Also, whereas in the *AOX1* promoter-based system using the Mut^s strain, q_p maximum values are reached after 20 hours of methanol induction, in the P*FLD1*-based system these were achieved only 4~5 hours after methylamine induction. This indicates that q_p profiles over cultivation time (i.e. the kinetics of product secretion) are significantly different. Overall, ROL expression and secretion levels over the cultivation time seem to be substrate (C-source and/or N-source) dependent. This has also been observed for other proteins expressed in *P. pastoris* (Hohenblum et al., 2004).

In terms of process productivity, the comparison of the results obtained with the P*FLD1*-based system cultivation at near μ_{max}, 0.025 h^{-1} with those obtained with both Mut^s and Mut^+ P*AOX*-based strains clearly indicate that the performance of the P*FLD1* system is better than the classic P*AOX* system (1.9- and 1.5-fold higher productivity than the Mut^s and Mut^+ system, respectively).

The q_p profiles observed in all cultivations could reflect possible effects of protein turnover by proteolysis, which have also been reported to be a potential bottleneck for protein production in *P. pastoris* (Curvers et al., 2001, Jahic et al., 2003, Kobayashi et al., 2000). Further, ammonium limitation has been shown to trigger proteolytic degradation of ROL in high cell density cultivations using the classic *AOX1* promoter-based systems with Mut^+ strains (Cos et al., 2005). Notably, we could not detect significant product degradation nor extracellular protease levels in our cultivations, even in the case where methylamine levels were very low or below detection limits (cultivation at 0.005 h^{-1}).

	μ mean (h^{-1})	μ max (h^{-1})	q_p mean (AU X^{-1} h^{-1})	q_p max (AU X^{-1} h^{-1})	μ mean (h^{-1})
P*AOX*[a] Mut^S	0.005	0.014	83	170	0.005
P*AOX*[a] Mut$^+$	0.036	0.051	130	244	0.036
P*FLD1* μ = 0.005 h^{-1}	0.005	0.009	29	88	0.005
P*FLD1* μ = 0.01 h^{-1}	0.010	0.014	132	206	0.010
P*FLD1* μ = 0.02 h^{-1}	0.020	0.029	244	388	0.020

[a] data taken from Cos et al. (2005)

Table 2.. Mean and maximal specific growth rate (μ), and specific lipase production rate (q_p) obtained in fed-batch cultivations of P*FLD1* and P*AOX*-based *P. pastoris* strains expressing ROL. X: dry cell weight ($g \cdot L^{-1}$), S: substrate ($g \cdot L^{-1}$), μ: specific growth rate (h^{-1}), UA: units of activity.

Thus, the q_p and intracellular lipase profiles could reflect an adaptation of cell's physiological state to ROL overexpression along the cultivation time leading to a down regulation of the *ROL* transcription levels. Remarkably, maximum intracellular lipase levels in growth-limited cultivations were very similar, i.e. suggesting that a threshold level of intracellular ROL may exist. Interestingly, down regulation of secreted recombinant protein expression levels in stressed cells has recently been shown to be related to the triggering of the unfolded protein response (UPR) in the fungus *Trichoderma reesei* (Pakula et al., 2003). Further, the UPR has been also described in yeast such as *S. cerevisiae* (Valkonen et al., 2003) and *P. pastoris* (Hohemblum et al., 2004).

Besides, a parameter that should be taken into account is the oxygen requirements during fed-batch fermentations, oxygen might be a limiting factor when performing high density cultures. In our case, in the *FLD1* promoter based-fermentations performed with sorbitol and methylamine as carbon and nitrogen source respectively, the airflow used to maintain a constant dissolved oxygen concentration above 30% was 1.5 $L \cdot m^{-1}$ along the whole culture, similar results were obtained with the *AOX1*-promoter based fermentations with Mut^S strains. In contrast, in the *AOX1*-promoter fermentations with

Mut^+ strain, airflow rate steadily increased throughout the fed-batch phase from 10 to 20 $L \cdot m^{-1}$ becoming limiting at the 60-70 hours of culture (Cos O. PhD thesis).

4.4. Conclusions

In this chapter a series of experiments were performed in order to test the viability of a new strategy for high cell density cultures of *P. pastoris* using the *FLD1* promoter to control the expression of the *R. oryzae* lipase (ROL). The main conclusions obtained during this work are:

- A new fed-batch strategy has been developed for production of recombinant proteins in *P. pastoris* using the *FLD1* promoter.
- Methanol-free cultures based in the use of sorbitol and methylamine as carbon and nitrogen source, respectively, has been carried out. High levels of extracellular ROL were achieved using this strategy.
- The specific growth rate has demonstrated to be a key factor in process productivities using the *FLD1* promoter under the conditions mentioned above.
- A fed-batch culture strategy, under carbon-excess conditions (i.e. growing near to maximum specific growth rate) based on the maintenance of a constant sorbitol concentration in the culture broth has shown to be the most effective of the here tested approaches.
- In order to improve the system's performance, further studies should be performed to evaluate the effect of folded and misfolded product accumulation inside the cells and its potential relationship with decrease of product synthesis or secretion rate and cell viability.

4.5. References

Cos O, Serrano A, Montesinos J, Ferrer P, Cregg J and Valero F (2005) Combined effect of the methanol utilization (Mut) phenotype and gene dosage on recombinant protein production in *Pichia pastoris* fed-batch cultures. Journal of Biotechnology 116: 321-35.

Cos, O. Monitorització i control del procés de producció de proteïnes heteròlogues en el llevat metilotròfic *Pichia pastoris*. 2005. PhD Thesis.

Curvers S, Brixius P, Klauser T, Thommes J, Weuster-Botz D, Takors R and Wandrey C (2001) Human chymotrypsinogen B production with *Pichia pastoris* by integrated development of fermentation and downstream processing. Biotechnology Progress 17: 495-502.

d'Anjou MC and Daugulis AJ (2001) A rational approach to improving productivity in recombinant *Pichia pastoris* fermentation. Biotechnology and Bioengineering 72: 1-11.

Hohenblum H, Gasser B, Maurer M, Borth N and Mattanovich D (2004) Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris*. Biotechnology and Bioengineering 85: 367-375.

Inan M and Meagher MM (2001) Non-repressing carbon sources for alcohol oxidase (AOX1) promoter of *Pichia pastoris*. Journal of Bioscience and Bioengineering 92: 585-589.

Jahic M, Wallberg F, Bollok M, Garcia P and Enfors S (2003) Temperature limited fed-batch technique for control of proteolysis in *Pichia pastoris* bioreactor cultures. Microbial cell factories 2: 6.

Kobayashi K, Kuwae S, Ohya T, Ohda T, Ohyama M and Tomomitsu K (2000) Addition of oleic acid increases expression of recombinant human serum albumin by the AOX2 promoter in *Pichia pastoris*. Journal of Bioscience and Bioengineering 89: 479-484.

Pakula T, Laxell M, Huuskonen A, Uusitalo J, Saloheimo M and Penttila M (2003) The effects of drugs inhibiting protein secretion in the filamentous fungus *Trichoderma reesei* - Evidence for down-regulation of genes that encode secreted proteins in the stressed cells. Journal of Biological Chemistry 278: 45011-45020.

Shen SG, Sulter G, Jeffries TW and Cregg JM (1998) A strong nitrogen source-regulated promoter for controlled expression of foreign genes in the yeast Pichia pastoris. Gene 216: 93-102.

Sinha J, Plantz BA, Zhang WH, Gouthro M, Schlegel V, Liu CP and Meagher MM (2003) Improved production of recombinant ovine interferon-tau by Mut(+) strain of *Pichia pastoris* using an optimized methanol feed profile. Biotechnology Progress 19: 794-802.

Thorpe ED, d'Anjou MC and Daugulis AJ (1999) Sorbitol as a non-repressing carbon source for fed-batch fermentation of recombinant *Pichia pastoris*. Biotechnology Letters 21: 669-672.

Valkonen M, Penttila M and Saloheimo M (2003) Effects of inactivation and constitutive expression of the unfolded-protein response pathway on protein production in the yeast *Saccharomyces cerevisiae*. Applied and Environmental Microbiology 69: 2065-2072.

Zhang WH, Bevins MA, Plantz BA, Smith LA and Meagher MM (2000) Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A. Biotechnology and Bioengineering 70: 1-8.

CHAPTER 5

Analysis and engineering of bottlenecks in *R. oryzae* lipase production in *P. pastoris* using the nitrogen source-regulated *FLD1* promoter

---

5.1. Introduction

As seen in the previous chapter, the intracellular accumulation of soluble (biologically active) product within the cell during the fermentation was detected. Intracellular lipase was quantified by cell disruption and subsequent enzymatic assay, suggesting that some processed lipase was retained at some stage of the secretion process and/or by the cell wall. Moreover the specific product formation rate in all performed cultures experienced a drastic decrease soon after the transition phase. This observation, together with the intracellular product accumulation, suggested the presence of a bottleneck throughout the synthesis or/and secretion process of the heterologous lipase.

In this context, several studies have pointed out the presence of an effect produced by the accumulation of misfolded proteins in the endoplasmatic reticulum (ER), namely the unfolded protein response (UPR) of yeast (Mori et al., 1997), filamentous fungi (van Gemeren et al., 1997) and higher eukaryotes (Kozutsumi et al., 1988). In figure 1 the UPR activation signal transduction pathway in yeast is depicted. In the yeast *Saccharomyces cerevisiae* the protein Ire1p, a transmembrane serine-threonine kinase, oligomerizes when the ER-resident chaperone Hsp70 (BiP protein) is removed from it to bind the unfolded proteins. Ire1p activates its endonuclease activity in the cytosolic tail domain. The endonuclease Irep1 cuts the *HAC1* mRNA at two specific sites, *HAC1* mRNA is religated then by Rlg1p. After Ire1p splicing, *HAC1* mRNA can enter the ribosome and is efficiently translated into Hac1p, a transcriptional factor that

upregulates the UPR related genes encoding ER-chaperones such as BiP and other proteins (Patil and Walker 2001).

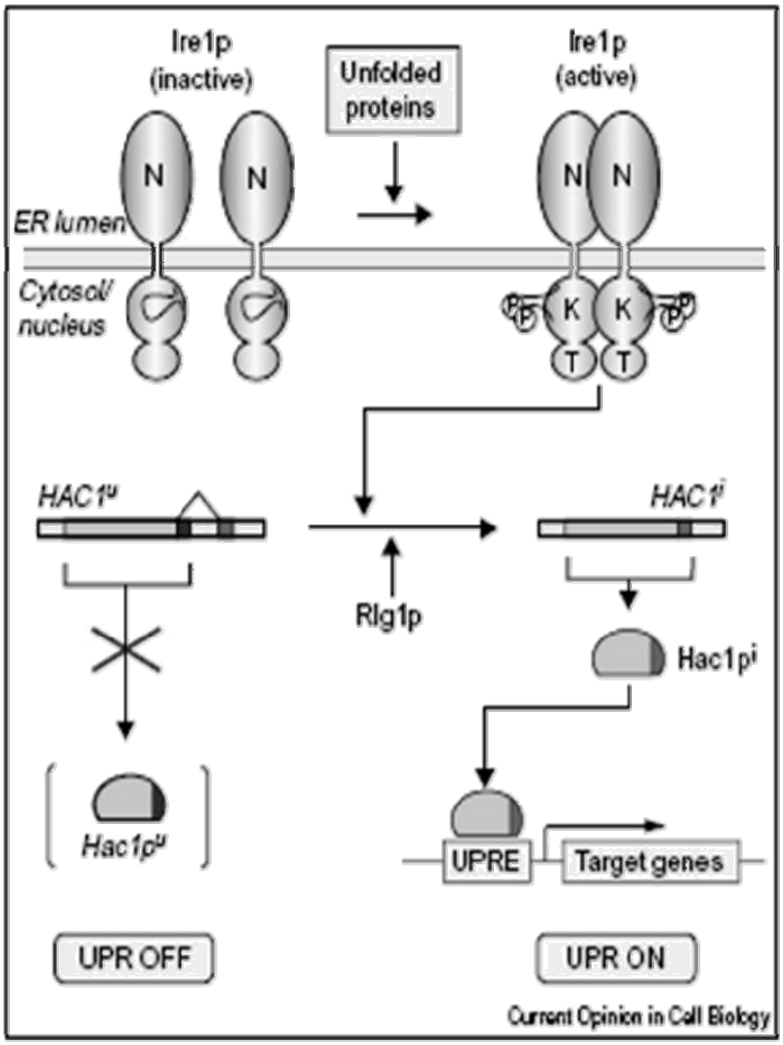


Figure 1. Schematic representation of the unfolded protein response in yeasts. Ire1p is a transmembrane serine-threonine kinase, oriented with the amino terminus in the ER lumen and the carboxyl terminus in the cytosol. Once unfolded proteins accumulate in the ER, Ire1p oligomerizes, trans-autophosphorylates via the cytosolic kinase domain (K) and activates the endonuclease in the tail domain (T). The endonuclease Ire1p cuts *HAC1* mRNA at two sites, removing a non-classical intron, the two exons are rejoined by Rlg1p (tRNA ligase). *HAC1*^{u} (uninduced) is not translated due to the presence of the intron, and Hac1p^{u} is not produced. After Ire1p-mediated splicing, *HAC1*^{i} mRNA enters the ribosome and is efficiently translated in Hac1p^{i}, a transcriptional factor that upregulates the UPR target genes after binding to the unfolded protein response element (UPRE) in the promoter of genes encoding ER-resident chaperones and other proteins (Patil and Walker, 2001).

In *P. pastoris*, Hohenblum et al. (2004) have observed the activation of the UPR in a X-33 strain overexpressing and secreting human trypsinogen under the control of two different promoters, *GAP* and *AOX1*. They analyzed the chaperone BiP, which is a signal for the unfolded protein response, as well as the intracellularly retained trypsinogen during fed-batch cultivations. The results obtained in the cited work suggest that the accumulation of misfolded heterologous proteins in the ER is substrate-dependent and not promoter-dependent, and that this phenomenon is a major bottleneck in the heterologous protein expression and secretion process.

Some approaches have been made in order to relax this bottleneck by engineering the UPR signal pathway. For instance the disruption of the *S. cerevisiae HAC1* gene resulted in a decrease of the production of *Bacillus amyloliquefaciens* α-amylase and of *Trichoderma reesei* endoglucanase (EGI) expressed in *S. cerevisiae* (Valkonen et al., 2003a). Notably, the constitutive overexpression of *HAC1* gene caused a 70 % increase of the α-amylase expression. Furthermore, overexpression of the active form of *T. reesei hac1* in *S. cerevisiae* resulted in a 2.4-fold increase of α-amylase production, as well as the total protein production. Also, the combination of both *S. cerevisiae HAC1* and *T. reesei hac1* caused an increase of the BiP content. The same strategy was used on *Aspergillus niger* var. *awamori* obtaining an increase of the secreted *Trametes versicolor* laccase and bovine preprochymosin (Valkonen et al., 2003b)

In addition, overexpression of foldases and chaperones in yeast has obtained different results depending of the expressed protein. In *S. cerevisiae*, overexpression of protein disulfide isomerase (PDI), but not BiP, resulted in the increase of *Pyrococcus furiosus* β-glucosidase (Smith et al., 2003). Recently, protein disulfide isomerase (PDI) has been co-overexpressed in a *P. pastoris* strain expressing extracellularly the *Necator americanus* secretory protein (Na-ASP1) (Inan et al., 2005), both genes being under control of the *PAOX1* promoter. An increase in the secreted Na-ASP1 was observed in strains with increasing of *PDI* gene copy number, i.e. the secretion levels of Na-ASP1 were correlated with PDI levels. However, when the Na-ASP1 gene copy number was increased, despite of the increase in PDI content, intracellular product accumulation was observed, suggesting the system capacity was exceeded. The expression of human antibody Fab fragments in *P. pastoris* was improved almost three-fold by co-

overexpression of PDI, while co-expreesion of *HAC1* had only a minor effect (Gasser et al., 2006).

In the present study our aim is to elucidate whether the ROL expression in *P. pastoris* triggers the UPR as a result of the accumulation of unfolded ROL protein in the ER. We have used flow cytometry, a technique that gives high sensitivity and robustness to obtain the relative amounts of intracellular BiP and ROL during fed-batch cultures at different growth rates. Hence, this strategy also allows studying the relationship between the specific growth rate (i.e. physiological state of the cell) and the UPR activation under induction conditions. Besides, we investigated the potential effect of ROL overexpression combined with a poor nitrogen source such as methylamine on cell viability.

On the other hand, a metabolic engineering strategy based on the constitutive overexpression of the *S. cerevisiae HAC1* gene in *P. pastoris* has been tested. The *P. pastoris* GS115 strain was transformed with a plasmid containing the constitutive promoter *GAP* and the *his4* gene as selectable marker and also the expression vector pPICZαFLDROL which expresses ROL under the control of the *FLD1* promoter. Fed-batch cultivations have been carried out to compare the intracellular ROL and BiP profiles in X-33 and GS115 strains expressing ROL.

5.2. Materials and methods.

5.2.1. Strains

The *P. pastoris* X-33-derived strain containing the expression vector pPICZFLDαROL (for construction details see chapter 3) integrated in its genomic *FLD1* locus was used throughout this study. Also, the wild type *P. pastoris* X-33 strain (Invitrogen) was used as a negative control for UPR analysis.

Additionally, an histidine auxotrophic strain, the *P. pastoris* GS115 was transformed with a vector containing the induced variant of the *S. cerevisiae HAC1* gene under the transcriptional control of the constitutive *PGAP* and the *his4* gene as a selection marker (pGAPHAC1, Gasser et al., 2006). Transformants were selected on minimal medium, and subsequently re-transformed with the pPICZFLDαROL vector (containing the *ROL* gene fused to the *S. cerevisiae* α-mating factor under the transcriptional control of the *PFLD1*) and selected again on YPD medium containing zeocin. Several transformants

were isolated and further tested for lipase production in shake flask cultivations using BMS (buffered minimal sorbitol) medium, containing 1% (w/v) sorbitol, 1.34 % YNB without aminoacids and ammonium sulphate, 0.4 % (w/v) methylamine hydrochloride, $4 \cdot 10^{-5}$ % (w/v) biotin and 100 mM potassium phosphate pH 6.0. The highest lipase producer, the *P. pastoris* GS115H / [pGAPHAC1 + pPICZFLDαROL] clone 11 was selected for further cultivation studies.

5.2.2. Flow cytometry

5.2.2.1. Immunofluorescence staining

Samples for flow cytometry were prepared as follows: a volume between 1000 μL and 25 μL of sample from culture were centrifuged at 12000 rpm in a microcentrifuge. After removing supernatant, 1000 μL of 70% ethanol were added while vortexing. Fixed samples were stored at -20ºC for further analyses.

Staining was performed according to the protocol used by Hohenblum et al. (2003). For staining, 200 μL fixed sample was centrifuged at 12000 rpm and washed with 1 mL of wash buffer (100 mM Tris-HCl buffer pH 7.4 containing 0.1% Triton X-100 and 2 mM $MgCl_2$). Once the cells were washed, the pellet was resuspended in 200 μL of wash buffer containing 1% BSA and mouse anti-ROL antiserum or rabbit anti-HSP70 (BiP) antiserum (StressGen, Vancouver, BC, Canada) both diluted 1:100. After 1 hour of incubation at room temperature and agitation, samples were centrifuged and washed with 1 mL wash buffer. Pellets were resuspended in 200 μL wash buffer containing 1% BSA and goat anti-mouse or goat anti-rabbit IgG-FITC conjugate (Sigma), diluted 1:100. Cells were analyzed on a FACS Calibur (Becton Dickinson) with a 488 nm Argon laser, and 10^4 cells were measured per analysis. Immunofluorescence staining was measured using a 530/30 nm BP filter (FL1).

Representative dot plots from immunofluorescence staining are shown in figure 2. Immunofluorescence data were normalized to the cell size by dividing the fluorescence by the respective volume-corrected forward scatter signals (FSC). As forward scatter is commonly assumed to correlationate with the cross sectional area of the cell, and presupposing that all cells are similar in shape, the FSC can be used to estimate the relative cell size. Thus, a size factor is calculated from the arithmetical mean of FSC making the following assumptions:

$$\text{mean FSC} \propto \text{Area} \quad (1)$$

$$\text{size factor} \propto \text{volume} \quad (2)$$

The size factor is calculated for each sample taking the arithmetical mean of FCS values:

$$\text{Size factor} = \text{mean } FSC^{\left(\frac{3}{2}\right)} \quad (3)$$

The geometric mean of the immunofluorescence values was divided by the size factor, and the blank was subtracted. The blank was obtained from the same experiment. In the retained product immunofluorescence values, a sample from the batch phase was used as blank. Then, the relative fluorescence normalized by cell size was:

$$\text{Relative fluorescence} = \frac{geo.mean \quad FL1}{size \ factor} - \text{blank} \quad (4)$$

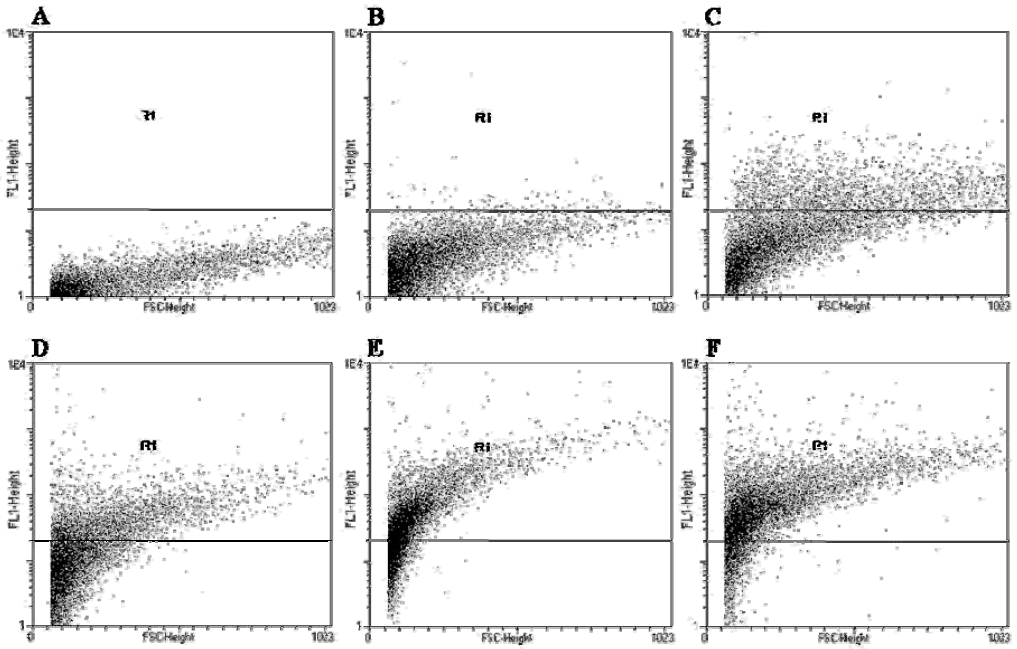


Figure 2. Immofluorescence staining of fed-batch culture samples. Intracellular lipase fluorescence (FL1-H) versus forward scatter (FSC-H). A region was set to observe the increment of fluorescent signal from the blank sample. (A) 14.0 hours of fermentation. (B) 23.5 hours of fermentation. (C) 37.0 hours of fermentation. (D) 44.5 hours of fermentation. (E) 68.7 hours of fermentation. (F) 91.0 hours of fermentation.

5.2.2.2. Cell viability staining

For viability staining a culture sample of 2 µL was diluted with PBS to 300 µL before adding 2 µL of propidium iodide (Sigma, 1 mg mL^{-1}) and samples were analyzed within 10 minutes. PI fluorescence was measured through a 670 nm LP filter (FL3) (Hohenblum et al., 2003).

In order to perform viability controls, fresh samples from shake flask cultures and killed cells by heat treatement over a flame, were stained to separate the two populations by its PI flouresence. Also, a sample without PI staining was analyzed. The fresh cells showed a similar pattern to the non stained ones, on the other hand the previously killed cells showed a clear and higher fluorescence (figure 3).

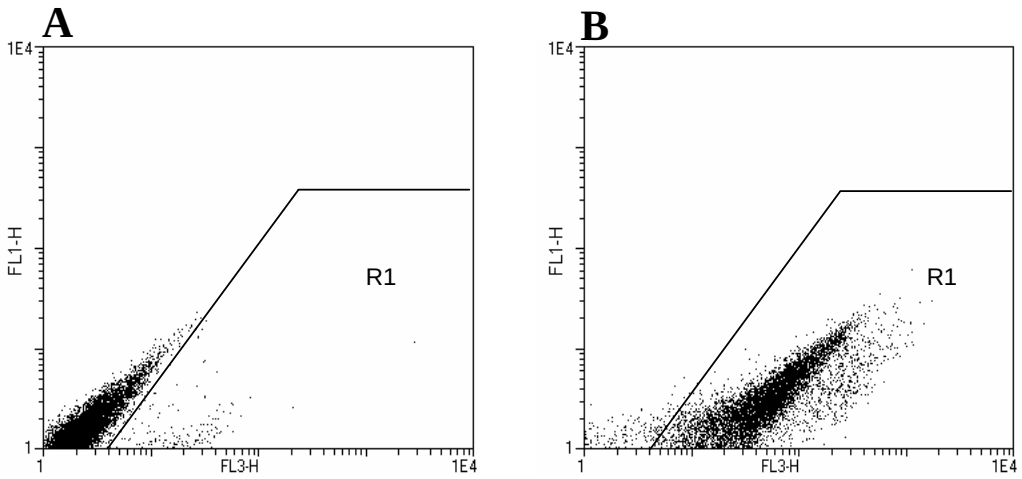


Figure 3. Controls for viability staining performed with fresh cultured cells (A) and previously killed cells (B). FL3-H: PI staining fluorescence; FL1-H: cell autofluorescence.

Viability measurements were represented using the PI fluorescence versus the cells autofluorescence (FL1). A region was drawn between the viable cells and the non viable to quantify the percentage of viability. In figure 4 the evolution of typical viability analyses from fed-batch samples is shown.

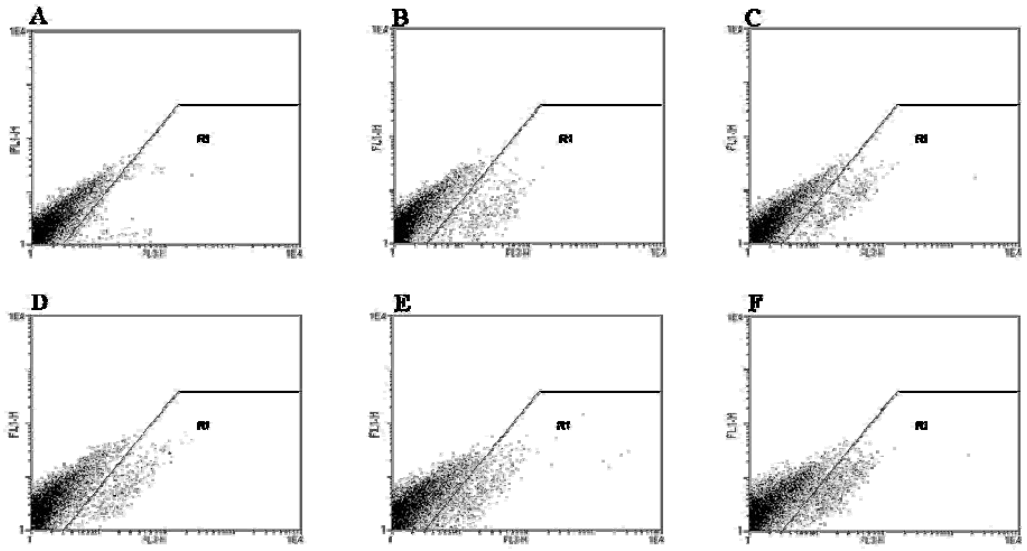


Figure 4. Viability staining of samples from a fed-batch fermentation using methanol and methylamine as carbon and nitrogen source respectively. FL3-H: PI staining fluorescence; FL1-H: cell autofluorescence. (A) 14.0 hours of fermentation. (B) 23.5 hours of fermentation. (C) 37.0 hours of fermentation. (D) 44.5 hours of fermentation. (E) 68.7 hours of fermentation. (F) 91.0 hours of fermentation.

5.3. Results

5.3.1. Monitoring cell's viability in fed-batch cultivations of *P. pastoris* expressing a *Rhizopus oryzae* lipase (ROL) under the control of the *PFLD1*.

Recent studies have proven that *P. pastoris* high cell density cultivations are susceptible to stress, which may lead to decreased viability and increased cell lysis/proteolysis. This may have a major negative impact on process productivities of secreted proteins produced in *P. pastoris*. Cells may be stressed by starvation, change of carbon sources (e.g. from glycerol to methanol), temperature and pH changes (Jahic et al., 2003; Sinha et al., 2005; Hohenblum et al 2003). Further, toxic by-products of methanol metabolism have been recently shown to cause oxidative stress in *P. pastoris* (Xiao et al 2005).

The high cell density cultivation studies described in chapter 4 revealed that ROL specific production rates (q_p) decreased along the induction phase. Notably, no significant extracellular product proteolysis nor protease levels could be detected under the tested cultivations conditions. As suggested by previous studies (see Introduction), a possible explanation for such a decrease in q_p could be the result of ROL intracellular

accumulation, which could trigger a cell's stress response potentially leading to a loss of cell's viability along the induction phase of fed-batch cultivations.

Hence, in order to assess the impact of ROL overexpression on *P. pastoris* cells, two fed-batch fermentations were initially performed using methanol and methylamine as carbon and nitrogen sources, respectively, during the induction phase (that is, using the carbon/nitrogen source combination for maximum induction of the *FLD1* promoter). Methanol concentration was kept at limiting concentration along the induction phase. The first cultivation was carried out with the X-33-derived strain expressing ROL under the control of the *FLD1* promoter, whereas the second cultivation was performed with a untransformed wild-type X-33 strain. Moreover, cell viability during a fed-batch cultivation using sorbitol and methylamine growing at a near to μmax specific growth rate was determined. The results are summarized in figure 5.

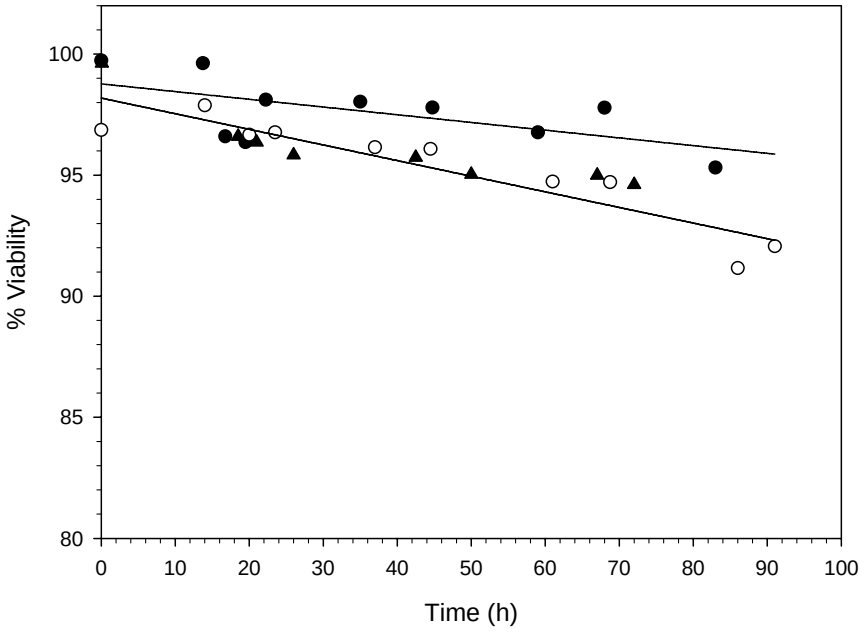


Figure 5. Percentage of viable cells in fed-batch cultures. Fed-batch culture using methanol and methylamine (▲) and X-33 wild type strain. Fed-batch culture using methanol and methylamine (○) and X-33 strain expressing ROL. Fed-batch culture using sorbitol and methylamine (●) and X-33 strain expressing ROL.

Remarkably, the fraction of viable cells analyzed by flow cytometry along the fed-batch induction phase was always above 91.0 % in the ROL-expressing strain. Cell viability

in the control culture showed a similar pattern. Both cultures reached a 92.0 % of viable cells at 70 hours of culture, suggesting that ROL overexpression under the cited conditions does not have any deleterious effects on cell viability. Furthermore, although a phospholipase activity has been described for the mature ROL as being most likely toxic for *Escherichia coli* cells accumulating this enzyme intracellularly (Beer et al., 1996) our results indicate that active ROL potentially retained in the cell's periplasm/cell wall and/or accumulated in the extracellular medium does not seem to be toxic for *P. pastoris*.

Analogous results were obtained when using sorbitol and methylamine as carbon and nitrogen sources, respectively. Moreover, the viability values in these cultures were slightly higher than in the methanol and methylamine cultures, indicating that methanol may have some negative effect on the cell's viability under the tested growth conditions.

Notably, sustained growth on methylamine as a sole nitrogen source does not seem to have an important impact on cell's viability, even though several early studies suggest that cells growing on this nitrogen source may undergo a physiological state analogous to nitrogen limitation (Zwart and Harder, 1983).

Following these results, specific growth rate, specific lipase production and specific substrate consumption rates in subsequent cultivations were calculated taking into account the percentage of non-viable cells within the total biomass.

5.3.2. Monitoring of intracellular BiP and intra/extracellular ROL levels in fed-batch cultivations of *P. pastoris* expressing ROL under the control of the *PFLD1*

The specific growth rate has proven to be an important parameter for the productivity of secreted heterologous proteins in *P. pastoris* (Sinha et al., 2003; Zhang et al., 2000) and, in particular, of *PFLD1*-based systems. In this study, two fed-batch fermentations using the *P. pastoris* X-33-derived strain expressing ROL under the transcriptional control of the *PFLD1* were performed following the standard scheme developed in chapter 4. The induction phase of the first cultivation was performed under growth rate-limited conditions (μ of about 0.005 h^{-1}), whereas a near-μ_{max} growth rate of about 0.02 h^{-1} was maintained during the induction phase of the second cultivation by keeping a constant residual sorbitol concentration of about 8 $g \cdot L^{-1}$.

The overall results in terms of biomass concentration, extracellular lipase levels and residual substrate concentrations were similar to those observed in the previous chapter. The evolution of the main fermentation parameters during the fed-batch processes at a μ of 0.005 h^{-1} and 0.02 h^{-1} is shown in figures 6A and 6B, respectively. In both fermentations extracellular ROL activity was rapidly detected in the medium after the transition phase started. However, whereas in the lower specific growth rate cultivation, the detected lipase activity levels in the medium stayed constant around 30~40 AU mL^{-1} throughout the induction phase, extracellular lipase levels steadily increased throughout the induction phase performed at about 0.02 h^{-1} up to 280 AU mL^{-1} (i.e., 7.0-fold higher than the 0.005 h^{-1} culture).

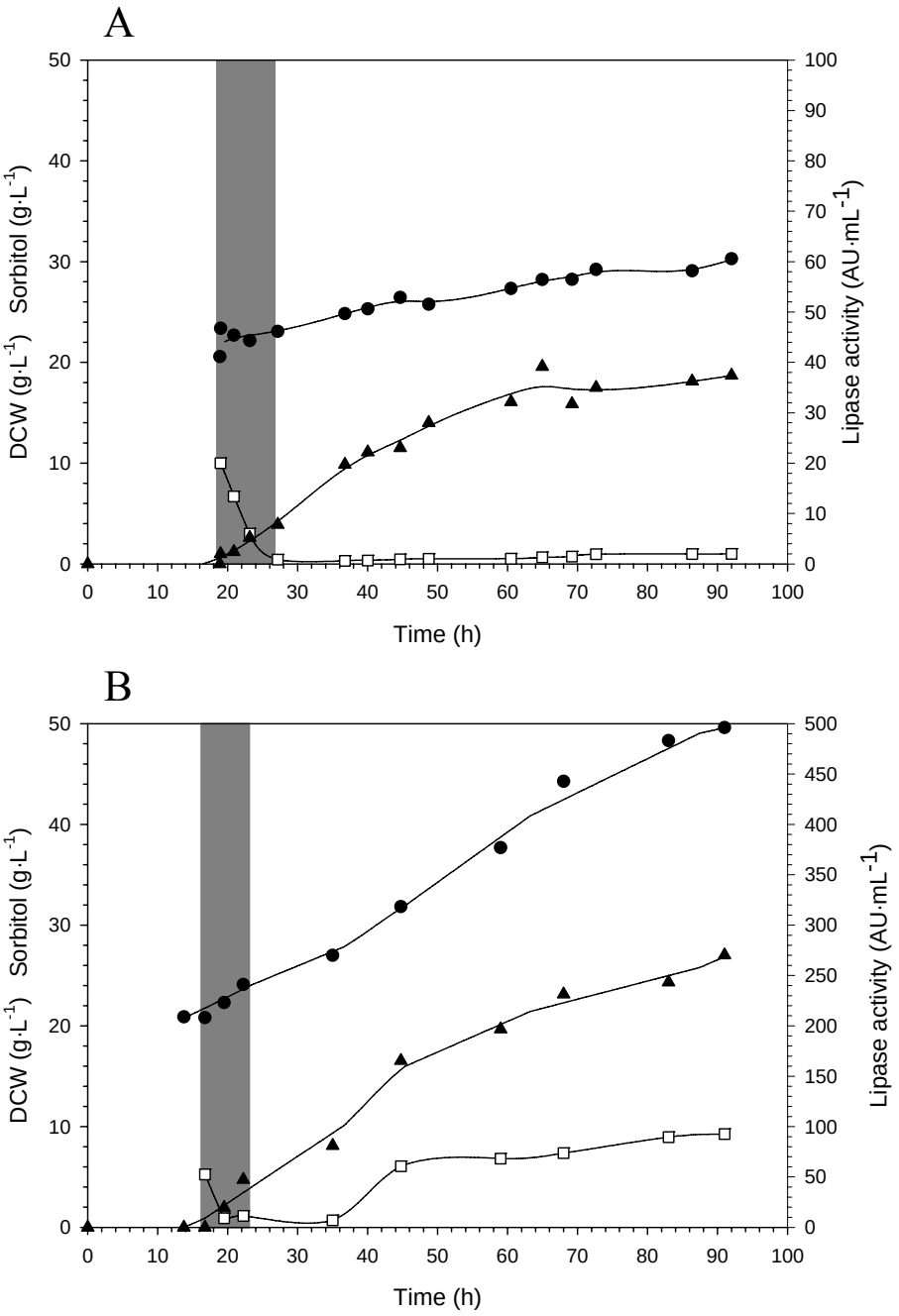


Figure 6. Fed-batch cultivations of *P. pastoris* X-33 strain expressing ROL at a controlled specific growth rate of 0.005 h^{-1} (**A**) and at a mean specific growth rate of about 0.02 h^{-1} (near μ_{max}) (**B**). Biomass (dry cell weight) (●), sorbitol concentration (□) and extracellular lipase activity (▲) are indicated.

Flow cytometry analyses of intracellular ROL levels confirmed that a fraction of the product was retained within the cell. Further, this intracellular product accumulation

was concomitant with an increase of the BiP protein, an ER chaperone which is activated by the UPR (figure 7). A negative control was performed using the wild type strain X-33 growing on methanol and methylamine as carbon and nitrogen source respectively. In this control experiment (figure 8), BIP amounts remained at a constant low level throughout the fermentation (relative fluorescence values were always well below 1), indicating no activation of the UPR.

Notably, a clear increase in BiP and ROL intracellular content (relative to basal levels detected in the control cultivation) was detected immediately after the start of the transition phase in both cultivations. In the growth-limited cultivation, a moderate but steady increase in both BiP and ROL intracellular relative levels is observed during the first 40 h of the induction phase; after this point, ROL levels tend to decrease, whereas BiP levels stay constant. Conversely, in the high specific growth rate cultivation, BIP and ROL levels remain very low along the first 10 h of the induction phase; after this point, BiP and ROL levels experience a clear increase, reaching a maximum after 30 h of induction; this stage is followed by a steady decrease to basal levels towards the end of the cultivation (figure 7B).

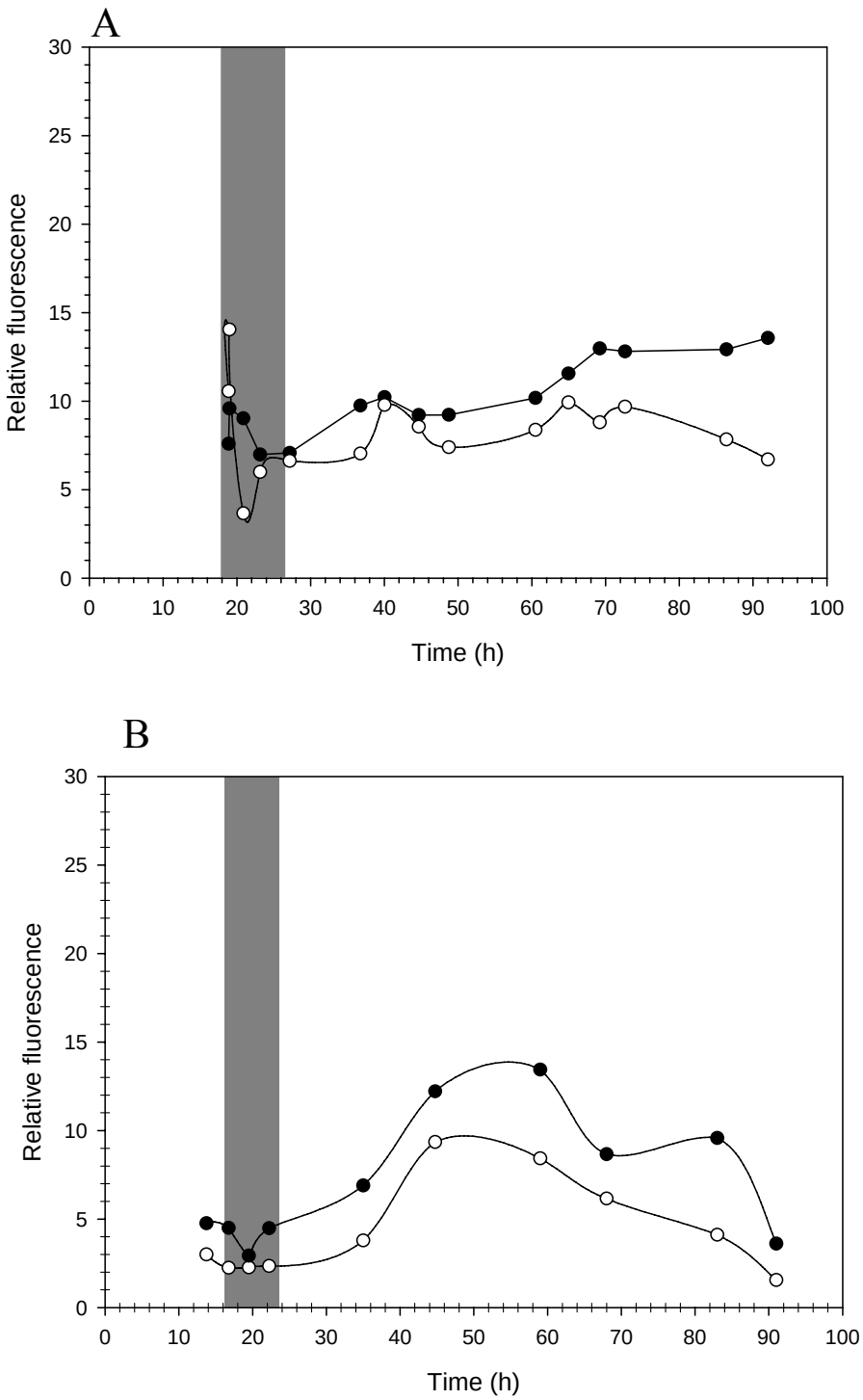


Figure 7. Relative immunofluorescence of intracellular BiP and ROL during induction phase of fed-batch cultivations of *P. pastoris* X-33 strain expressing ROL at a controlled specific growth rate of about 0.005 h^{-1} (**A**) and, at a mean specific growth rate of about 0.02 h^{-1} (near μ_{max}) (**B**). BiP (●) and ROL (○).

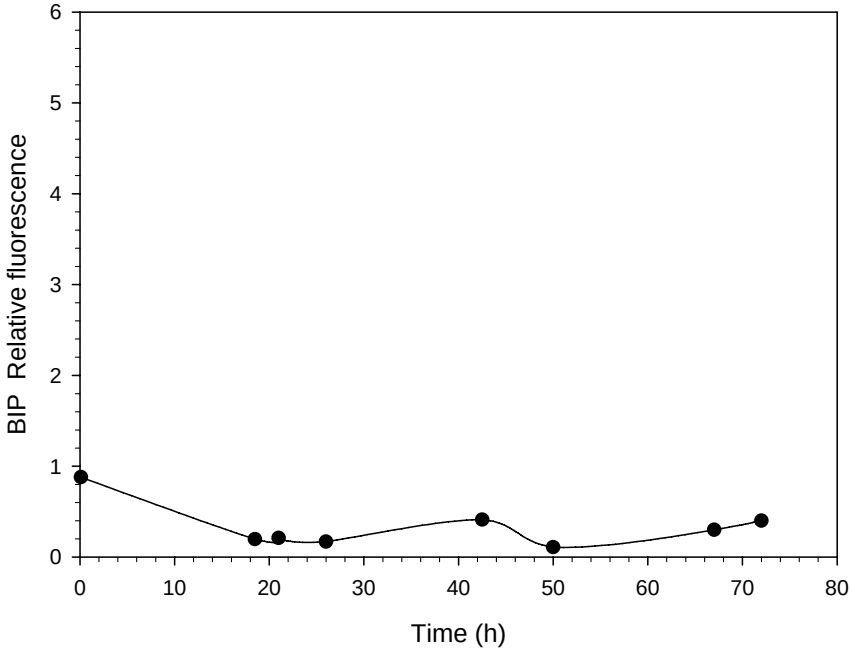


Figure 8. Control experiment performed with a X-33 wild type *P. pastoris* strain where BIP levels were monitored along the cultivation time.

5.3.3. Monitoring of intracellular BiP and intra/extracellular ROL levels in fed-batch cultivations of *P. pastoris* co-expressing HAC1 constitutively and the ROL under the control of the *PFLD1*

Two fed-batch fermentations were performed using the selected *P. pastoris* GS115H / [pGAPHAC1 + pPICZFLDαROL] strain, using the same cultivation scheme and operational conditions as for the ROL-expressing X-33-derived strains, i.e. one at growth-limiting conditions (growth rate of about 0.005 h^{-1}) and the other at a near-maximum specific growth rate of about 0.02 h^{-1}. The evolution of cultivation parameters is shown in figure 9. As observed in this figure, the extracellular ROL accumulation profiles are very different between the two fermentations. In the growth-limited fed-batch culture (figure 9A) the extracellular accumulation of lipase ended after 36 hours of cultivation, reaching a maximum of 70 AU mL^{-1}. In contrast, in the cultivation at about 0.02 h^{-1} (figure 9B), extracellular lipase levels continued increasing steadily during the whole induction phase until the end of the process, reaching a

maximum of 450 AU mL^{-1}, i.e. 6.4-fold higher than the maximum lipase activity levels obtained during the fed-batch cultivation performed at low specific growth rate.

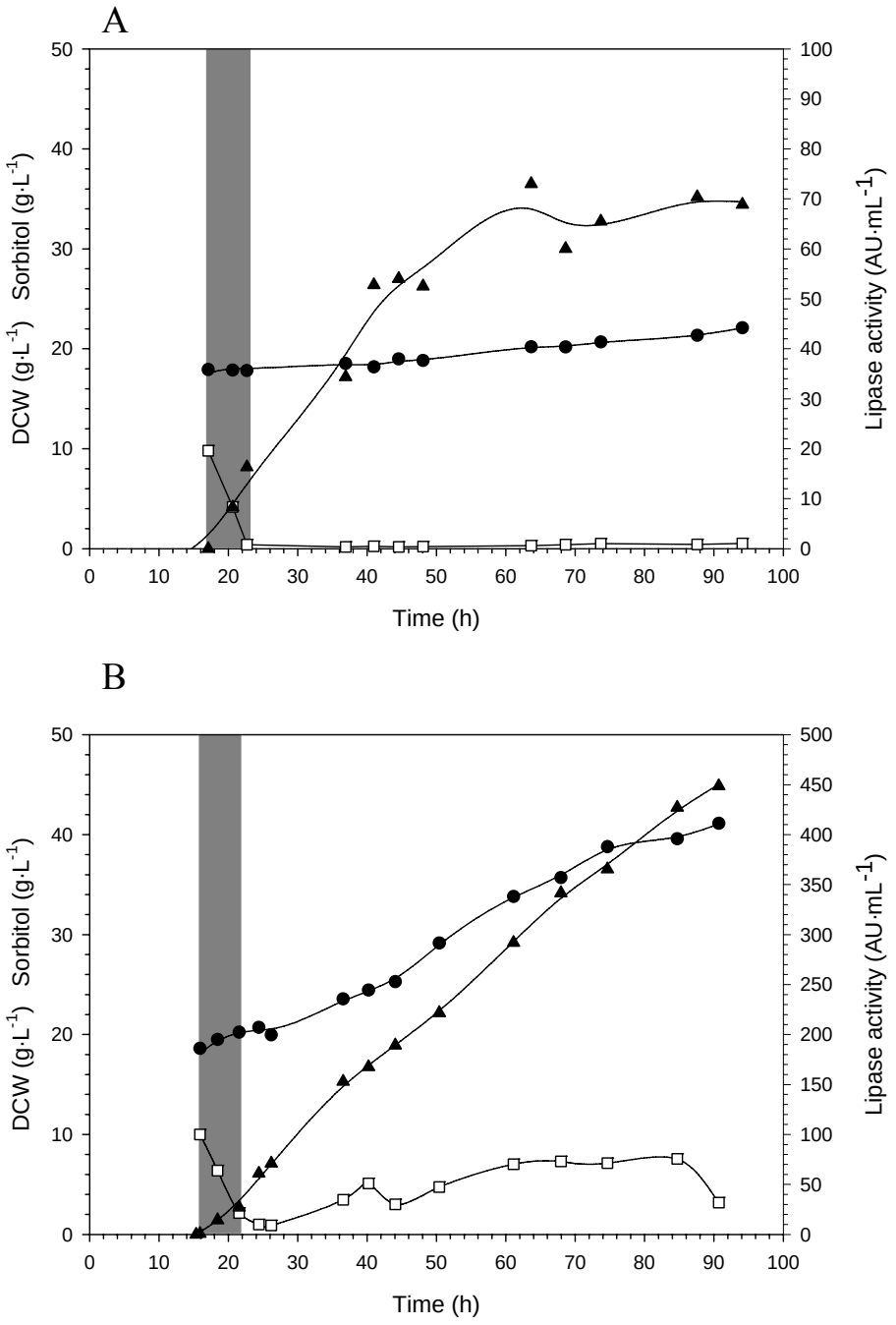


Figure 9. Fed-batch cultivations of *P. pastoris* GS115H/HAC1 strain expressing ROL at a controlled specific growth rate of 0.005 h^{-1} (**A**) and at a mean specific growth rate of about 0.02 h^{-1} (near μ_{max}) (**B**). Biomass (dry cell weight) (●), sorbitol concentration (□) and extracellular lipase activity (▲) are indicated.

Regarding intracellular BIP and ROL content (figure 10), the profiles of intracellular BIP and ROL content are very different between both cultivation conditions, as well as to the corresponding profiles observed with the X-33-derived strain.

The decrease of growth rate in the growth-limited cultivation towards the end of the transition phase and start of the induction phase leads to a rapid ROL intracellular accumulation, as well as a sharp increase in BiP levels. In contrast, in the higher growth rate cultivation, intracellular ROL and BiP relative levels remain very low during the late transition phase and start of the induction phase.

In the growth limited fed-batch cultivation, after reaching a maximum soon after the start of the induction phase, intracellular ROL levels stay relatively constant throughout this phase, with some fluctuations. In parallel, BiP levels reach a maximum towards the end of the transition phase and subsequent onset of the induction phase, followed by a sharp and steady decrease until 20 h after the induction phase start; thereafter, BiP levels remain constant and high relative to basal levels (relative fluorescence values of about 9~10, figure 10A).

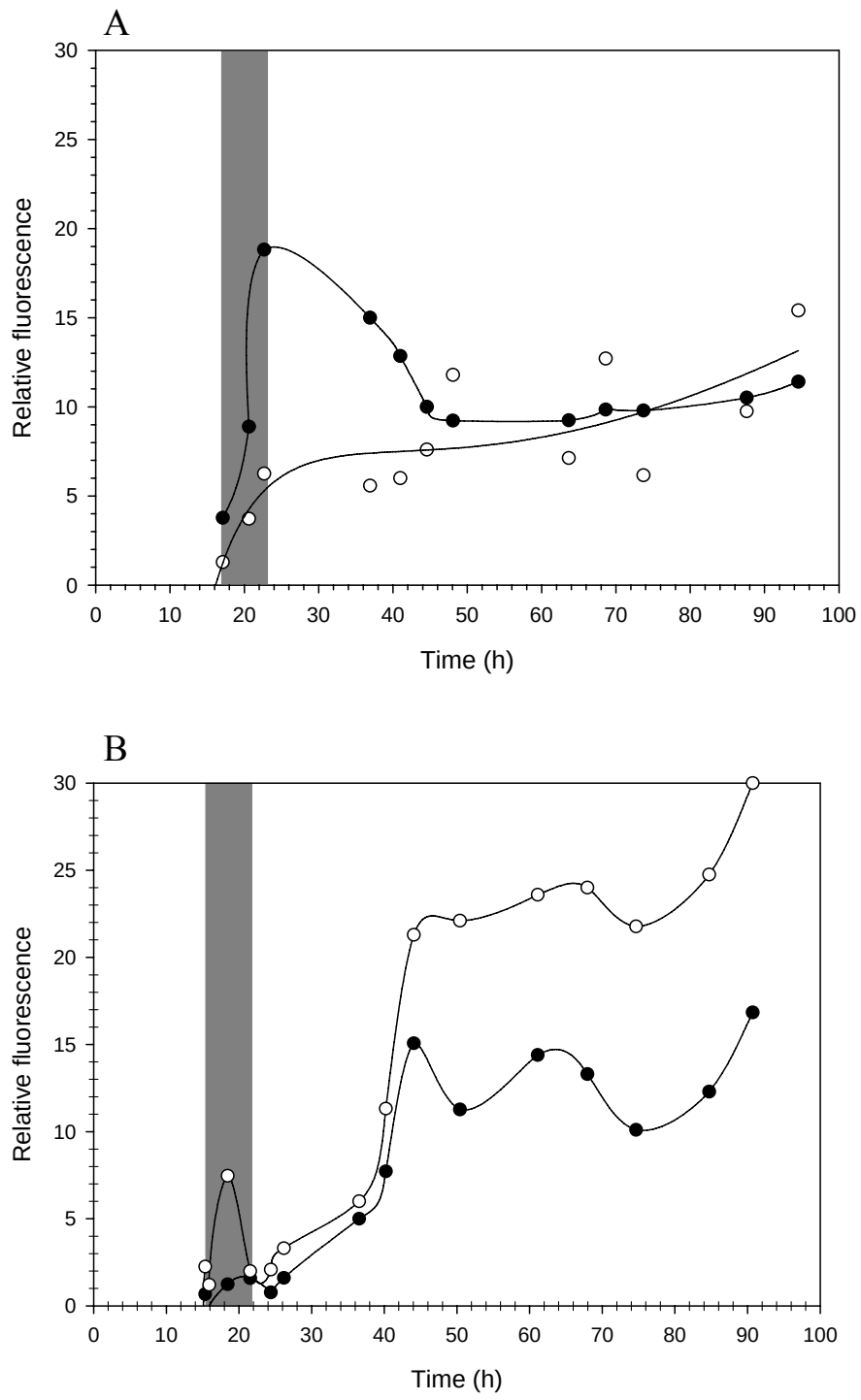


Figure 10. Relative immunofluorescence of intracellular BiP and ROL during induction phase of fed-batch cultivation of *P. pastoris* GS115H/HAC1 strain expressing ROL at a controlled specific growth rate of 0.005 h^{-1} (**A**) and, at a mean specific growth rate of about 0.02 h^{-1} (near μ_{max}) (**B**). BiP (●) and ROL (○).

In the higher growth rate cultivation, intracellular ROL and BiP levels stay relatively low (below relative fluorescence values of ca. 5) until 12 h after the start of the induction phase; remarkably, at this point, there is a dramatic increase of ROL and BiP levels; after this shift, these values remain constant and relatively high with some fluctuations (BiP relative fluorescence values of about 12 ± 2, figure 10B). That means, in contrast to the corresponding cultivation with the X-33 derived strains, there is no decrease in BiP and ROL levels in the later stages of the fed-batch phase; moreover, the ROL relative fluorescence values during this phase are significantly higher (22~25) than the maximum values obtained in the corresponding cultivation with the X-33-derived strain (ca. 10).

5.3.4. Comparison of X-33 versus GS115H/HAC1 strains producing ROL.

Maximal lipase activity, lipase yield, mean specific growth rate and productivity values obtained in fed-batch cultivations of *PFLD1*-based *P. pastoris* X-33 and GS115H/HAC1 strains expressing ROL are summarized in table 1. Overall, the results clearly indicate that ROL overexpression in a *P. pastoris* strain expressing constitutively the induced form of the *S. cerevisiae* UPR transcription factor Hac1p resulted in about a 3-fold and 2-fold increase in the overall process specific productivity at the growth-limiting and higher growth rate conditions, respectively.

The specific production rate (q_p) profiles were similar for both strains growing at the same corresponding growth rate. In the X-33-derived strain cultures, the q_p values reached a maximum at 25 hours of cultivation, i.e. soon after the end of the transition phase; thereafter, a constant decrease was observed, and values stabilized around 70 hours of culture (figure 11). Nevertheless, differences in the q_p max values were important: while in the 0.005 h^{-1} specific growth rate fermentation the q_p max value reached 38 UA g^{-1} h^{-1}, in the 0.02 h^{-1} specific growth rate culture the q_p max was 236 UA g^{-1} h^{-1}, i.e. 6-fold higher than in the growth-limited cultivation.

	AU max (AU mL^{-1})	**$Y_{P/X}$** (AU gX^{-1})	**μ mean** (h^{-1})	**Productivity** (AU L^{-1} h^{-1})	**Specific productivity** (AU gX^{-1} h^{-1})
P*FLD1* μ 0.005 h^{-1}	39	1396	0.006	602	21
P*FLD1* μ 0.02 h^{-1}	270	5448	0.017	2970	60
P*FLD1 HAC1* μ 0.005 h^{-1}	73	3630	0.005	1147	57
P*FLD1 HAC1* μ 0.02 h^{-1}	449	10928	0.017	4946	120

Table 1. Maximal lipase activity, lipase yield, mean specific growth rate and productivities obtained in fed-batch cultivations of *PFLD1*-based *P. pastoris* X-33 and GS115H/HAC1 strains expressing ROL. X: dry cell weight ($g \cdot L^{-1}$), S: substrate ($g \cdot L^{-1}$), μ: specific growth rate (h^{-1}), UA: units of activity.

Such decrease in the ROL secretion rates is correlated with an increasing intracellular BiP and ROL content (figure 7). Western blot analyses performed with culture broth samples showed no presence of product degradation (figure 12). In addition, extracellular protease activity levels were very low or below detection limit (data not shown). These observations lead us to conclude that the observed decrease of q_p values could not be primarily caused by proteolytic degradation of the product in the cultivation broth.

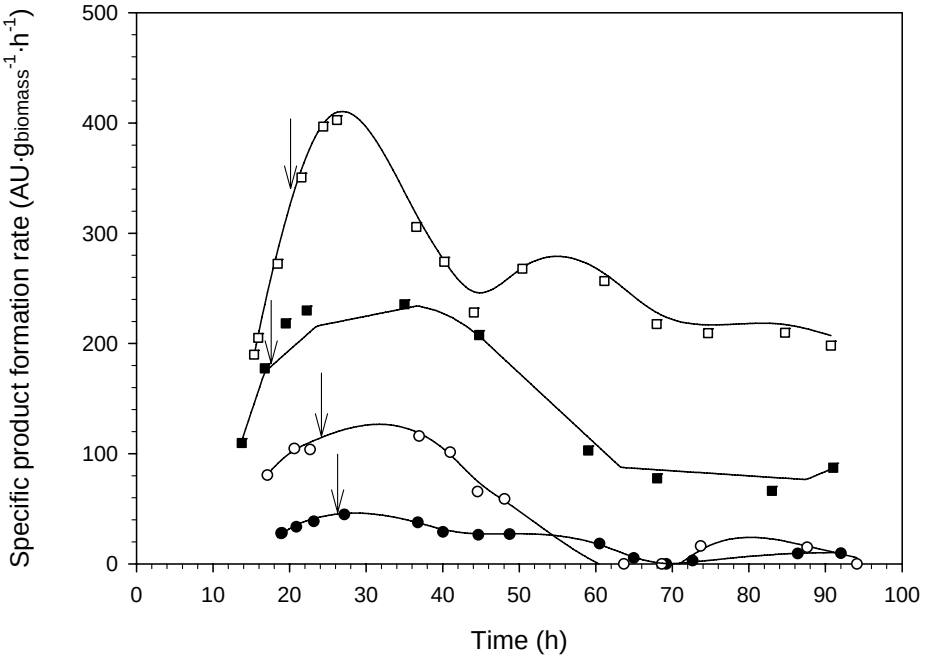


Figure 11. Specific product production rate (q_p) profiles of four different fed-batch cultures of *P. pastoris* expressing ROL at different specific growth rate and strains. X-33 strain at a mean specific growth rate of about 0.005 (h^{-1}) (●), X-33 strain at a mean specific growth rate of 0.017 (h^{-1}) (■). GS115H/HAC1 strain at a mean specific growth rate of about 0.005 (h^{-1}) (○) and, GS115H/HAC1 strain at a mean specific growth rate of about 0.02 (h^{-1}) (□). The vertical arrows indicate the start of the induction phase.

The same trend in the q_p profiles was observed in both fermentations performed with the HAC1-overexpressing strain (figure 11), i.e. both cultures reached a maximum q_p value at an early stage of the induction phase (ca. 25 h of cultivation) and, after these maxima, an important decrease in q_p was observed. The q_p max value in the higher specific growth rate was 4-fold higher than in the lower specific growth rate culture. In the cultivation at the higher growth rate, such strong decrease in q_p is clearly parallel to a significant increase in the intracellular ROL and BiP levels (figure 10). Furthermore, sustained higher intracellular ROL levels in the later phase of this cultivation were correlated with the stabilization of q_p to higher values than in the corresponding cultivation phase with the X-33-derived strain.

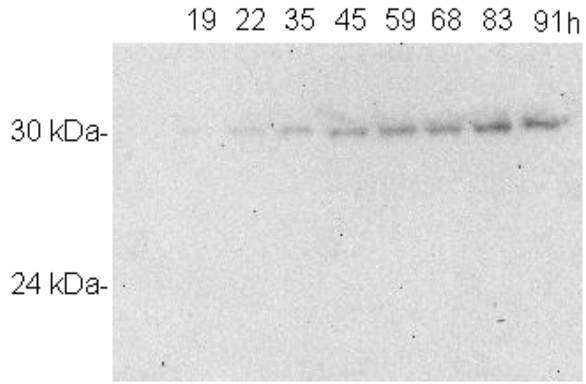


Figure 12. Western blot analysis of culture supernatants from fed-batch cultivation of *P. pastoris* X-33 expressing ROL at a mean specific growth rate of about 0.02 h^{-1}. Western blot was performed using anti-ROL mouse polyclonal antibodies diluted 1:100 and secondary peroxidase-conjugate anti-mouse antibodies developed in goat diluted 1:1000 following the protocol detailed in materials and methods (page 22). Lanes indicate fermentation time (hours).

Overall, the effect of the constitutive expression of the UPR transcription factor HAC1 on ROL production and secretion rates profiles, as well as on the dynamics of the cellular responses to ROL induction depend on the specific growth rate (i.e. the energetic state of the cells). For instance, the higher accumulation of BiP upon the start of the induction phase in growth-limited HAC1-expressing cells may indicate that energy-limiting conditions may pose a further limitation in the ROL folding and secretion processes. In addition, in both cultivations performed under carbon excess conditions (i.e. fed-batch at a near μ_{max} growth rate) a clear shift in the intracellular BiP and ROL levels is observed after about 24 h of growth on methylamine as sole nitrogen source. This could indicate that methylamine metabolism cannot support the nitrogen flow needed to sustain the carbon and energy demands needed for simultaneous cell growth and ROL production.

Interestingly, the GS115H/HAC1 strain expressing the ROL showed significantly higher mean specific sorbitol consumption rates (q_s) during the induction phase of cultivations (table 2). This fact might be explained by an increase in the energy demand

for maintenance due to the constitutively expression of the UPR related proteins, many of them, especially the Hsp70 family, with ATPase activity (Sakaguchi et al., 1997).

	q_p mean (AU gX^{-1} h^{-1})	**q_p max** (AU gX^{-1} h^{-1})	**q_s mean** (gS gX^{-1} h^{-1})	**q_s max** (gS gX^{-1} h^{-1})
P*FLD1* μ 0.005 h^{-1}	24	39	0.022	0.024
P*FLD1* μ 0.02 h^{-1}	123	236	0.037	0.050
P*FLD1HAC1* μ 0.005 h^{-1}	52	119	0.031	0.034
P*FLD1HAC1* μ 0.02 h^{-1}	272	411	0.071	0.107

Table 2. Mean and maximal specific lipase production rate (q_p) and substrate consumption rate (qs) obtained in fed-batch cultivations of *PFLD1*-based *P. pastoris* X-33 and GS115H/HAC1 strains expressing ROL. X: dry cell weight ($g \cdot L^{-1}$), S: substrate ($g \cdot L^{-1}$), μ: specific growth rate (h^{-1}), UA: units of activity.

5.4. Conclusions

In the current study, a metabolic engineering strategy has been developed to avoid or circumvent the effect produced by the accumulation of misfolded proteins in *P. pastoris* cells. The key conclusions extracted from this work are:

- Flow cytometry has demonstrated to be a useful tool for studying process development. Intracellular product and a chaperone involved in cellular stress have been monitored along the culture by combining immunofluorescence staing and flow cytometry. Moreover, cell viability along fermentation has been measured as well.
- The overexpression of ROL in *P. pastoris* under the transcriptional control of the *FLD1* promoter does not have any toxic effect on cells as observed by viability staining.
- The observations made using immunofluorescence staining has proved that the unfolded protein response is activated in *P. pastoris* when expressing the fungal

lipase ROL. Extracellular and intracellular product accumulation as well as BIP content showed to be dependent on the specific growth rate.

- The constitutive upregulation of the UPR has had a positive effect on process productivity of *P. pastoris* expressing ROL and may be a valuable tool to overcome the possible bottlenecks produced by the overexpression of foreign proteins.

5.5. References

Beer HD, McCarthy JEG, Bornscheuer UT and Schmid RD (1998) Cloning, expression, characterization and role of the leader sequence of a lipase from *Rhizopus oryzae*. Biochimica et Biophysica Acta-Gene Structure and Expression 1399: 173-180.

Gasser B, Mauer M, Gach J, Kunert R and Mattanovich D (2006) Engineering *Pichia pastoris* for improved production of antibody fragments. Biotechnology and Bioengineering 94: 353-361.

Hohenblum H, Borth N and Mattanovich D (2003) Assessing viability and cell-associated product of recombinant protein producing *Pichia pastoris* with flow cytometry. Journal of Biotechnology 102: 281-290.

Hohenblum H, Gasser B, Maurer M, Borth N and Mattanovich D (2004) Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris.* Biotechnology and Bioengineering 85: 367-375.

Inan M and Meagher MM (2001) Non-repressing carbon sources for alcohol oxidase (AOX1) promoter of *Pichia pastoris*. Journal of Bioscience and Bioengineering 92: 585-589.

Jahic M, Wallberg F, Bollok M, Garcia P and Enfors S (2003) Temperature limited fed-batch technique for control of proteolysis in *Pichia pastoris* bioreactor cultures. Microbial cell factories 2: 6.

Kozutsumi Y, Segal M, Normington K, Gething MJ and Sambrook J (1988) The presence of malfolded proteins in the endoplasmic-reticulum signals the induction of glucose-regulated proteins. Nature 332: 462-464.

Mori K, Sant A, Kohno K, Normington K, Gething MJ and Sambrook JF (1992) A 22 Bp cis-acting element is necessary and sufficient for the induction of the yeast Kar2 (Bip) gene by unfolded proteins. Embo Journal 11: 2583-2593.

Patil C and Walter P (2001) Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Current Opinion in Cell Biology 13: 349-356.

Sakaguchi M (1997) Eukaryotic protein secretion. Current Opinion in Biotechnology 8: 595-601.

Sinha J, Plantz B, Inan M and Meagher M (2005) Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast *Pichia pastoris*: case study with recombinant ovine interferon-tau. Biotechnology and Bioengineering 89: 102-12.

Smith JD, Tang BC and Robinson A.S (2004) Protein disulfide isomerase, but not binding protein, overexpression enhances secretion of a non-disulfide-bonded protein in yeast. Biotechnology and Bioengineering 3: 340-350.

Valkonen M, Penttila M and Saloheimo M (2003) Effects of inactivation and constitutive expression of the unfolded-protein response pathway on protein production in the yeast *Saccharomyces cerevisiae*. Applied and Environmental Microbiology 69: 2065-2072.

Valkonen M, Ward M, Wang HM, Penttila M and Saloheimo M (2003) Improvement of foreign-protein production in *Aspergillus niger* var. *awamori* by constitutive induction of the unfolded-protein response. Applied and Environmental Microbiology 69: 6979-6986.

vanGemeren IA, Punt PJ, DrintKuyvenhoven A, Broekhuijsen MP, vantHoog A, Beijersbergen A, Verrips CT and vandenHondel CAMJ (1997) The ER chaperone encoding bipA gene of black *Aspergilli* is induced by heat shock and unfolded proteins. Gene 198: 43-52.

Xiao A, Zhou X, Zhou L and Zhang Y (2006) Improvement of cell viability and hirudin production by ascorbic acid in *Pichia pastoris* fermentation. Applied Microbiology and Biotechnology.

Zhang WH, Bevins MA, Plantz BA, Smith LA and Meagher MM (2000) Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A. Biotechnology and Bioengineering 70: 1-8.

Zwart KB and Harder W (1983) Regulation of the metabolism of some alkylated amines in the yeasts *Candida utilis* and *Hansenula polymorpha*. Journal of General Microbiology 129: 3157-3169.

CHAPTER 6

Quantitative analysis of specific mRNAs of *P. pastoris* cells growing in fed-batch cultivations using a sandwich hybridization assay

---

6.1. Introduction

As discussed in previous chapters, the expression of heterologous proteins in *P. pastoris* may provoke a cellular stress response produced by the accumulation of unfolded proteins within the cells. The results obtained in the previous chapter strongly suggest that this is the case for ROL. Moreover, the constitutively activation of the UPR in *P. pastoris* strain expressing ROL has allowed for an improvement in the process productivity. However, it is still unclear why the specific production rates (q_p) decrease after the initial hours of induction; a possible explanation to this observation might be related to an UPR-activated transcriptional downregulation of genes coding for secreted proteins, recently reported in fungi and yeast, also affecting the transcriptional levels of heterologous genes coding for secreted products (Pakula et al., 2003). In addition, a downregulation of genes coding for ribosomal components and tRNAs has been observed in *S. cerevisiae* when the secretory pathway is blocked (Li et al., 2000).

The study at the transcriptional level of the effects produced by heterologous protein expression and culture conditions on *P. pastoris* physiology is becoming an increasingly relevant topic in the field of recombinant protein production (Sauer et al., 2004). Moreover, the full *P. pastoris* genome sequence has been recently elucidated by Integrated Genomics (USA). In this context, new techniques have been recently developed for the quantitative analysis of bioprocess-relevant genes transcriptional levels. For instance, a novel technique has been developed by Rautio et al. (2003). This technique consists in the detection of hybridization events between two specific

oligonucleotide probes and the target nucleic acids. A capture probe is used to immobilize the target sequence on a solid support and the detection probe is labelled with a detectable marker. A helper probe is used as well and contributes to stabilize the mRNAs secondary structure (Barken et al., 2004).

The use of this technique offers some advantages over the more conventional methods, Northern blot or slot blot, which are usually less sensitive and time consuming, and offer only a relative quantification. Other techniques such as real time RT-PCR may give a quantitative result but are more expensive and susceptible to PCR-related and reverse transcription related errors.

Our aim in this study is to quantify the amount of specific mRNAs species during fed-batch fermentations. In particular, we wish to monitor the evolution of the expression levels of key genes involved in protein maturation and central metabolism.

First, a series of preliminary shake flask experiments was performed in order to test the suitability of the method. Three different carbon sources were used, glycerol as a repressing carbon source, methanol as an inducing carbon source and sorbitol as a non-repressing carbon source of the P*FLD1* promoter, respectively. Methylamine was used as inducing nitrogen source in the cultures containing methanol and sorbitol. Dithiothreitol (DTT) was added in a culture containing glycerol and ammonium sulphate. DTT is a reducing agent that inhibits disulphide bonds formation, thus preventing the correct folding of proteins in the ER and subsequently inducing the UPR (Pakula et al., 2003, Simonen et al., 1994).

After these preliminary experiments, a series of fed-batch fermentations were carried out at two different specific growth rates, namely under growth-limiting conditions (0.01 h^{-1}) and under carbon-excess conditions (0.02 h^{-1}). The *P. pastoris* X-33 strain, expressing ROL under the transcriptional control of the *FLD1* promoter was employed. Additionally, a fed-batch fermentation was performed with the genetically modified *P. pastoris* strain GS115H co-expressing constitutively the *HAC1* gene and ROL (see chapter 5). All fermentations were performed under the same culture conditions and strategy developed in chapter 4.

The following bioprocess-relevant marker genes were selected to monitor their transcriptional levels during these fed-batch cultures:

The ***KAR2*** gene (GenBank accession number: AY965684) that codifies for the BiP protein, an ER resident chaperone that is upregulated when UPR is activated (Hohemblun et al. 2004).

The protein disulphide isomerase gene (***PDI***) (GenBank accession number: AJ302014), which codifies for a lumenal ER enzyme that catalyses the mechanism of disulphide bond formation (Ferrari et al., 1999).

The ***AOX1*** gene (GenBank accession number: U96967), codifying for the alcohol oxidase enzyme AOX1, the first enzyme in the methanol oxidation pathway, which is highly induced in methanol growth conditions but strongly repressed on glycerol or glucose culture conditions (Cregg and Madden, 1987).

The ***FLD1*** gene (GenBank accession number: AF066054), which is responsible for the synthesis of the formaldehyde dehydrogenase enzyme (Shen et al., 1998), an enzyme implied both in the methanol oxidation pathway and methylamine assimilation metabolism (see chapter 3).

The ***26S*** **rRNA** (GenBank accession number: D43818). Ribosomal RNA represents the 80% of total RNA in the cell, and its content is thought to be closely related with the specific growth rate.

In addition, the transcriptional levels of the product-encoding gene (**ROL**) (GenBank accession number: AF229435) were monitored.

6.2. Materials and methods

6.2.1. Strains

A *P. pastoris* X-33-derived strain containing the expression vector pPICZFLDαROL integrated in its genome *FLD1* locus (chapter 3) and the *P. pastoris* GS115H-derived strain constitutively expressing the *HAC1* gene and co-transformed with the pPICZFLDαROL vector (chapter 5) were also used in this study.

6.2.2. Shake flask cultures

Four shake flask experiments were performed in parallel in 500 mL shake flasks containing 50 mL of culture medium. Shake flasks were inoculated from a pre-culture

of the *P. pastoris* X-33 strain containing the pPICZFLDROL expression vector. Four different media were used:

BMG (buffered minimal glycerol), containing 1% (w/v) glycerol, 1.34 % YNB (w/v) without aminoacids, $4 \cdot 10^{-5}$ % (w/v) biotin and 100 mM potassium phosphate, pH 6.0.

The BMG + DTT medium, including the same components as BMG plus addition of 10 mM DTT.

BMM (buffered minimal methanol), containing 0.5 % (w/v) methanol, 1.34 % YNB (w/v) without aminoacids and ammonium sulphate, 0.4 % (w/v) methylamine hydrochloride, $4 \cdot 10^{-5}$ % (w/v) biotin and 100 mM potassium phosphate, pH 6.0.

BMS (buffered minimal sorbitol), containing 1% (w/v) sorbitol, 1.34 % YNB (w/v) without aminoacids and ammonium sulphate, 0.4 % (w/v) methylamine hydrochloride, $4 \cdot 10^{-5}$ % (w/v) biotin and 100 mM potassium phosphate pH 6.0.

All cultivations were performed at 30ºC and 150 rpm. Cells were grown for 8 hours, and 1 mL samples were withdrawn every 20 minutes.

6.2.3. Sample preparation

Samples from shake flask cultures and fed-batch fermentations (1 mL) were withdrawn and immediately added in a tube containing 100 µL of ice-cool inhibition solution (ethanol:phenol 95:5 v/v) and centrifuged for 1 minute at 12000 rpm using a bench top centrifuge at 4ºC and 12000 rpm. Supernatants were discarded and pellet were resuspended in 1 mL of RNALater solution (Ambion) and stored at -70ºC.

6.2.4. Yeasts lysis and RNA extraction

Total RNA extractions from yeast samples were performed using the RNA extraction kit (Qiagen) according to the manufacturer instructions. Samples were diluted in DEPC water to achieve the same optical density and cells were disrupted with a FastPrep cell homogenizer (ThermoSavant) following the manufacturer's instructions. Total extracted RNA was quantified using RiboGreen RNA Quantification Kit (Molecular Probes). Ribosomal RNA (16S and 23S rRNA from *E. coli;* component C) was used as standard for total RNA quantification assays.

6.2.5. Oligonucleotide probes design and synthesis

Primers for DNA amplification and oligonucletide probes for sandwich hybridization were designed using the software Clone Manager 5.0 (SE Central). Primers and probes

used in this study are shown in table 1. Oligonucleotides were synthesised by Sigma Genosys. Capture probes were 5' biotin labeled by Sigma Genosys and detection probes were labeled with digoxigenin at 3' using the DIG Oligonucleotide Tailing kit (Roche) according to the manufacturer instructions. The primers and probes designed and used in this study are summarized in table 1.

	Sequence 5'→3'	*Position*	*Tm ºC*	*Th ºC*
AOX1				
Forward primer	AGC AGG TGA GAA CAA CCT CAA C	583	66	
Reverse primer	AAG GTC CAC CGT AGG CAT TAG A	1988	66	
Detection	ACC ATT GGC GTA CCA TTG	1510	54	55
Capture	GAA TCT CAG CAT CAC CAC	1475	54	55
Helper	GAC TCG TAC TGA GGC TTG	1440	56	55
26S rRNA				
Forward primer	AAT GAG TGG TGA CAC GAG AC	8	60	
Reverse primer	GGA TGT CGG AGA TGC GTA G	168	62	
Detection	GCG TAG ACG CAC CAA CT	155	54	50
Capture	ACT CCT CCT ACG CCT TC	61	54	50
Helper	TTG GCC GTC TCG TGT CA	33	54	50
PDI				
Forward primer	TGT CCG CTC TCA CAC TAG CA	726	62	
Reverse primer	TTC CTC GAC TGG TCT GAG TG	2182	62	
Detection	CCG ACT AGC TTG AAG ACT	1827	54	55
Capture	CCT CTC CTG CGA TGA ATT	1760	54	55
Helper	TCT CAA TCA GCT CGG TCA	1742	54	55
FLD1				
Forward primer	TCA CTG GTG TAT GCC ACA CT	839	60	
Reverse primer	GAT GTT GTC CAG GTC ATG TC	1788	60	
Detection	AGC AGT GTT GAT AGC AGC AC	1278	60	55
Capture	AG GAG CTT CGT CTT GGA CT	1213	60	55
Helper	TAA CCA CT GAGA TGT CAG CC	1192	60	55
KAR2				
Forward primer	GCC ATG CTA TTG GTC GTA GT	49	60	
Reverse primer	GGT AGG CTC GTT CAC AAT TC	633	60	
Detection	TTG AAG TAG GCT GGA ACG GT	554	60	50
Capture	GTG GCT TGA CGT TGA GCG T	575	60	50
Helper	CGA TGA GAC CGG CAT CCT T	595	60	50
ROL				
Forward primer	CTG ATG GTG GTA AGG TTG T	1271	56	
Reverse primer	AAG GAA CGA TAG AGT TAC TG	2020	56	
Detection	TGC ACC ACC GAG TGA GTG A	1716	60	50
Capture	AGA TCC ATA CCG GCA AGC AA	1742	60	50
Helper	ACA GTC TTG GTT CAC GTT GG	1765	60	50

Table 1. Primers and probes utilized in this work. Tm: melting temperature. Th: hybridization temperature. Position: First nucleotide localization within the corresponding gene sequence at GeneBank

6.2.6. Synthesis of *in vitro* transcripts for standard values

For *in vitro* transcription, the specific PCR products containing the T7 promoter sequence were used as template for *in vitro* transcription performed according to the MAXIscript protocol (Ambion). The concentrations of the *in vitro* transcripts were determined by measuring the absorbance at 260 nm and using Ribogreen RNA quantification assay (Molecular Probes). To calculate the molecular weight of each transcript the web-based Biopolymer Calculator of the Schepartz Laboratory of Chemical Biology (Yale University, U.S.) was used. The *in vitro* transcripts were diluted in DEPC water and used as standards in sandwich hybridization assays.

6.2.7. Sandwich hybridization assay

In the sandwich hybridization assay, the target RNA is hybridized with three DNA probes, complementary to the target RNA sequence (see figure 1). A capture probe is labelled with biotin at its 5' site,which immobilizes the target RNA on a streptavidin-coated MagneSphere particle (Promega). Prior to sandwich hybridization, the detection probe is tailed with digoxigenin. Anti-DIG-alkaline phosphatase Fab-fragments (Roche) are added to the reaction after hybridization and the enzymatic reaction is performed with BBTP (2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole phosphate substrate) (AttoPhos, Promega), which is cleaved to inorganic phosphate (Pi) and BBT (2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole). The signal is detected by a fluorescence reader (Victo2, Wallac Instruments) according to the manufacturer's instructions.

The sandwich hybridization was carried out in 96-well plates, using a Thermomixer Comfort incubator (Eppendorf). The total volume of the hybridization reaction was 100 µL, containing 5 X SSC buffer (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 20 % (v/v) formamide, 2 % (v/v) Denhardts reagent, 3 % (v/v) dextran sulphate and 0,2 % (v/v) SDS. An amount of 5 pmol of biotin-labelled capture probe, 5 pmol of helper probe and 1 pmol of dig-tailed detection probe were added to the hybridization solution. Finally, 10 µL of the specific mRNA *in vitro* standards or extracted RNA from samples were added to each well.

The hybridization took place at 50ºC, for 30 minutes at 700 rpm shaking. After hybridization, 20 µL of streptavidin-coated magnetic beads (Promega) were added to the wells, followed by incubation at 37ºC for 30 minutes and 500 rpm. Before its addition, the magnetic particles were washed three times with SSC buffer and finally taken to the original volume with the same buffer. Once the immobilization took place, the MagnaBot 96 Magnetic Separation device (Promega) was used to separate the beads from the solution. The magnetic beads were washed twice with 130 µL of SSC buffer containing 0.05 % (v/v) SDS by incubating the plates at 25ºC for 2 minutes and 700 rpm. After washing the beads, 100 µL of Anti-DIG-alkaline phosphatase FAB fragments diluted 1:2000 in SSC buffer were added to each well and incubated at 25ºC for 30 minutes and 700 rpm. To remove the unbound antibody, the wells were washed four times with SSC buffer containing 0.05 % (v/v) SDS by incubating at 25ºC for 2 minutes and 700rpm. After the washing steps, the beads were transferred to a new microtiter plate and washed again. After removing the washing buffers, 100 µL of Attophos fluorescent substrate for alkaline phosphatase (2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole phosphate (BBTP) (Promega) were added to the wells and incubated at 37ºC and 700 rpm for 20 minutes. Alkaline phosphatase cleaves the BBTP to inorganic phosphate (Pi) and BBT (2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole).

When the reaction was completed, the beads were separated from the solution using the MagnaBot 96 Magnetic Separation device (Promega) and the supernatant was transferred to a black microtiter plate and measured using a fluorescence reader Victor2 Multilabel Counting (Wallac) at excitation wavelength 430 nm and emission wavelength 560 nm.

Fed-batch samples were analysed in duplicates, while for the shake flask experiment samples only one run for sample was performed due to the smaller sample volume.

The detection limit of the analysis was determined by Rautio et al. (2003) using 18S rRNA. The amount of 2 fmol of the target molecules gave a signal that was four-fold higher than the background and the reaction was linear up to 980 fmol of the target molecule. For each series of samples, a calibration curve using the *in vitro* synthesised

standards was performed and the amount of a given mRNA specie calculated taking the linear part of the resulting standard curve.

The amounts of the given mRNA species obtained from the sandwich hybridization assay were expressed in fmol of each specific mRNA, and were normalized by the total RNA of the corresponding sample.

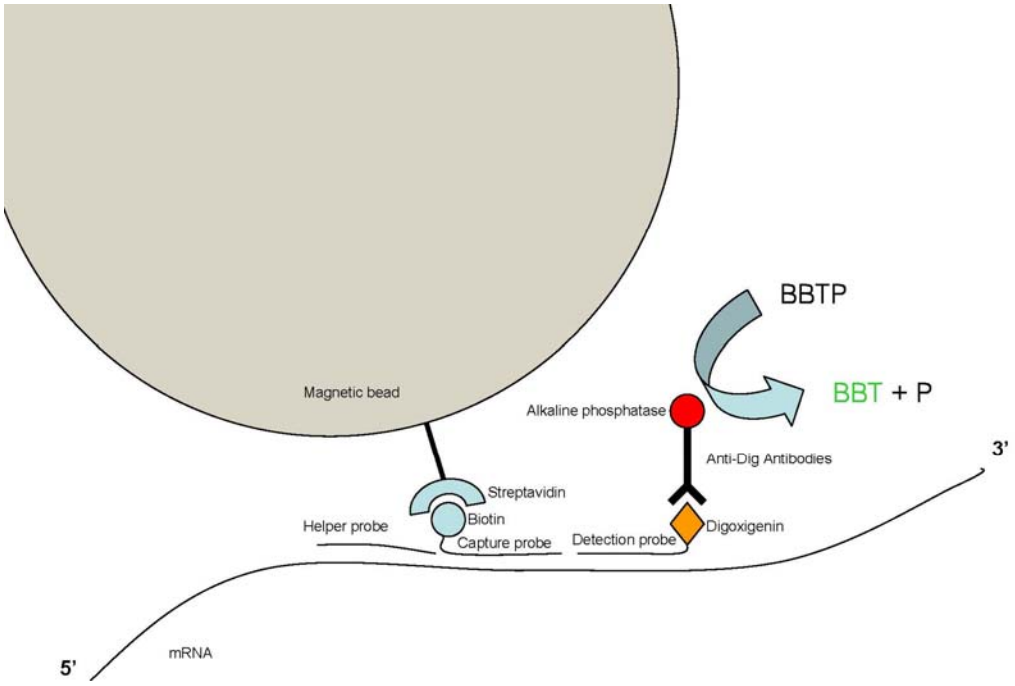


Figure 1. Schematic representation of the principle of sandwich hybridization assay.

6.3. Results

6.3.1. Preliminary shake flask experiments

A battery of shake flask cultures was initially performed in order to test the functionality of the designed probes and the transcriptional response of the genes of interest upon induction of ROL expression. Thus, cells were grown minimal medium (see materials and methods for details) containing different carbon sources (glycerol, methanol or sorbitol) and nitrogen sources (ammonium or methylamine) combinations. In addition cells were grown in the presence of DTT, as a positive control for UPR-triggered cells. The results of the transcriptional analysis of the *AOX1*, *FLD1*, *PDI*, *ROL* and *KAR2* genes are summarized in figure 2.

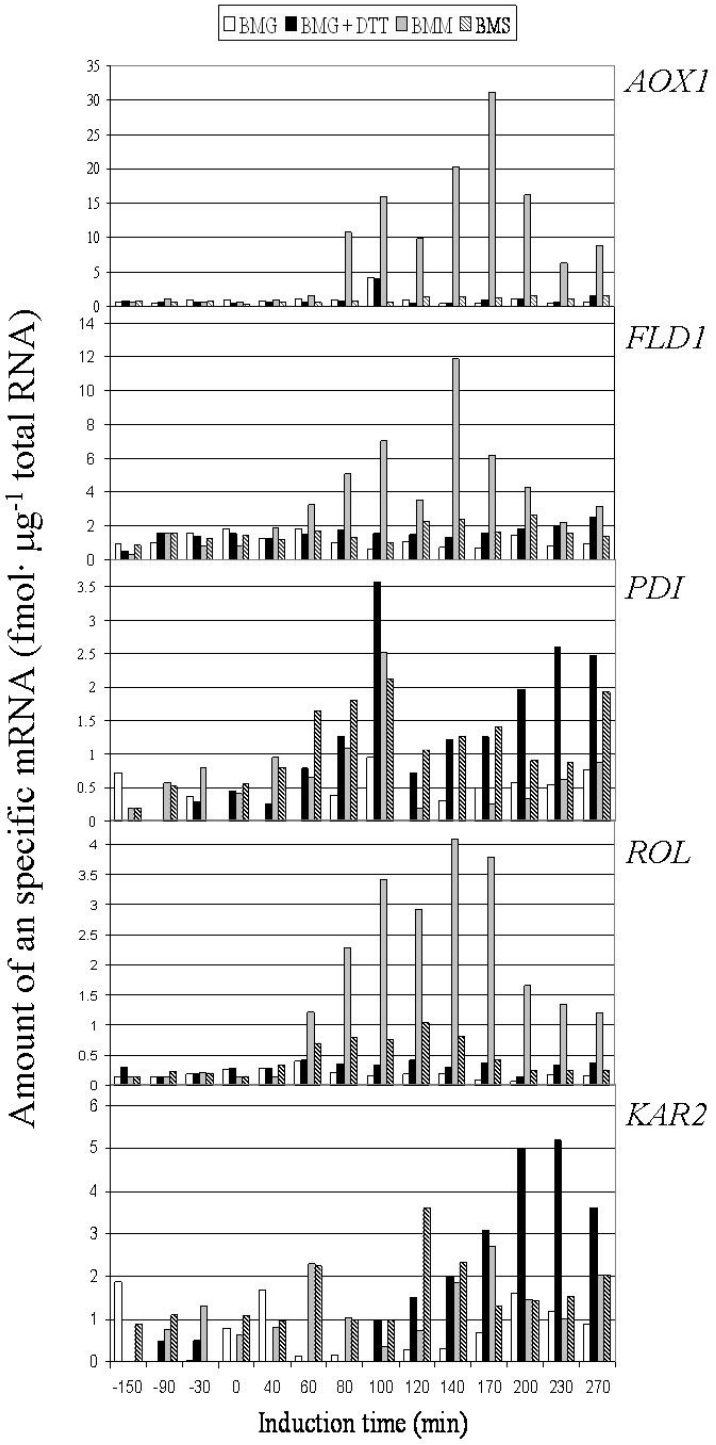


Figure 2. Transcriptional levels of *AOX1, FLD1, PDI, ROL* and *KAR2* expressed as fmol of mRNA per microgram of total RNA. Time= 0 hours corresponds to change to induction medium

As expected, in the culture containing methanol as a sole carbon source (BMM), *AOX1* transcriptional levels increased soon after the addition of methanol into the medium, reaching more than 30 fmol of *AOX1* mRNA per microgram of total RNA. Such drastic increase is consistent with the fact that the *AOX1* promoter is one of the strongest promoters known in yeast (*AOX1* mRNA levels in methanol-grown *P. pastoris* cells can reach about 5% of the total cell's mRNA, Cregg et al., 1993). As also expected, the glycerol-growncells did not show any significant *AOX1* induction, whilesome degree of derepression was observed in the sorbitol-containing medium.

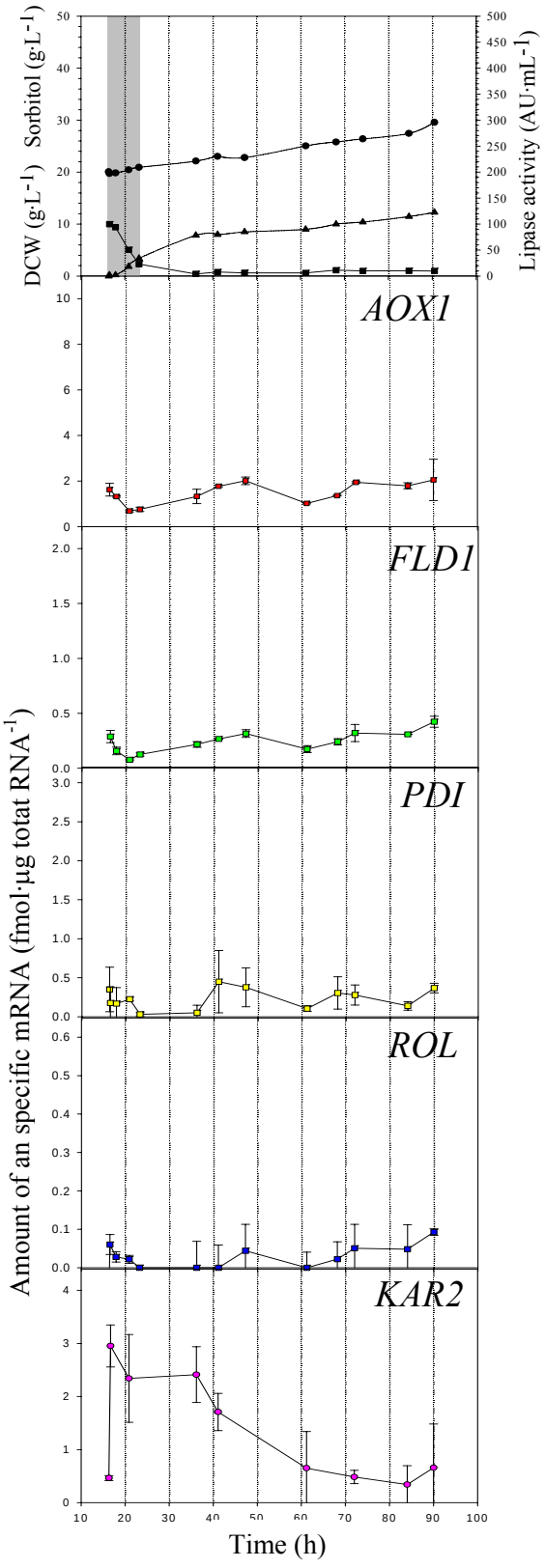
FLD1 showed a similar profile as *AOX1*, i.e. its transcriptional levels increased after 40 to 60 minutes of induction in the BMM culture, reaching about 12 fmols of *FLD1* mRNA per microgram of total RNA, whereas in the BMG and BMG+DTT only basal levels of *FLD1* transcripts were observed. The maximum *FLD1* transcriptional levels reached in the BMS culture, containing methylamine, were significantly lower than the corresponding values in the BMM, confirming the synergistic effect of methanol and methylamine on *FLD1* induction (see chapter 3).

ROL transcriptional levels followed an analogous pattern to the *FLD1* levels, that is, induction of ROL expression was only detected in BMM and BMS media. In particular, in BMM culture, *ROL* mRNA levels were 3-4 fold higher than in the BMS culture. However, although *FLD1* and *ROL* genes are under the control of the same promoter, their corresponding mRNAs levels were significantly different one to the other. This fact might be explained by a different degradation rate of both mRNAs within the cell.

Regarding *PDI* and *KAR2*, their transcriptional levels in cells growing on BMG+DTT were clearly induced, i.e. indicating that the experimental set-up was appropriate for detection of increased UPR-related genes transcriptional levels. In addition, induction of *PDI* and *KAR2* was detected in cells grown in BMM and BMS media, i.e. under ROL expression-inducing conditions. . Thus, this parallel upregulation of the *PDI* and *KAR2* genes caused by either DTT or ROL further supported that ROL overexpression triggers the UPR in *P. pastoris*.

6.3.2. Fed-batch fermentations

As discussed in previous chapters, the specific growth rate has proven to be an important parameter for the productivity of secreted heterologous proteins in the *P. pastoris* system (Sinha et al., 2003; Zhang et al., 2000) and, in particular, when using



the P*FLD1* for heterologous extracellular ROL production. Here, two fed-batch fermentations using the *P. pastoris* X-33-derived strain expressing ROL under the transcriptional control of the *FLD1* promoter were performed following the same scheme developed in chapter 4. The induction phase of the first cultivation was performed under growth rate-limiting conditions (μ mean of about 0.01 h^{-1}), whereas a near-μ_{max} growth rate of about 0.02 h^{-1} was maintained under carbon excess conditions during the induction phase of the second cultivation.

6.3.2.1. Fed-batch of X-33 strain at a low specific growth rate.

The main fermentations parameters as dry cell weight, lipase activity and substrate concentration followed essentially the same trend as obtained in previous fermentations under the same conditions (see chapter 4).

Figure 3. Fed-batch cultivations of *P. pastoris* X-33 strain expressing ROL at a controlled specific growth rate of 0.01 h^{-1}. Dry cell weight (●), sorbitol concentration (■) and extracellular lipase activity (▲) are indicated. mRNAs levels are expressed as fmol of each mRNA per µg of total RNA in

the sample.

In this cultivation, analyses of mRNAs levels of the 5 specific genes were carried out. The results for the fed-batch culture performed at a controlled specific growth rate of 0.01 h^{-1} are depicted in figure 3.

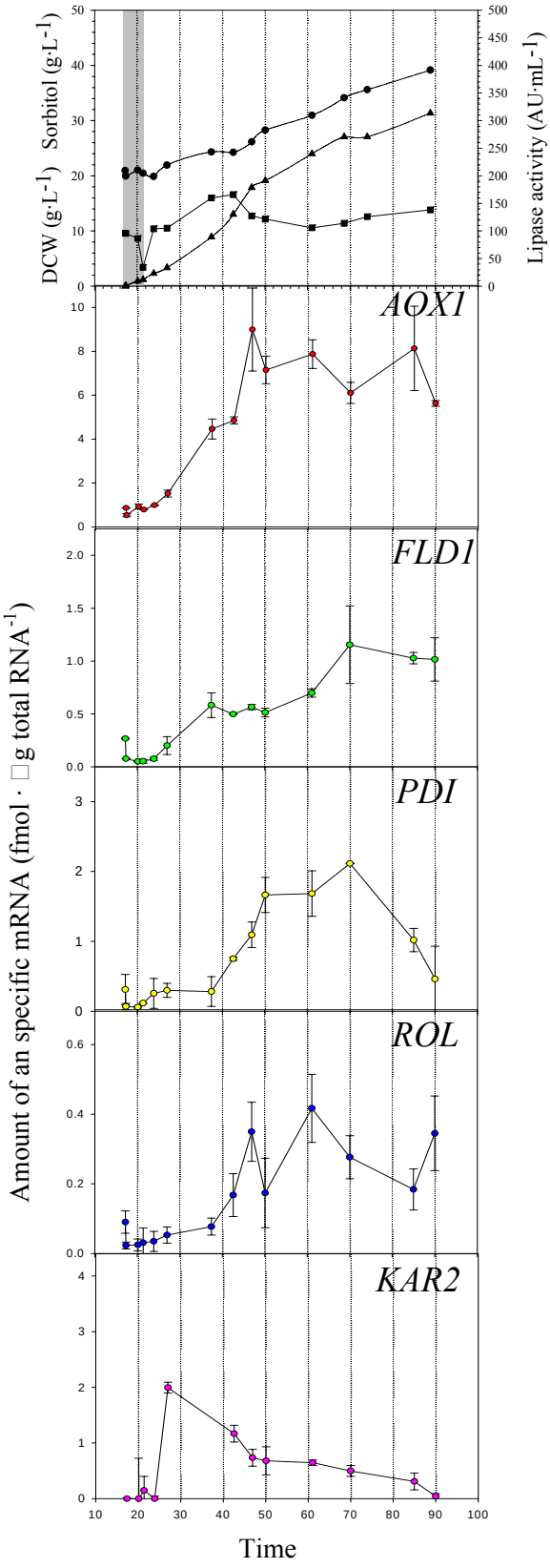
As observed in figure 3, the AOX1, FLD1, PDI, and ROL genes showed small variations in their expression levels when cells were switched to a medium containing sorbitol and methylamine at growth limiting conditions. Only in the case of the *KAR2* gene, a clear transient induction (about 6-fold) was detected at the early stage of fed-batch transition phase; these upregulated transcriptional levels lasted for about 20 h, thereafter decreasing steadily to basal levels after 35 h of induction phase.

As observed in the preliminary shake flask cultivations, there is some derepression of the *AOX1* gene in cells growing on sorbitol plus methylamine. Moreover, the *AOX1* transcriptional levels under de-repressed conditions were significantly higher than the corresponding levels for *FLD1*, i.e. around 2 fmol of *AOX1* mRNA per microgram of total RNA versus only 0.4 fmol per microgram of total RNA in *FLD1*.

6.3.2.2. Fed-batch of X-33 strain at specific growth rate near to μ maximum.

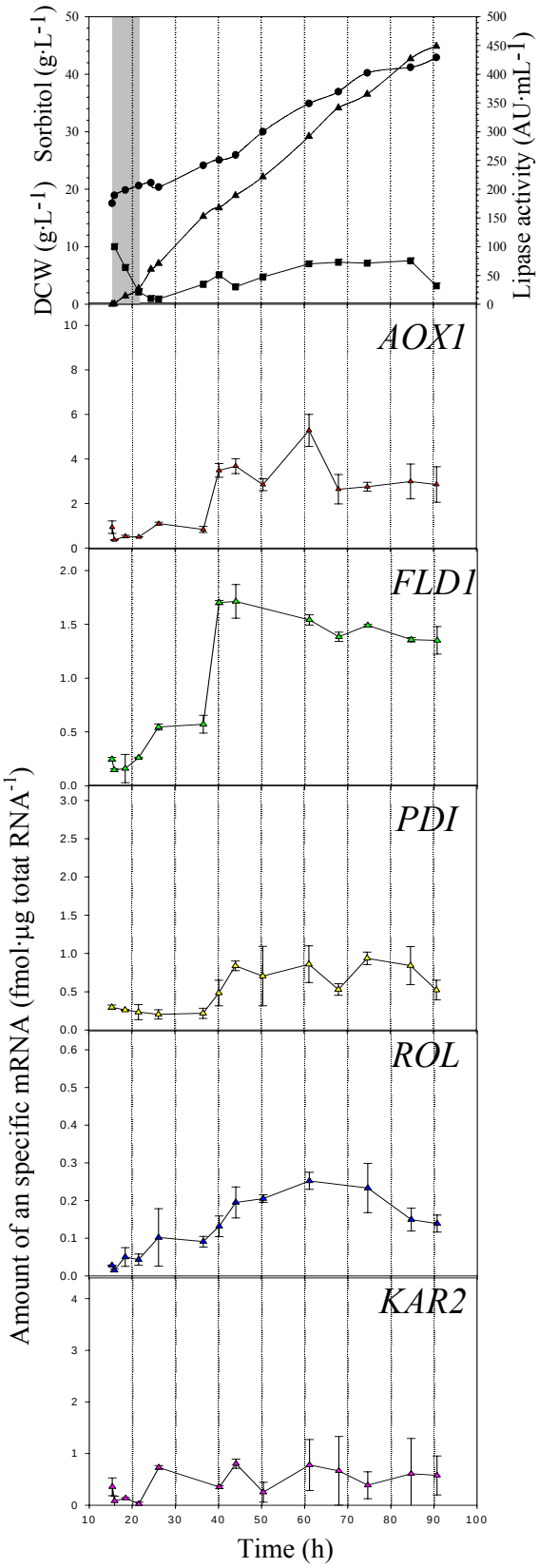
In the fed-batch performed under substrate excess conditions, i.e. growing at a near-μ_{max} of 0.02 h^{-1}, the results obtained from the transcriptional analyses are significantly different compared to the previous cultivation (figure 4).

The *AOX1* mRNA levels started increasing soon after transition phase, until the 50 hours of cultivation. Then, values stabilized at around 8 fmol per microgram of total RNA, which is a relative high value compared to the rest of analyzed genes. This further confirmed that the *AOX1* gene is derepressed to a significant extent in *P. pastoris* cells growing on sorbitol, as previously reported by some authors (Thorpe et al., 1999; Inan et al., 2001). However, in contrast to other methylotrophic yeasts such as Hansenula polymorpha, *P. pastoris* AOX1 transcriptional levels under sorbitol growth conditions are relatively lower compared to the corresponding levels found under methanol growth conditions, as observed in the shake flask experiment where *AOX1* mRNA levels reached about 30 fmol per microgram of total RNA (figure 2).



The *FLD1* mRNA levels started increasing steadily soon after the start of the induction phase, reaching about 1 fmol per microgram of total RNA after 70 hours of cultivation. After this point, *FDL1* mRNA levels stabilized. *PDI* and *ROL* mRNAs showed similar profiles; both genes were increasingly induced until 60 h (ROL) and 70 h (PDI) of cultivation; However, *PDI* mRNA levels clearly decrease soon after the 70 hour of culture, down to almost basal levels towards the end of the cultivation, whereas the decrease in ROL mRNA levels after 60 h of cultivation is not so pronounced. Regarding *KAR2*, its profile was completely different from the rest of the analyzed genes. As observed in the growth-limited fed-batch cultivation, there is a sharp increase in *KAR2* mRNA levels at the beginning of induction phase followed by a constant decrease, reaching basal levels towards the end of the cultivation.

Figure 4. Fed-batch cultivations of *P. pastoris* X-33 strain expressing ROL at a specific growth rate of about 0.02 h^{-1} and carbon excess conditions. Biomass (dry cell weight) (●), sorbitol concentration (■) and extracellular lipase activity (▲) are



indicated. mRNA levels are depicted, expressed as fmol of each mRNA per microgram of total RNA in the sample.

6.3.2.3. Fed-batch of the GS115H/HAC1 strain at specific growth rate near to µ maximum.

A third fed-batch fermentation was carried out using the *P. pastoris* GS115H / [pGAPHAC1 + pPICZFLDαROL] strain under carbon-excess conditions, i.e. at a near-maximum specific growth rate of about 0.02 h^{-1}. The evolution of cultivation parameters is shown in figure 5. As seen in the corresponding fed-batch performed with the X-33 strain, the *AOX1* gene is derepressed when sorbitol is used as carbon source. However, in this case the *AOX1* transcriptional levels were significantly lower than in the corresponding cultivation with with the X-33 strain (maximum of 5.3 ± 0.71 fmol per microgram of total RNA in the GS115H/HAC1 strain vs. 8 fmol per microgram of total RNA in the X-33 strain).

Figure 5. Fed-batch cultivations of *P. pastoris* GS115H/HAC1 strain expressing ROL at a controlled specific growth rate of about 0.02 h^{-1}. Biomass (dry cell weight) (●), sorbitol concentration (■) and extracellular lipase activity (▲) are indicated. Analyzed mRNAs are depicted,

expressed as fmol of each mRNA per microgram of total RNA in the sample.

In contrast the *FLD1* gene showed higher mean mRNA levels along the induction phase than in the corresponding cultivation with the X-33 strain. However, the mean mRNA *ROL* levels were slightly lower than in the X-33 fed-batch culture performed at the same conditions, again revealing important differences in the mRNA's half-lives of *FLD1* and *ROL* transcripts. Notably, the maximum specific lipase activity in GS115H/HAC1 cells was higher than in the X-33 cells (10928 AU·g biomass^{-1} vs. 8008 AU·g biomass^{-1}, respectively). This observation might be explained by an improved efficiency of the protein folding and secretion machinery in the GS115H/HAC1 strain. This improved efficiency could be the result of the combination of lower ROL synthesis rates with pre-conditioning of the cells to ER stress.

On the other hand, an unexpected behavior was observed in the transcriptional levels of the *PDI* and *KAR2* genes. In principle, the constitutive overexpression of the HAC1 gene up regulates the synthesis of more than 300 genes involved in the UPR, including *KAR2* and *PDI* (Travers et al., 2000). However, the basal *KAR2* or *PDI* transcriptional levels measured at the end of the batch of the GS115H/HAC1 cultivation were essentially the same as those measured for the X-33 strain at the same stage of the cultivation. Moreover, the *PDI* mRNA levels showed lower induction levels than in the X-33 strain, reaching a maximum after 44 h of culture, thereafter maintaining a constant value of about 0.9 fmol per microgram of total RNA along the rest of the induction phase. *KAR2* had a similar pattern, i.e. levels kept a relatively constant value below 1 fmol per microgram of total RNA along the whole induction phase, in clear contrast with the *KAR2* mRNA profiles observed in the fermentations carried out with the X-33-derived strain.

Overall, these results suggest that the constitutive expression of the *S. cerevisiae* HAC1 gene in *P. pastoris* allows for a "pre-conditioning" of the host cells, which results to a significantly lower induction of UPR-related genes (*PDI* and *KAR2*) upon ROL induction. Nevertheless, further systematic studies are needed at the transcriptional level to unravel the complex physiological dynamic responses of *P. pastoris* to ROL overexpression.

6.3.2.4. *26S* rRNA analyses

Figure 6 shows the evolution of the 26S rRNA content. The 26S rRNA levels normalized by the total RNA content of the samples remain mainly constant throughout the whole fermentation time. This indicates that there is not a variation between total extracted RNA and the 26S rRNA ratio, as the main part of total RNA is ribosomal RNA, 26S rRNA is a good indicator of the ribosomal RNA content.

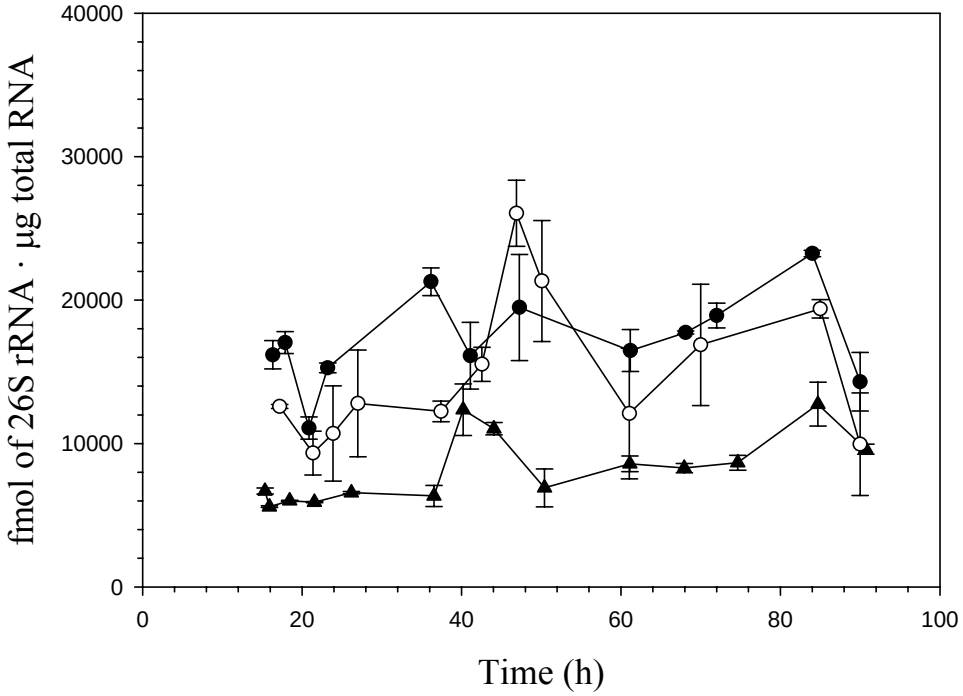


Figure 6. *26S* rRNA content of three different fed-batch cultures of *P. pastoris* expressing ROL at different specific growth rate and strains. X-33 strain at a mean specific growth rate of about 0.01 (h^{-1}) (●), X-33 strain at a mean specific growth rate of 0.02 (h^{-1}) (○). GS115H/HAC1 strain at a mean specific growth rate of about 0.02 (h^{-1}) (▲).

6.3.3. Discussion

The results from the transcriptional analysis of the ROL gene are generally consistent with the results presented in chapter 5, where intracellular ROL protein levels were analyzed by immunofluorescence-based techniques combined with flow cytometry.

However, whereas intracellular ROL levels showed a similar profile to its corresponding mRNA levels in X-33 cells growing at a low specific growth rate, *KAR2* transcriptional levels did not correlate with the observed intracellular BiP content (see chapter 5), as no clear pick of BiP was detected at the start of the induction phase.

In X-33 cells growing under carbon excess conditions, ROL intracellular levels were in good concordance with its corresponding mRNA levels, i.e. showing a clear increase, both in intracellular ROL protein and *ROL* mRNA content at 40 hours of cultivation, and reaching a maximum between the 50 and 60 hours of cultivation. Regarding *KAR2*, a delay of approximately 10 hours is shown between the increase in its mRNA content and the subsequent increase in intracellular BiP protein content.

The same correlation between intracellular ROL levels and *ROL* mRNA is found in GS115H/HAC1 cells growing carbon excess conditions. However, *KAR2* transcriptional levels and BiP protein levels did not correlate, as the increase in BiP levels during the induction phase of the cultivation did not appear to be reflected at the transcriptional level. .

Overall, our results support the hypothesis that increased transcriptional levels of the overproduced heterologous protein do not always result in more extracellular product. In fact, some authors have pointed out the existence an optimal specific growth rate for synthesis and secretion of proteins (Pakula et al., 2005).

In principle, a bottleneck in protein production may be the consequence of a limitation provoked either by a low transcription rate or low protein synthesis and secretion rates. In our case, the results suggest that, at low specific growth rates (0.005-0.01 h^{-1}), transcription seem to be responsible for the low product synthesis, as deduced from the low *ROL* transcriptional levels. However, *ROL* mRNA levels in the GS115H/HAC1 performed at 0.02 h^{-1} specific growth rate were lower than in the X-33 growing at an equivalent growth rate, although extracellular product was higher in the GS115H/HAC1 strain. Thus, a higher extracellular expression level but lower or similar transcriptional level indicate that enhance in the GS115H/HAC1 strain ROL expression is not provoked by an upregulation transcription of *ROL* but by an improvement in secretion or folding rate. Thus, in cells growing at specific growth rate of about 0.02 h^{-1} protein folding and secretion appears to be a limiting step.

6.4. Conclusions

In this chapter the influence of the specific growth rate, carbon and nitrogen sources and the induction of ROL expression on the transcriptional level of a reduced set of bioprocess-relevant genes has been quantitatively studied. The main conclusions obtained are summarized as follows:

- The bead-based sandwich hybridization assay has proved to be a reliable instrument for quantification of specific mRNA species in *P. pastoris* fermentation samples, although further improvement of cell lysis protocols and probe design for some specific genes such as *KAR2* is needed.
- Specific growth rate has shown to be a key factor in the evolution of the transcriptional levels of the analyzed genes following induction of ROL expression. Transcriptional levels of the analyzed set of genes were generally higher at higher growth rates.
- ROL overexpression in the X-33-derived strain clearly induces the expression of UPR-related genes such as *KAR2* and *PDI*. In contrast, when ROL is overexpressed in a strain having the UPR constitutively activated (GS115H/HAC1 strain), significantly lower induction levels of these marker genes were detected.
- The transcriptional levels of the *FLD1* gene under ROL-inducing conditions (i.e. sorbitol and methylamine as substrates) appear to be significantly lower than the *AOX1* levels. However, secretion, but not transcription, appears to be the major limiting factor in ROL production processes when using the *P. pastoris* P*FLD1*-based system

6.5. References

Barken KB, Gabig-Ciminska M, Holmgren A and Molin S (2004) Effect of unlabeled helper probes on detection of an RNA target by bead-based sandwich hybridization. Biotechniques 36: 124+.

Cregg JM and Madden KR (1989) Use of site-specific recombination to regenerate selectable markers. Molecular and general genetics 219: 320-3.

Cregg JM, Vedvick TS, Raschke WC (1993) Recent advances in the expression of foreign genes in *Pichia pastoris*. Bio-Technology 8: 905-910.

Ferrari DM and Soling HD (1999) The protein disulphide-isomerase family: unravelling a string of folds. Biochemical Journal 339: 1-10.

Hohenblum H, Gasser B, Maurer M, Borth N and Mattanovich D (2004) Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris.* Biotechnology and Bioengineering 85: 367-375.

Pakula T, Laxell M, Huuskonen A, Uusitalo J, Saloheimo M and Penttila M (2003) The effects of drugs inhibiting protein secretion in the filamentous fungus *Trichoderma reesei* - Evidence for down-regulation of genes that encode secreted proteins in the stressed cells. Journal of Biological Chemistry 278: 45011-45020.

Pakula T, Salonen K, Uusitalo J and Penttila M (2005) The effect of specific growth rate on protein synthesis and secretion in the filamentous fungus *Trichoderma reesei*. Microbiology 151: 135-143.

Rautio J, Bundvig Barken K, Lahdenperä J, Breitenstein A, Molin S and Neubauer P (2003) Sandwich hybridization assay for quantitative detection of yeast RNAs in crude cell lysates. Microbial Cell Factories 2.

Shen SG, Sulter G, Jeffries TW and Cregg JM (1998) A strong nitrogen source-regulated promoter for controlled expression of foreign genes in the yeast *Pichia pastoris*. Gene 216: 93-102.

Simonen M, Jamsa E and Makarow M (1994) The role of the carrier protein and disulfide formation in the folding of beta-lactamase fusion proteins in the

endoplasmic-reticulum of yeast. Journal of Biological Chemistry 269: 13887-13892.

Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS and Walter P (2000) Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell 101: 249-258.

Zhang WH, Bevins MA, Plantz BA, Smith LA and Meagher MM (2000) Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A. Biotechnology and Bioengineering 70: 1-8.

CHAPTER 7

Deletion of the *P. pastoris GAS1* gene and its application in ROL secretion

---

7.1. Introduction

In previous chapters, a metabolic engineering strategy based on the constitutive expression of the *S. cerevisiae HAC1* gene, has been carried out to alleviate the possible bottlenecks that the cell might undergo when overexpressing ROL. The obtained results indicate that the increased efficiency in protein folding and maturation in the modified *HAC1* overexpressing strain leads to increase ROL production levels. However, we still observe the same profiles in the specific production rates during fed-batch cultivations, i.e. a sharp decrease in q_p values after the maximum levels obtained at initial stages of the induction phase. Moreover, active ROL was measured intracellularly, indicating an additional limitation after the folding and maturation process. Thus, a further limitation must exist in the protein secretion pathway to the extracellular medium. In yeast, many secreted proteins are retained in the periplasmic space between the plasma membrane and the cell wall, while other are efficiently excreted to the growth medium (Venturini et al., 1997). The *S. cerevisiae* yeast cell wall composition by dry cell weigth is about 60% glucans (β-1,3 and β-1,6 glucans), 40 % mannoproteins and 1-2 % chitin (Popolo et al., 1999) which are gathered forming an extracellular matrix which is essential for maintenance of cell integrity.

In studies performed by Vai et al (2000), the secretion of human insuline-like growth factor 1 was improved 7-fold by the deletion of a cell wall cross linking enzyme (Gas1p) in *S. cerevisiae*. The Gas1p is a glycoprotein anchored to the outer layer of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. The enzymatic function of

Gas1p is thought to be a β-1,3-glucanosyltransglycosylase activity, a cross-linking enzyme that catalyses a transglycosylation with β-1,3-glucan as substrate (Popolo et al., 1999). The deletion of the *GAS1* gene revealed a reduction of specific growth rate of 15 to 40 % in the Δ*GAS1* mutants (Popolo et al., 1993). Moreover, disruption of the GAS1 gene results in a lower β-glucan content of the cell wall, swollen more spherical cells and defective bud maturation and cell separation. These mutants show a higher amount of chitin and mannan content compared to the wild type cells (Valdivieso et al., 2000; Popolo et al., 1997). It is thought that the cell wall weakening induces a general response to compensate the loss in strength of the cell wall in attempt to prevent cell lysis (Ram et al., 1998).

In this chapter, the deletion of a GAS1 homologe gene in *P. pastoris* expressing ROL is detailed. The disruption of the Gas1p, which is responsible for the cell wall structural integrity, was expected to alleviate the bottleneck produced by the cell wall barrier, thus improving the extracellular recombinant protein content.

7.2. Materials and methods

7.2.1. Strains

P. pastoris X-33 transformed with pPICZFLDαROL (expresses secreted ROL under the transcriptional control of the *PFLD1* promoter) was used for comparative studies.

The *S. cerevisiae* WB2d (*gpp1::LEU2*) generated from strain W303-1B by a one-step gene disruption (Vai et al., 1991) was used to verify the functionality of the GAS1 gene of *P. pastoris*.

7.2.2. Cloning and disruption of the GAS1 homologue of *P. pastoris*

P. pastoris Δ*GAS1* mutants strains used throughout this chapter were constructed by Hans Marx at Dr Diethard Mattanovich's laboratory in the Institute of Applied Microbiology, Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Vienna, Austria.

The *P. pastoris GAS1* homologue was isolated by PCR. In order to design the primers for amplification of the unknown nucleotide sequence of *P. pastoris GAS1* an alignment of already known *GAS1* coding sequences homologues of *S. cerevisiae*, *Neurospora*

crassa, *Candida dubliniensis*, *Candida glabrata*, *Candida maltosa* and *Candida albicans* was carried out. Regions showing the highest homology were chosen for primer design. Based on the sequences of the PCR products obtained, the full cDNA sequence of *P. pastoris GAS1* could be determined (Genebank accession DQ444263).

Further, the *P. pastoris GAS1* homologue functionality was tested in *S. cerevisiae* by transforming a *S. cerevisiae* strain WB2d, deficient in GAS1, with the plasmid pYX212 containing the *P. pastoris GAS1* insert. The transformed cells showed a wild type like morphological phenotype. Additionally, resistance to Congo Red and SDS were tested, proving the functionality of the *P. pastoris GAS1* gene in *S. cerevisiae*.

Finally, a marker cassette conferring resistance to G418 was inserted in the *Box*I restriction site of the *P. pastoris GAS1* homologue, interrupting the gene.

The *P. pastoris* strains X-33 + pPICZFLDαROL and the GS115 + pGAP-HAC1 + pPICZFLDαROL (GS115H/HAC1), were transformed by electroporation with the linearized deletion cassette and plated on YPDS-agar containing Geneticin (G418; 250μg/mL).

7.3. Results

7.3.1. Shake flask experiment

Several clones from the X-33 + pPICZFLDαROL *ΔGAS1* and from the GS115H/HAC1 *ΔGAS1* strains were selected from YPDS + Geneticin plates. Four clones of X-33 + pPICZFLDαROL strain and one from the GS115H/HAC1 *ΔGAS1* strain were pre-cultivated in shake flasks using BMS medium, and methylamine as inducer substrate. The untransformed parent strain X-33 + pPICZFLDαROL was used to compare expression levels. The tested transformants from the GS115H/HAC1 *ΔGAS1* strain did not show any growth or lipase activity in shake flask.

The amount of secreted recombinant ROL in culture supernatants was tested after 24 hours of cultivation. The *ΔGAS1* transformants showed an average increase of 20% in specific activity levels (0.12±0.031 AU/OD vs 0.10±0.006 AU/OD). The X-33 + pPICZFLDαROL *ΔGAS1* with the highest specific activity was selected for further expression studies in fed-batch cultivations.

7.3.2. Bioreactor fed-batch cultivation

The previous results seen in chapter 4 have shown that ROL production and productivity levels in high cell density fed-batch cultivations are higher when the

inducing phase (when cells are growing on sorbitol and methylamine as carbon and nitrogen source, respectively) is carried out under carbon-excess conditions, i.e. near μ_{max} (about 0.025 h^{-1}). Therefore, we performed a fed-batch cultivation with the new strain using the same conditions as utilized in chapter 4. In figure 1 the results from the X-33 + pPICZFLDαROL *ΔGAS1* strain fed-batch and a fedbacth performed with the untransformed parent strain X-33 + pPICZFLDαROL are summarized.

The *P. pastoris* X-33 *ΔGAS1* strain performed significantly different when compared with the corresponding untransformed strain grown under the same conditions. First, the maximum specific growth rate achieved by the *ΔGAS1* strain during the induction phase was significantly lower compared to the untransformed strain (0.01 vs 0.02 h^{-1}). This negative effect of the *GAS1* deletion on cell growth has also been previously described for *S. cerevisiae* (Popolo et al., 1993).

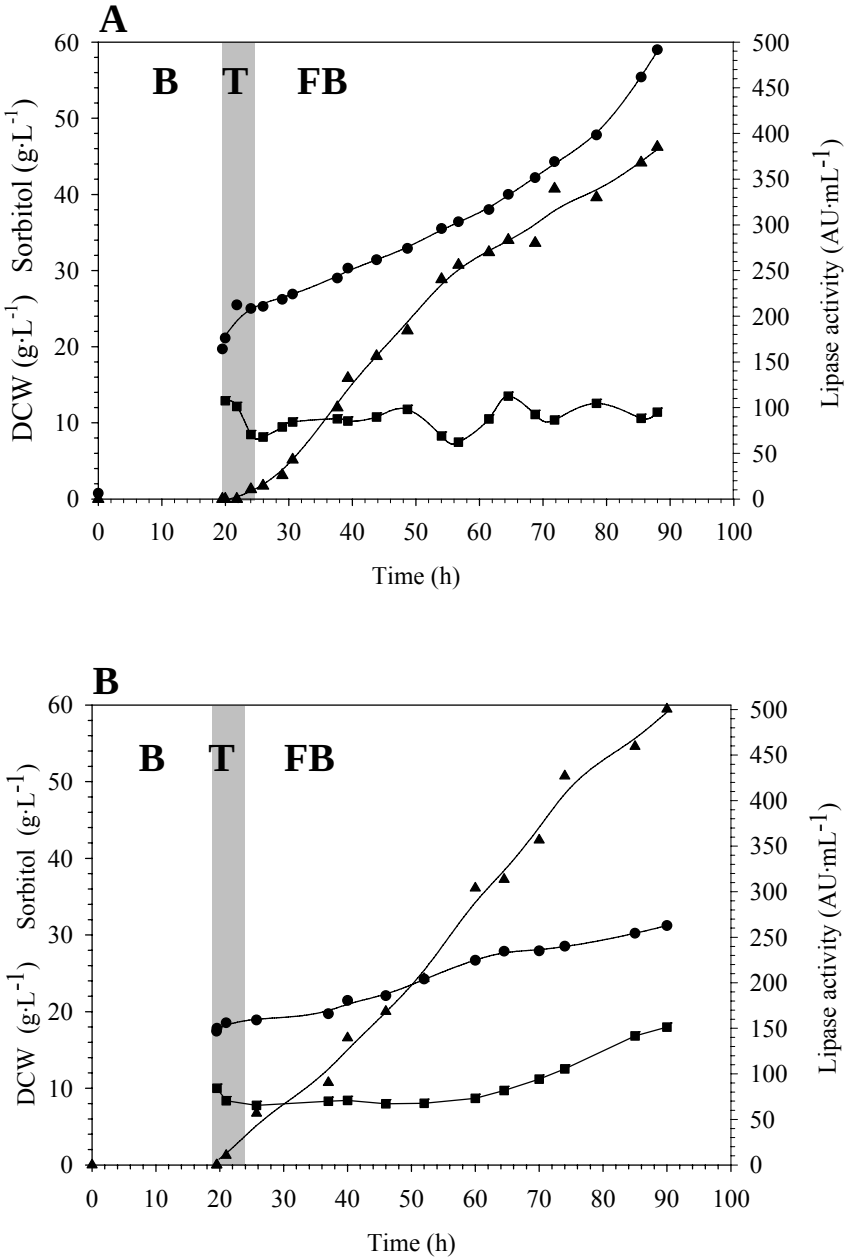


Figure 1. Fed-batch fermentations performed with a X-33 + pPICZFLDαROL *ΔGAS1* strain (A) and with the untransformed parent strain X-33 + pPICZFLDαROL (B) under the same cultivation conditions. (●) biomass (dry cell weight), (■) sorbitol concentration, (▲) extracellular lipase activity.

Second, the final extracellular lipase activity levels were about 20% higher after 90 h of cultivation with the *ΔGAS1* strain. Notably, intracellular activity levels remained consistently lower (ca. 0.25 vs. 0.5 AU· μg^{-1}) (figure 2) after 60 h of cultivation.

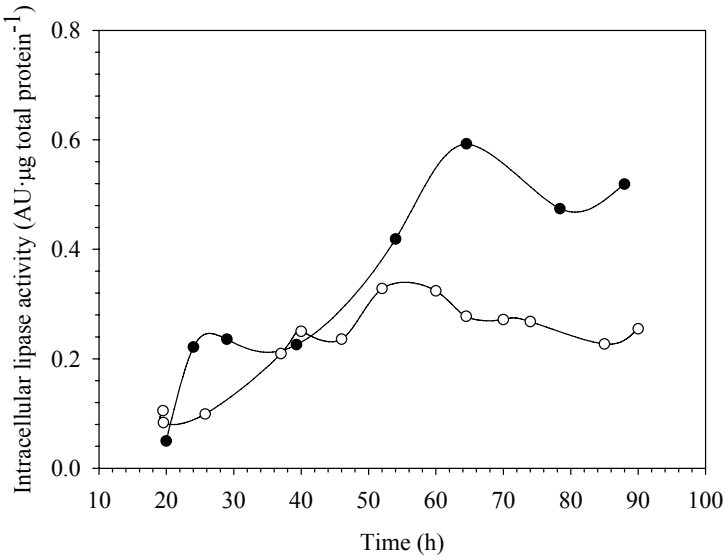


Figure 2. Intracellular lipase activity measured in untransformed parent strain X-33 + pPICZFLDαROL (●) and in the X-33 + pPICZFLDαROL *ΔGAS1* strain (○).

Third, the specific production rates (q_p) (figure 3) remained mainly constant, with a slight increase, during the major part of the induction phase of the *ΔGAS1* strain cultivation, opposite to the situation in the cultivations performed with the untransformed strain, where a sharp decrease in q_p values was observed shortly after the start of the induction phase.

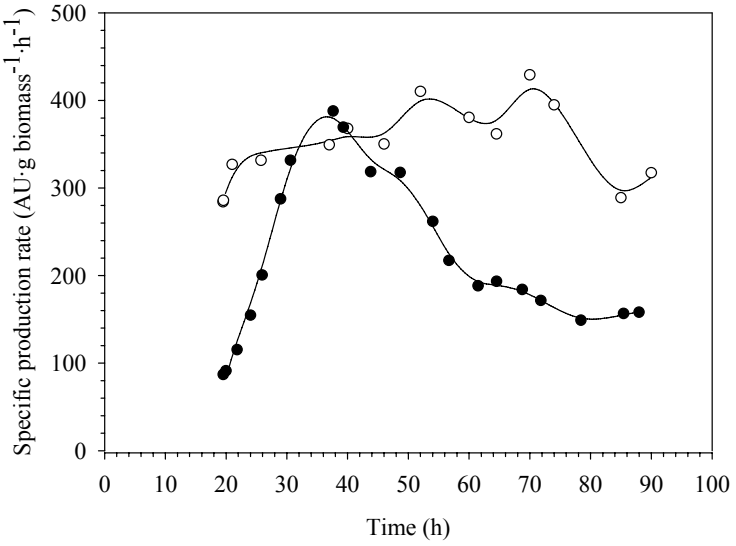


Figure 3. Specific extracellular production rate (q_p) profile in untransformed parent strain X-33 + pPICZFLDαROL (●) and in the X-33 + pPICZFLDαROL *ΔGAS1* strain (○).

A comparative analysis of the fed-batch cultivations carried out with the untransformed and Δ*GAS1* strains in terms of $Y_{P/X}$, productivity and specific productivity (table 1) reveals that the overall efficiency of the system increased significantly in the Δ*GAS1* strain: 2-fold in terms of $Y_{P/X}$ and specific productivity, and 1.3-fold in terms of productivity. The performance of the Δ*GAS1* strain was compared to that obtained with two strains expressing ROL under the classical *PAOX1* promoter (Cos et al., 2005) (table 1). The best $Y_{P/X}$, productivity and specific productivity levels were achieved with the Δ*GAS1* strain, strongly suggesting that the cell wall poses a critical bottleneck on ROL secretion.

Table 1: Comparison of different strains of P. pastoris expressing ROL. PAOX Muts: ROL under control of the AOX1 promoter, Muts phenotype. PAOX Mut+: ROL under the AOX1 promoter, Mut+ phenotype. PFLD1: untransformed GAS1 positive strain expressing ROL under the FLD1 promoter. PFLD-ΔGAS1: strain deleted in GAS1, experssing ROL under the FLD1 promoter. **AU max**: maximal concentration of ROL achieved. **$Y_{P/X}$**: maximal specific activity of ROL per biomass. **μ mean**: average specific growth rate. **Productivity**: ROL activity per volume and per time. **Specific productivity**: ROL activity per biomass and per time. X: dry cell weight ($g \cdot L^{-1}$), μ: specific growth rate (h^{-1}), UA: units of activity.

	AU max ($AU\ mL^{-1}$)	$Y_{P/X}$ ($AU\ gX^{-1}$)	μ mean (h^{-1})	Productivity ($AU\ L^{-1}\ h^{-1}$)	Specific productivity ($AU\ gX^{-1}\ h^{-1}$)
P*AOX1* [a] Mut^s	205	5775	0.005	2246	63
P*AOX1* [a] Mut^+	150	2470	0.036	3000	49
P*FLD1* [b] $\mu = 0.02\ h^{-1}$	385	7634	0.020	4379	87
P*FLD1-ΔGAS1* $\mu = 0.01\ h^{-1}$	500	16033	0.010	5561	178

[a] Data taken from Cos et al. (2005)
[b] Data taken from chapter 4.

7.4. Conclusions

The cloning and knock out of the *GAS1* gene of *P. pastoris* has been carried out in order to test whether the deficiency in Gas1p has a phenotypic effect on protein secretion or not. Gas1p is thought to perform cross links between β1,3-glucans in the cell wall and therefore its inactivation would result in an increase of the cell wall porosity and permeability allowing for a higher release of the recombinant products. The major conclusions of this study are summarized as follows:

- The isolated and cloned *GAS1* homologue of *P. pastoris* was successfully truncated in the X-33 strain expressing ROL under the transcriptional control of the *FLD1* promoter. In the Δ*GAS*1 strain, although growing under carbon and nitrogen excess conditions, specific growth rate experienced a decrease of the 50% compared to the wild type strain when growing under equal culture conditions.
- The effect of the *GAS1* disruption seems to produce a severe negative effect on ROL expression and cell growth in the GS115H/HAC1 strain. The high number of genes controlled under the transcription factor *HAC1* should be taken into account when introducing additional modifications into the *P. pastoris* genome.
- Overall, the deletion of *GAS1* in the *P. pastoris* X-33 strain expressing ROL showed an improvement of specific productivity in 2-fold compared with the *GAS1* non-disrupted strain. These results suggest the presence of a bottleneck between the plasmatic membrane and the cell wall, as previously described in the yeast *S. cerevisiae*.

7.5. References

Cos O, Serrano A, Montesinos JL, Ferrer P, Cregg JM and Valero F (2005) Combined effect of the methanol utilization (Mut) phenotype and gene dosage on recombinant protein production in *Pichia pastoris* fed-batch cultures. Journal of Biotechnology 116: 321-35.

Popolo L, Vai M, Gatti E, Porello S, Bonfante P, Balestrini R and Alberghina L (1993) Physiological analysis of mutants indicates involvement of the *Saccharomyces cerevisiae* Gpi-anchored protein Gp115 in morphogenesis and cell-separation. Journal of Bacteriology 175: 1879-1885.

Popolo L, Gilardelli D, Bonfante P and Vai M (1997) Increase in chitin as an essential response to defects in assembly of cell wall polymers in the ggp1 Delta mutant of *Saccharomyces cerevisiae*. Journal of Bacteriology 179: 463-469.

Popolo L and Vai M (1999) The Gas1 glycoprotein, a putative wall polymer cross-linker. Biochimica et Biophysica Acta-General Subjects 1426: 385-400.

Ram AFJ, Kapteyn JC, Montijn RC, Caro LHP, Douwes JE, Baginsky W, Mazur P, Van den Ende H and Klis FM (1998) Loss of the plasma membrane-bound protein Gas1p in *Saccharomyces cerevisiae* results in the release of beta 1,3-glucan into the medium and induces a compensation mechanism to ensure cell wall integrity. Journal of Bacteriology 180: 1418-1424.

Vai M, Gatti E, Lacana E, Popolo L and Alberghina L (1991) Isolation and deduced aminoacid sequence of the gene encoding Gp115, a yeast glycophospholipid-anchored protein containing a serine-rich region. Journal of Biological Chemistry 266: 12242-12248.

Vai M, Brambilla L, Orlandi I, Rota N, Ranzi BM, Alberghina L and Porro D (2000) Improved secretion of native human insulin-like growth factor 1 from gas1 mutant *Saccharomyces cerevisiae* cells. Applied and Environmental Microbiology 66: 5477-5479.

Valdivieso MH, Ferrario L, Vai M, Duran A and Popolo L (2000) Chitin synthesis in a gas1 mutant of *Saccharomyces cerevisiae*. Journal of Bacteriology 182: 4752-4757.

Wach A, Brachat A, Pohlmann R and Philippsen P (1994) New heterologous modules for classical or pcr-based gene disruptions in *Saccharomyces cerevisiae*. Yeast 10: 1793-180.

CHAPTER 8

Cloning and expression of a GFP-ROL fusion protein in *P. pastoris*

8.1. Introduction

The *Aequorea victoria* green fluorescence protein (GFP) has attracted a huge interest as a molecular reporter. GFP has the advantage that its chromophore is formed in an autocatalytic cyclization that does not require a cofactor. Moreover, GFP usually maintains intact the properties of the proteins which is fused to. Many applications have been developed using this protein as a reporter of gene expression, protein localization or folding (reviewed in Zimmer et al., 2002). In heterologous protein production, the main interest has focused in the fusion of the GFP to proteins of interest as a reporter protein. In some cases, this strategy allows the on-line monitoring of the heterologous product with *in-situ* methods (Reischer et al., 2004; Jones et al. 2004; Hisiger et al., 2005). So far, only few examples of the extracellular expression of fusion GFP-proteins in *P. pastoris* have been reported using the *S. cerevisiae* α-mating factor (Cha et al., 2005; Passolunghi et al., 2003). Also, some positive results attempting to secret GFP in *P. pastoris* have been accomplished by using alternative secretion factors such as the viral secretion signal derived from the K28 virus preprotoxin (Eiden-Plach et al., 2004) or the *Phaseolus vulgaris* agglutinin secretion signal (Raemaekers et al., 1999).

The aim of this chapter is to obtain a fusion of *R. oryzae* lipase (ROL) with GFP as a tracer in ROL subcellular trafficking and to identify possible bottlenecks during protein secretion. Although in previous studies (Passolunghi et al., 2003) better secretion levels were obtained when fusing GFP to the N-terminus of the *Candida rugosa* lipase 1, we can not predict which fusion construct (ROL-GFP or GFP-ROL) will result in higher secretion levels. Hence, the GFP has been fused with ROL either at its N-terminus or at

its C-terminus. A GFP S65T mutant containing a threonine in position 65 instead of a serine (Waldo et al., 1999) was employed because this mutant has a higher brightness than the wild type protein (Zimmer et al., 2002). GFP S65T has a maximum absorbance peak at 489 nm and an emission peak at 509 nm.

This study was performed in collaboration with Anna Surribas (Dept Chemical Engineering, UAB), i.e. also contributing to her Ph.D. research project focused on the application of fluorimetric techniques for the on-line monitoring of *P. pastoris* physiological state during fermentation processes..

8.2. Materials and methods

8.2.1. Strains

Escherichia coli DH5α was used for plasmid construction and amplification. The wild-type phenotype *Pichia pastoris* X-33 strain (Invitrogen Co.) was used as a host strain for expressing a *Rhizopus oryzae* lipase gene (*ROL*) fused to the *Aequoria victoria* GFP S65T variant, under the transcriptional control of the *FLD1* promoter. The *P. pastoris* X-33/pPICZFLDα_ROL strain expressing and secreting the *ROL* gene under the *FLD* promoter (see chapter 3) was used for comparative cultivation experiments.

8.2.2. Media composition

E. coli strains were cultured in Luria broth medium supplemented with 50 μg mL^{-1} zeocin (Invitrogen Co.) as required. *P. pastoris* strains were cultured in YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) supplemented with 100 $\mu g \cdot mL^{-1}$ of zeocin when required. Shake flask cultures were performed using a minimal medium BMS (buffered minimal sorbitol) containing 1% (w/v) sorbitol, 1.34 % YNB without aminoacids and ammonium sulphate, 0.4 % (w/v) methylamine hydrochloride, $4 \cdot 10^{-5}$ % (w/v) biotin and 100 mM potassium phosphate pH 6.0. Bioreactor batch cultivations were carried out using a mineral medium (d'Anjou et al., 2001) with the following composition: KH_2PO_4 4.8 $g \cdot L^{-1}$, $MgSO_4 \cdot 7H_2O$ 1.88 $g \cdot L^{-1}$, $CaCl_2 \cdot 2H_2O$ 0.144 $g \cdot L^{-1}$, sorbitol 20 $g \cdot L^{-1}$, methylamine chloride 6 $g \cdot L^{-1}$, 0.1 $mL \cdot L^{-1}$ of antifoam Mazu DF 7960, 1 $mL \cdot L^{-1}$ of a biotin solution (400 $mg \cdot L^{-1}$), and 1 $mL \cdot L^{-1}$ of trace salts solution (0.2 mM $CuSO_4 \cdot 5H_2O$, 1.25 mM KI, 4.5 mM $MnSO_4 \cdot 4H_2O$, 2 mM $Na_2MoO_4 \cdot 2H_2O$, 0.75 mM H_3BO_3, 17.5 mM $ZnSO_4 \cdot 7H_2O$, 44.5 mM $FeCl_3 \cdot 6H_2O$). The

biotin and trace salts components were sterilised separately by micro filtration. The medium used for starter cultures was YPD medium.

8.2.3. Shake flask and bioreactor cultivation conditions

Small scale cultures were carried out at a working volume of 20 mL in 100 mL baffled shake flasks, in an orbital shaker (Infors) at 30ºC and 200 rpm for 48 h. Cultures were carried out in duplicate. A starter culture of 50 mL was cultivated in 500 mL baffled shake flasks at 200 rpm, 30ºC for 24h. Cells were harvested by centrifugation and resuspended in sterile water prior to the inoculation of the shake flasks to the desired initial optical density.

Bioreactor batch cultivations were carried out at a working volume of 1 L in a 2 L bench-top bioreactor (BioStat B, Braun) at 30ºC, for 72 h. The pH of the culture was maintained at 5.5 by automatic addition of 5 M KOH. The airflow was maintained at 2 L min^{-1}, assuring a minimum of 30% dissolved oxygen along the cultivation. The agitation speed was set to 500 rpm. The exhaust gas of the bioreactor was cooled in a condenser at 2-4°C (*Frigomix R*, B. Braun Biotech Int). Starter cultures (100 mL) were grown in 1 L baffled shake flasks at 200 rpm, 30ºC for 24h. Cells were harvested by centrifugation and resuspended in fresh medium prior to the inoculation of the bioreactor. At 75 hours of culture 15 g of sorbitol plus 4.5 g of methylamine were added into the bioreactor to maintain the culture growth.

8.2.4. Plasmid and strain construction

Two constructions coding for the fusion protein were performed, one coding for GFP protein fused to the ROL N-terminal end and one fused to the ROL C-terminal, as shown in figure 1. The constructions ROL-GFP and GFP-ROL were obtained by SOE-PCR (Horton et al., 1989). The primers used for each reaction are summarized in table 1.

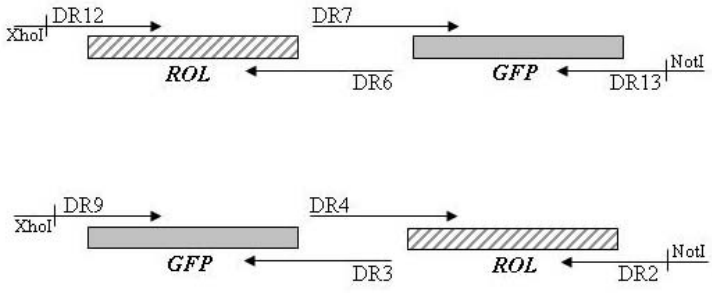


Figure 1. Schematic representation of the construction of the GFP-ROL and ROL-GFP fusions.

The construction involved two steps: first, the two genes were separately amplified introducing a 12 aminoacid linker between the two sequences (Waldo et al., 1999; Passolunghi et al., 2003). The GFP gene was amplified from the plasmid pPICZ-GFP, kindly provided by Stefania Brocca from the Dipartamento di Biotecnologie e Bioscienze, Università degli studi di Milano-Bicocca. For the GFP-ROL fusion, the forward primer DR9 and the reverse primer DR3 were used for amplification. Primer DR9 introduced a *Xho*I restriction site at the 5' of the sequence for further cloning of the construct into the expression plasmid. Primer DR3 introduced a linker sequence codifying for 12 aminoacids (marked in italics in the primer table). For ROL amplification, the plasmid pPICZFLDα_ROL was used as template. Primers DR4 and DR2 were used for PCR, primer DR4 contained the linker sequence (in italics) and primer DR2 a restriction site for *Not*I.

Name	*Sequence*	*Tm ºC*
DR2	gtagagcggccgccaaacagcttccttcgttgatatcaaagtaactca	55.8
DR3	*aaattcaccagaaccagcagaaccagcagaacc*tttgtatagttcatccatgccatgtgtaatccc	55.8
DR4	*ccaagacgaccaagacgaccaagaccacttaaa*tctgatggtggtaaggttgttgctgctactactg	59.3
DR6	*aaattcaccagaaccagcagaaccagcagaacc*caaacagcttccttcgttgatatca	56.5
DR7	*ggttctgctggttctgctggttctggtgaattt*agtaaaggagaagaacttttcactggagt	53.0
DR9	ctctcgagaaaagagaggctgaagctgaattcatgagtaaaggagaagaacttttca	55.5
DR10	gtatctctcgagaaaagagaggctgaagctagtaaaggagaagaactt	55.5
DR11	gcggccgcttattacaaacagcttccttcgttgatatca	56.5
DR12	gtatctctcgagaaaagagaggctgaagcttctgatggtggtaaggtt	56.5
DR13	gcggccgcttattatttgtatagttcatccatgccatgtg	56.8
DR14	gtatctctcgagaaaagagaggctgaagct	61.3
DR15	gcggccgcttattatttgtatagttcatc	61.2

Table 1. Primer employed in the fusion of GFP and ROL genes.

For ROL-GFP fusion, first DR12 and DR6 were utilized for ROL amplification. DR12 contained a *Xho*I restriction sequence and DR6 introduced a linker sequence identical to the used for GFP-ROL fusion, showed in italics (table 1). GFP was also amplified using the primers DR7 and DR13. DR7 included the linker sequence and DR13 introduced a restriction site for the *Not*I enzyme.

In a second step, PCR products containing the GFP and ROL genes were fused using the complementary linker regions as PCR primers.

In the third step, primers DR14 and DR15 were added into the reaction tube to amplify the product of the SOE-PCR for the ROL-GFP fusion, DR10 and DR11 were utilized in GFP-ROL reaction.

The SOE-PCR conditions for construction of the GFP and ROL fusions are detailed in table 2.

ROL-GFP		
1st reaction		
98 ºC	10 minutes	
98 ºC	15 seconds	
51 ºC	30 seconds	30 cycles
72 ºC	20 seconds	
72º C	5 minutes	
2nd reaction		
98 ºC	10 minutes	
98 ºC	30 seconds	10 cycles
51 ºC	30 seconds	
72 ºC	30 seconds	
72º C	5 minutes	
3rd reaction		
98 ºC	10 minutes	
98 ºC	30 seconds	20 cycles
56 ºC	30 seconds	
72 ºC	30 seconds	
72º C	5 minutes	
GFP-ROL		
1st reaction		
98 ºC	10 minutes	
98 ºC	15 seconds	
50 ºC	30 seconds	20 cycles
72 ºC	20 seconds	
72º C	5 minutes	
2nd reaction		
98 ºC	10 minutes	
98 ºC	30 seconds	10 cycles
51 ºC	30 seconds	
72 ºC	30 seconds	
72º C	5 minutes	
3rd reaction		
98 ºC	10 minutes	
98 ºC	30 seconds	20 cycles
56 ºC	30 seconds	
72 ºC	30 seconds	
72º C	5 minutes	

Table 2. SOE-PCR reaction conditions used for the fusion of the *GFP* and *ROL* genes.

For a 50 μL reaction, the following components were added into an ice-cold 0.5 mL PCR tube prior to reaction: 28.6 μL PCR grade water, 5 μL 10 X Reaction Buffer for KOD HiFi polymerase (Novagen), 5 μL dNTPs (final concentration 0.2 mM), 2 μL $MgCl_2$ (final concentration 1 mM), 1 μL template DNA, 4 μL forward primer (final concentration 0.4 μM), 4 μL reverse primer (final concentration 0.4 μM), 0.4 μL KOD HiFi DNA polymerase (2.5 $U \cdot \mu L^{-1}$).

The resulting fusion fragments, GFP-ROL and ROL-GFP, both consisting of 1613 base pairs, were cut with *XhoI* and *Not*I and ligated into *Xho*I- and *Not*I-digested pPICZFLDα backbone. The DNA ligation reaction was then transformed into *E. coli* and transformants were selected on LB plates containing zeocin. Plasmidic DNA was extracted from several colonies and sequenced. Once the correct constructions for each gene fusion were confirmed, the expression vector constructs were linearized and used for transformation of *P. pastoris* X-33 cells via electroporation, as described in (Cregg and Russell, 1998).

8.2.5. Fluorescence measurement

GFP fusion protein fluorescence in culture supernatant and cells samples were measured using a fluorimeter Perkin-Elmer LS55 equipped with a xenon lamp. Cells were centrifuged at 12000 rpm using a bench-top centrifuge and cells and supernatants were analysed independently. Fluorescence buffer (50 mM phosphate buffer, pH 7.0) was used to ensure the optimal GFP conditions for analysis (Patterson et al., 1997).

8.2.6. FITC staining for total protein

Cells were stained for measurement of total protein content by flow cytometry as follows: Prior to staining, samples from cultures were fixed using a 70% ethanol solution. Volumes of 200 to 500 μL of culture were withdrawn and centrifuged at 12000 rpm in a benchtop centrifuge. Supernatants were discarded and the pellets were resuspended in 1 mL of 70% (v/v) ethanol. Samples were stored at -20 ºC.

For FITC (fluorescein isothiocyanate) staining, fixed cells were firstly washed with 1mL of washing solution (100 mM Tris-HCl, pH 7.4, containing 0.1% Triton X-100 and 2 mM $MgCl_2$). Once washed, cells were resuspended in 200 μL FITC solution (FITC stock solution containing 4 mg · mL^{-1}, diluted 1:100 in wash solution) and incubated 30 minutes with shaking at room temperature and in the dark using a Thermomixer Comfort incubator (Eppendorf). After incubation, cells were washed with 1 mL wash solution and resuspended again in 500 μL of wash buffer.

8.2.7. Flow cytometry measurements

Previously fixed cells were analyzed on a FACS Calibur (Becton Dickinson) with a 488 nm Argon laser, and 10^4 cells were measured per analysis. GFP and FITC fluorescence was measured using a 530/30 nm BP filter (FL1). Fluorescence sample values were

normalized to the protein content by dividing the geometric means of the GFP fluorescence signals by the geometric mean of correspondent FITC signals. Alternately, GFP fluorescence was normalized by cell size using FSC signal, following the methodology described in chapter 5 (page 67).

8.2.8. Confocal microscopy

Samples for confocal microscopy were rapidly fixed using a solution of 4 % (v/v??) parafolmaldehyde (microscopy grade, Sigma) dissolved in PBS buffer.

Fixed cells were directly observed under a Leica TCS SP2 AOBS confocal microscope (Leica, Heidelberg, Germany) with a resolution of 0.2 μm with an oil immersion objective of 65X. GFP was excited with an Argon laser at 488 nm and the fluorescence acquired through an LP 510-590 nm filter.

8.2.9 Western blot analyses

Western blots were carried out according to the protocol detailed in chapter 2 (page 22). For ROL detection, a mouse anti-ROL antiserum was used (raised at the Institute of Applied Microbiology, BOKU – University of Natural Resources and Applied Life Sciences, Vienna, Austria) with a dilution 1:100. For GFP detection, a mouse anti-GFP (Sigma) was used with a dilution 1:500. Antimouse IgG horseradish peroxidase conjugate (Sigma) was used as a secondary antibody diluted 1:1000 in both cases.
Prior to western blot, samples from shake flask cultures were concentrated 10-fold using the Amicon Ultra-15 centrifugal filters units (Millipore).

8.3. Results

8.3.1. Constructions and isolation of transformants expressing GFP/ROL fusions.

Two fusions of the *GFP* and *ROL* genes, one with the *GFP* gene fused at the 5' end and one with the *GFP* gene fused to the 3' end of the *ROL* gene were carried out by SOE-PCR and inserted into the pPICZFLDα expression vector. *P. pastoris* transformants from GFP-ROL and ROL-GFP constructions were selected on YPD plates containing zeocin. For each construction several clones were selected and re-inoculated on fresh

selective plates for three successive passages to ensure the isolation of pure transformant colonies. After this isolation step, tranformants were inoculated into shake flasks containing 50 mL of BMS medium to test their fusion product expression levels. The *P. pastoris* X-33/pPICZFLDα_ROL strain expressing *ROL* gene under the *FLD1* promoter was used as a reference to compare expression levels. In figure 2, the specific lipase activity at 48 hours of culture for the different assayed clones is shown.

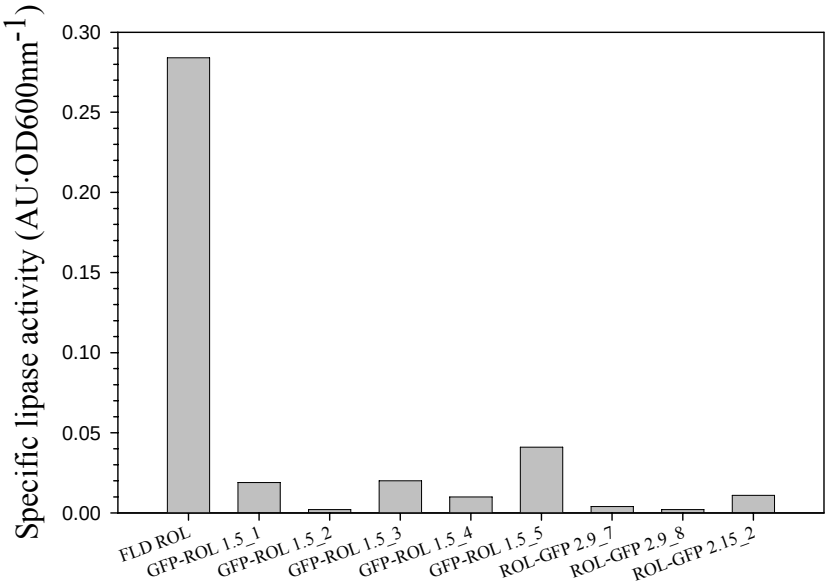


Figure 2. Specific lipase activity expressed as the amount of lipase activity per unit of optical density measured at 600 nm. FDLROL culture corresponds to the X33 strain transformed with pPICZαFLDROL vector.

Clones containing the ROL-GFP fusion showed lower expression levels than the transformants with GFP-ROL constructions. Moreover, in all tested clones, expression levels of the fusion protein, measured as units of lipase activity per millilitre of culture supernatant and normalized by biomass concentration were almost ten times lower than in the cultivation with the control strain expressing ROL. Thus, it is apparent that the fusion of GFP to ROL (or vice versa) results in a clear decrease in its expression levels.

8.3.2. GFP-ROL expression analysis in shake flask cultivations

Clones GFP-ROL 1.5.3, GFP-ROL 1.5.5 and ROL-GFP 2.15.2 were selected for further expression experiments in shake flasks. Cultivations were performed under the same

conditions utilized above. Figure 3 shows the results from these experiments; as observed in the preliminary cultivations for clone screening (see section 8.3.1), the strain expressing the ROL-GFP fusion achieved very low production levels. The GFP-ROL 1.5.3 and 1.5.5 transformants showed similar specific lipase activity. The ROL-GFP 2.15.2 transformant was discarded for subsequent experiments. The GFP-ROL 1.5.5 strain was selected for further experiments.

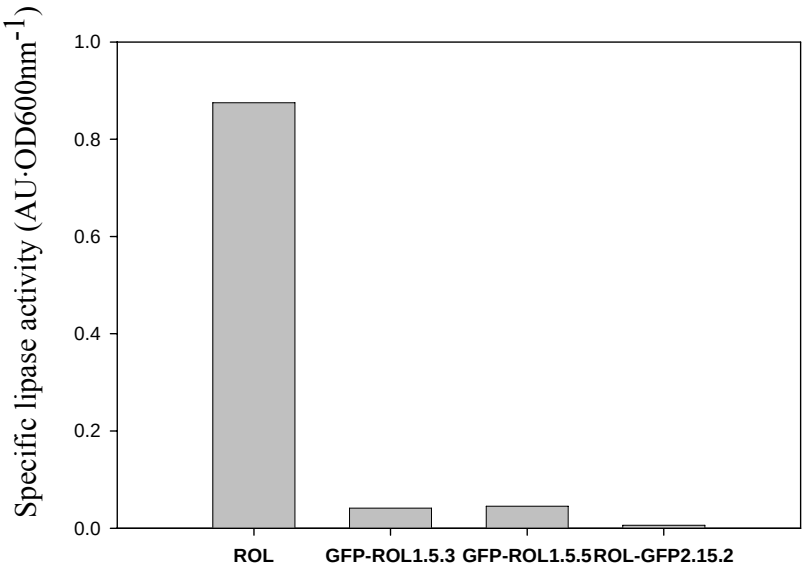


Figure 3. Specific lipase activity after 48 hours of cultivation expressed as the amount of lipase activity per unit of optical density measured at 600 nm. FDLROL culture corresponds to the X-33 strain transformed with pPICZFLDα_ROL vector

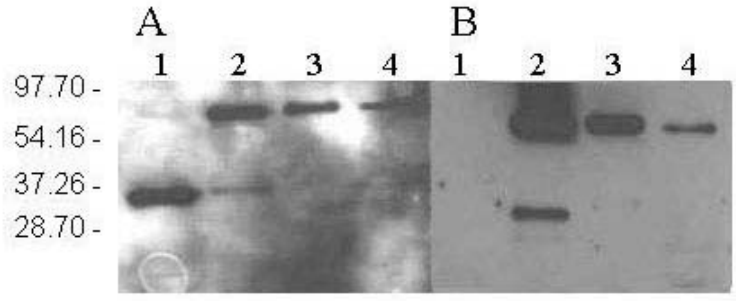


Figure 4. Western blots using anti-ROL antibodies (A) and anti-GFP antibodies (B). Lane 1: ROL positive control, lane 2: culture supernatant from clone GFP-ROL 1.5.5; lane 3: culture supernatant from clone ROL-GFP 2.15.2; lane 4: culture supernatant from bioreactor culture of clone GFP-ROL 1.5.5.

Also, western blots from supernatant samples of the shake flask experiment at 66 hours of culture were done to confirm product quality and the possible product proteolytic degradation (figure 4).

Western blot of samples from both GFP-ROL and ROL-GFP cultures supernatants showed the presence of fusion protein of about 60 kDa. However, in both westerns, samples from the GFP-ROL 1.5.5 cultivation showed a second a band of approximately 30 kDa that probably corresponds to the proteolytic cleavage of the fusion product into its two components, GFP and ROL. In contrast, no degradation was observed in the clone ROL-GFP 2.15.2 samples.

8.3.3. GFP-ROL expression analysis in a bioreactor batch culture

In order to obtain better cultivation conditions of controlled pH, aeration, and agitation, a batch culture was carried out in a 2 litres bioreactor with the growth medium and under the conditions mentioned in materials and methods. The *P. pastoris* X-33-derived strain transformed with the expression vector pPICZFLDαGFP-ROL clone 1.5.5. was employed. A parallel control batch cultivation was performed in shake flask with the X33 + pPICZFLDαROL strain expressing ROL. The evolution of cell density, expressed as the optical density measured at 600 nm, sorbitol concentration and the lipase activity along the cultivation is depicted in figure 5. In contrast to the previously performed shake flask cultures, no proteolytic degradation was observed in a western blot analysis (figure 4, lane 4)

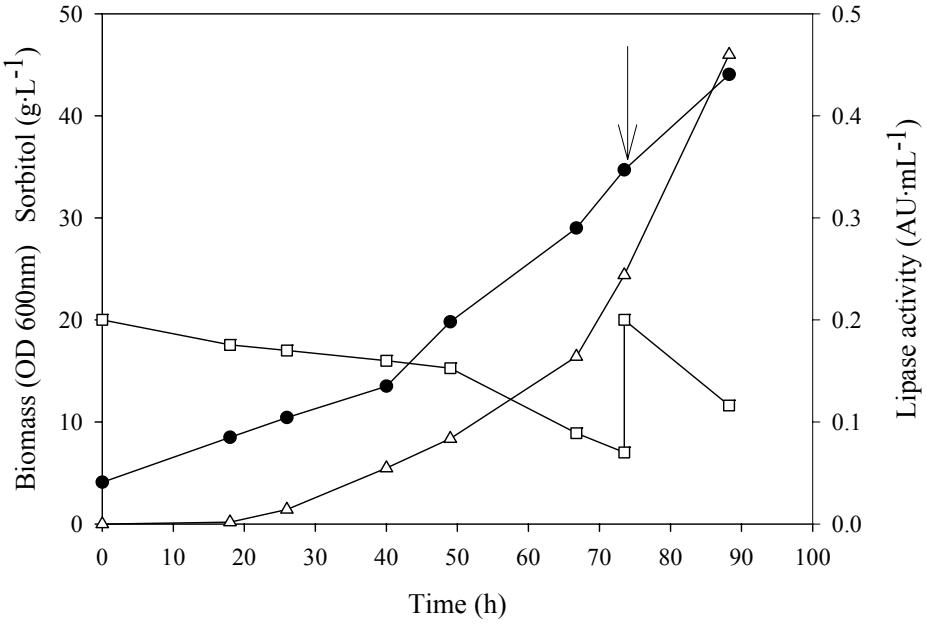


Figure 5. Bioreactor batch cultivation of *P. pastoris* expression the fusion protein GFP-ROL under the control of the *FLD1* promoter growing on sorbitol and methylamine. Biomass (●), sorbitol concentration(□) and lipase activity (Δ) are shown. Arrow indicates the addition of 15 $g \cdot L^{-1}$ of sorbitol.

8.3.3.1. Fluorimetric analyses

Several problems appeared when trying to perform direct fluorimetric measurements of culture samples. First, fluorescence scans at multiple emission wavelengths from culture supernatants and cell extracts were performed (figure 6). Cells samples from culture expressing GFP-ROL containing cells resuspended in fluorescence buffer showed a peak at the emission wavelength corresponding to GFP S65T (with a maximum emission wavelength at approximately 509 nm), where cells from control culture did not show any significative fluorescence at this wavelength (figure 6A). Nevertheless, GFP signal in cells was not detected until approximately 70 hours of culture.

In figure 6B, the emission spectra for the corresponding culture supernatants excited at 480 nm is shown; a peak appeared at 520 nm in the control samples and samples from the batch expressing the fusion protein. This peak increased during fermentation time, both in culture expressing GFP-fusion protein and in the control culture. Previous works in our Group showed that ROL did not have any fluorescence signal at the tested emission and excitation wavelength; thus, ROL was not responsible for this

fluorescence. This suggests that a fluorescent growth-related product was present in the culture medium produced by *P. pastoris*, with a very similar excitation and emission spectra to the GFP S65T.

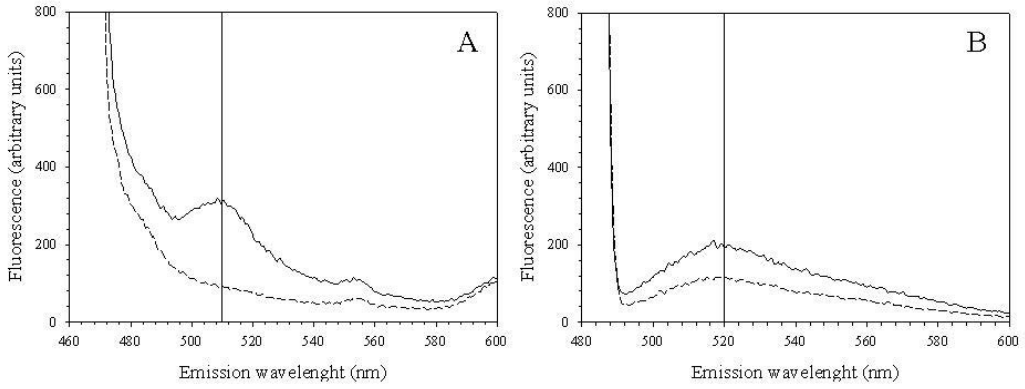


Figure 6. Emission spectrum of samples from batch culture containing cells (A) and supernatant (B). Dashed lines correspond to control culture samples and solid lines to batch culture expressing the GFP-ROL fusion. Vertical line indicates the maximum emission wavelenght.

In recent studies carried out by Surribas et al. (2006), the estimation of biomass, substrate and product during a *P. pastoris* production process, has been carried out by means of multi-wavelenght on-line fluorescence measurements. Riboflavin, with an excitation and emission spectra of 450/530, among other fluorophores, was used to predict biomass. Riboflavin (or vitamin B2) is a vitamin that works as a precursor for the synthesis of coenzymes like flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), needed as electron acceptors by oxidoreductases. Yeasts such as *Candida famata* are industrially-employed natural overproducers of riboflavin (more than 20 g/L) (Stahmann et al., 2000).

Hence, our results suggested that the riboflavin signal fluorescence could be overlapping with the GFP signal in culture supernatant samples. Surprisingly, no riboflavin signal was observed in cell samples. In order to remove riboflavin from culture supernatant samples, ultrafiltration units (Amicon Ultra-15 centrifugal filters, Millipore) with a 10 kDa cut-off membrane were utilized to separate the fusion protein from riboflavin. Ultrafiltrated and retained fractions were subsequently analysed. Samples of 15 mL of culture supernatant from the above mentioned batch cultivation

were ultrafiltered, and the final volume readjusted to 1.5 mL using fluorescence buffer. Figure 7A shows the retained fraction at 10 kDa, while figure 7B shows the filtrate fraction, presumably containing riboflavin.

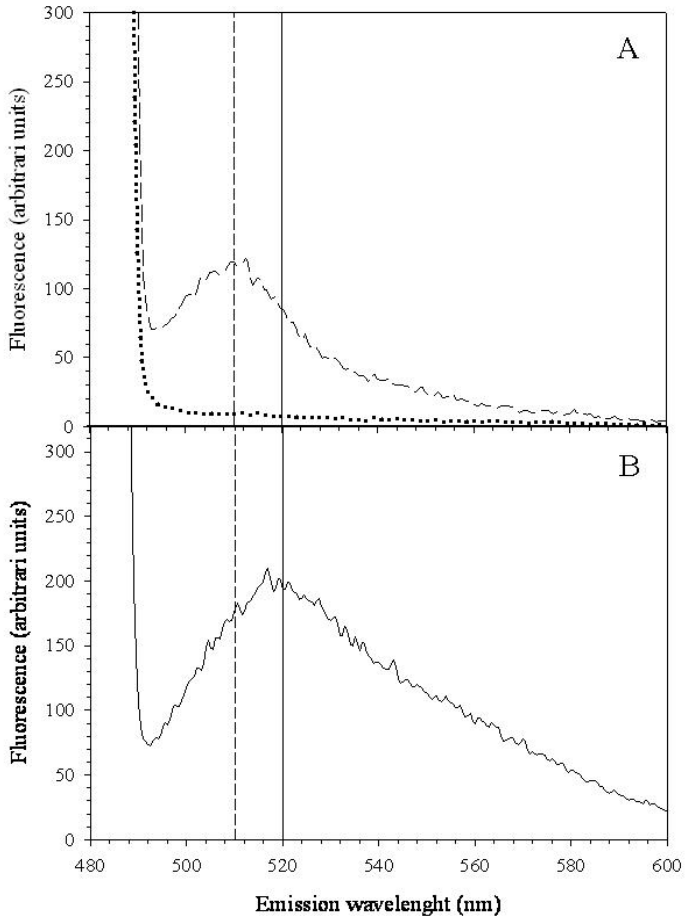


Figure 7. Emission spectrum, given as fluorescence arbitrary units, excited at 480 nm. Retained fraction of control culture samples (doted line) and GFP-fusion expressing samples (dashed line) (A), and filtrated fraction (B) from batch culture supernatant samples.

As expected, the filtrate fraction excited at 480 nm showed a peak at 520 nm, corresponding to riboflavin. Besides, in the retained fraction excited at 480 nm, a new peak appeared with a maximum at 510 nm, in concordance with the GFP emission spectrum. These results showed that the extracellular GFP fluorescence from batch samples could be efficiently measured after the ultrafiltration step.

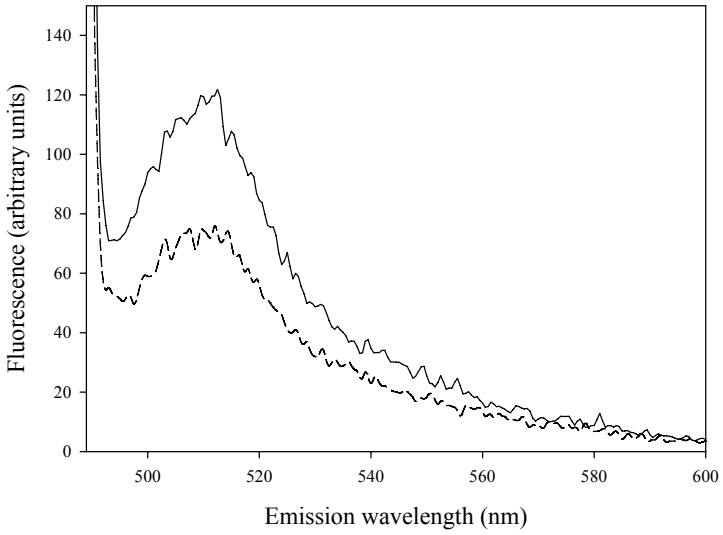


Figure 8. Emission spectra of retained fractions excited at 489 nm from ultrafiltred sample of batch culture. Dashed line corresponds to fraction resuspended in culture medium and solid line corresponds to fraction resuspended in fluorescence.

The effect of the culture medium on GFP fluorescence was tested by resuspending the retained fraction from batch cultivation supernatant samples in two different media: the same culture medium used for fermentation or in fluorescence buffer. The emission spectra of both samples revealed that GFP fluorescent activity is unstable (or undergoes an inactivation) in the culture medium (figure 8).

On the other hand, a fine correlation (over the tested range) was obtained between the measured extracellular lipase activity and the GFP fluorescence from the retained fraction of ultrafiltrated samples. This correlation is shown in figure 9.

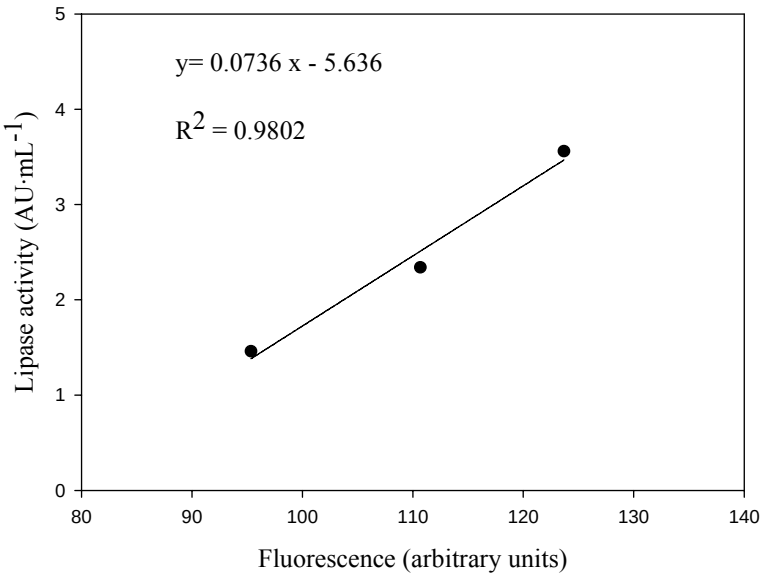


Figure 9. Correlation between the extracellular lipase activity and the GFP fluorescence of batch samples at 66, 73.5 and 88.5 hours of cultivation respectively.

8.3.3.2. GFP-ROL intracellular activity analyses

In order to investigate whether the low expression levels were directly related to an intracellular accumulation of the fusion protein, the following series of analyses were carried out. First, samples from the batch culture were mechanically disrupted using a Constant Systems cell disrupter. Lipase activity in cleared cell lysates was normalized by the optical density after disruption.

Intracellular lipase and GFP fluorescence normalized by cell density (i.e. the specific lipase activity and GFP fluorescence) showed a constant level throughout the whole cultivation, as depicted in figure 10, that is, the intracellular recombinant fusion protein levels were essentially constant along the cultivation time

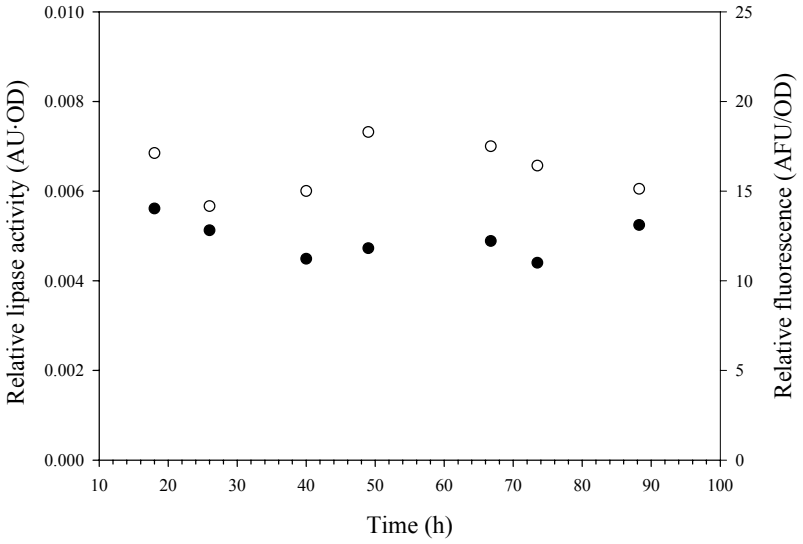


Figure 10. Relative lipase activity (○) and relative GFP fluorescence (●) from cleared lysates of disrupted samples from batch culture.

Fluorescence from the soluble fraction of cell extracts was also related to lipase activity. As shown in figure 11, the correspondence between lipase activity and fluorescence emission at 510 nm is linearly correlated, with a linear regression close to 1.0..

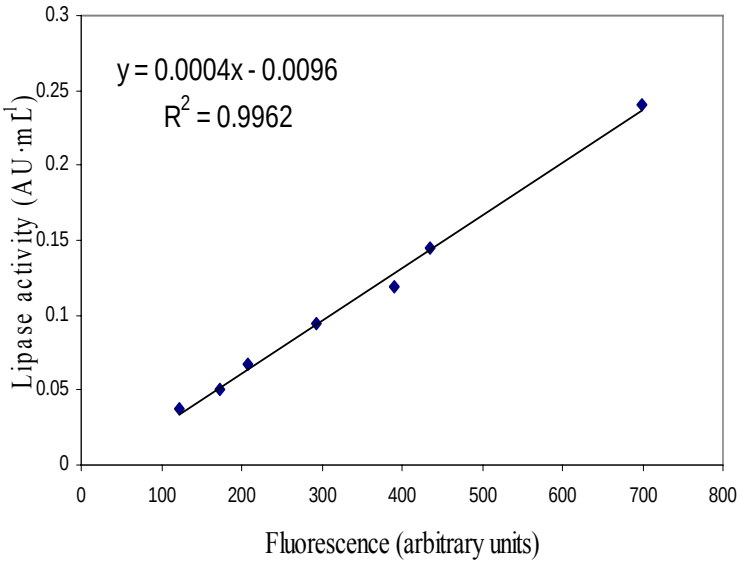


Figure 11. Correlation between the measured lipase activity and the relative fluorescence of cleared lysates.

When comparing the relationship between intracellular fluorescence and lipase activity (figure 11) and the extracellular fluorescence and lipase activity (figure 9) a significant difference is observed: the correspondence between absolute intracellular lipase activity values and absolute fluorescence values is different to that found for extracellular lipase activity values. These differences might respond either to an inactivation of the GFP fluorescence caused by an extracellular growth medium component (as suggested above, figure 8) or, to an inactivation of the intracellular lipase due to the presence of endogenous proteases in the cleared lysates.

8.3.3.3. Flow cytometry analyses

Parallel flow cytometry analyses were carried out in order to follow the intracellular fusion product content along the batch cultivation run by measuring intracellular GFP fluorescence. Cells were stained with FITC to normalize the cells' GFP fluorescence by their protein content.

Notably, the observed increase in GFP fluorescence along batch cultivation time was concominant with an increase in total protein cellular content in the GFP-ROL-expressing cells. This important increase in the protein content was not observed in the control culture and might be provoked by the intracellular accumulation of the fusion protein. Thus, normalisation of the cells' GFP fluorescence by their protein content does not allow to effectively monitor GFP intracellular accumulation. Instead, we decided to use, FSC (see chapter 5) to normalize the GFP fluorescence by cell size in flow cytometry measurements. The evolution of the GFP fluorescence normalized by cell size is illustrated in figure 12. GFP signal increased all along the fermentation time, in contrast with the previous observed results from intracellular disrupted samples, while in cells from control samples, fluorescence remained constant at a low level.

These observations may indicate that part of the fusion protein is retained in the insoluble fraction of cell lysates, thus not detectable in the analysed cleared lysates.

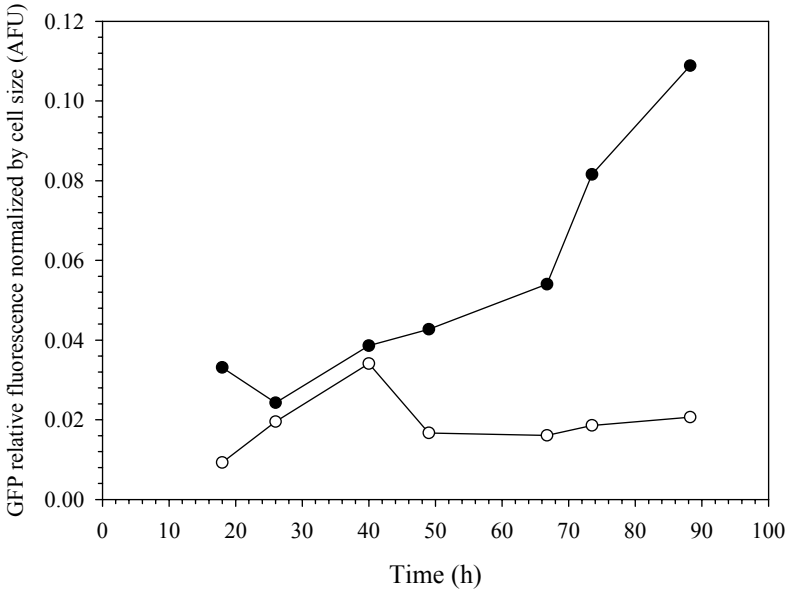


Figure 12. Relative fluorescence from GFP expressing cells during batch culture (●) and from control culture (○). AFU: arbitrary fluorescence units.

8.3.3.4. *In situ* analysis of product subcellular localization by confocal microscopy

Confocal microscopy techniques were employed to attempt to determine the subcellular localization of the fusion protein during its secretion process and the possible accumulation within an a subcellular compartment.

Figure 13 shows confocal microscopy images obtained from cells samples from the batch cultivation. Two different types of cells were detected during microscopic observation: A few of the visualized cells showed peripheral fluorescence (figure 13C), while most of the cells showed an almost homogeneous intracellular accumulation (or in big subcellular compartments), as shown in figure 13A and B.

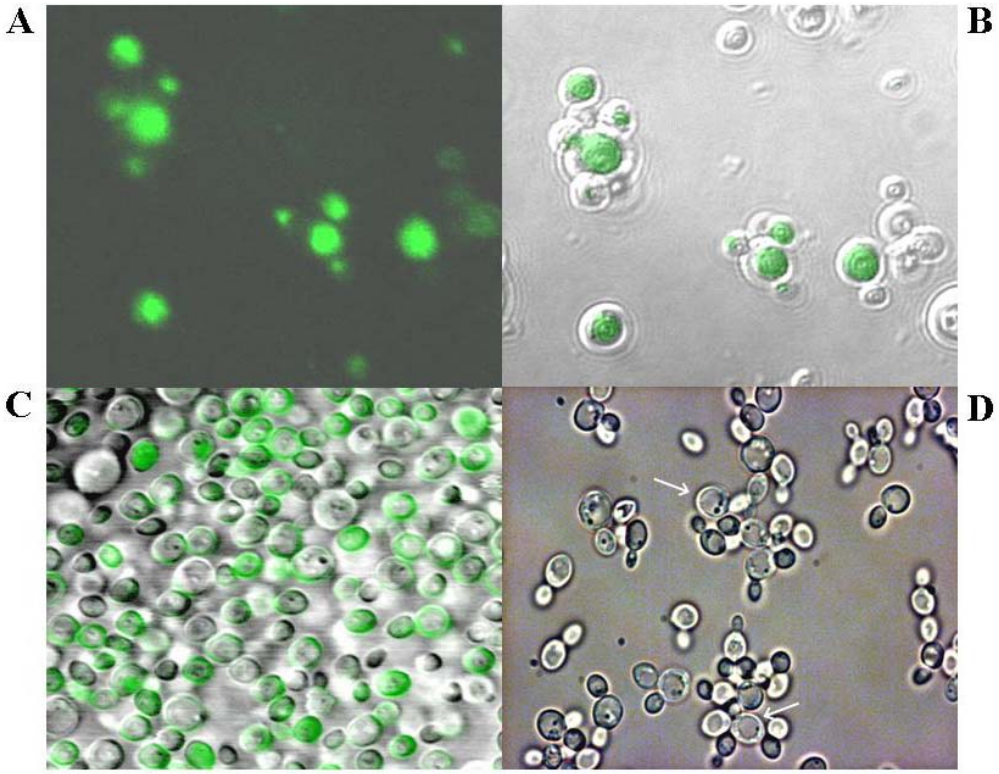


Figure 13. Images of *P. pastoris* cells expressing the fusion protein GFP-ROL in bioreactor batch cultivation. Fluorescence confocal microscopy (A) showing high levels of intracellular GFP-fusion protein localized in large compartments. (B) Overlay of confocal microscopy image with transmission optical microscope. (C) Overlay of confocal image with transmission microscope showing cells with peripheral fluorescence and other fluorescence in subcellular compartiments. (D) Transmission optical microscope image showing cells containing vacuoles which fills the main cell volume (arrows indicate vacuoles examples).

This presence of intracellular fluorescence into large compartment might reflect the cellular inability to efficiently secret the recombinant product. Some authors have pointed out the possible targeting of secreted GFP-fusion proteins to the yeast vacuole (Kunze et al., 1999; Li et al., 2002). The yeast vacuole has many functions, including the degradation of proteins by vacuolar resident proteases (Thumm et al., 2000). Studies by Kunze et al (1999) in *S. cerevisiae* suggested the existence of an unknown vacuolar signal in GFP that may lead this protein to the vacuole. However, when attempting to secrete GFP-fusion proteins, Li et al. (2002) found that such proteins were localized into different subcellular compartments such as ER, Golgi as well as vacuoles.

Proteins leaving the ER are packaged into COPII vesicles and targeted to the Golgi apparatus. Besides performing several post-translational modifications such as glycosilation, the Golgi apparatus functions as a final quality check point. Proteins targeted to the vacuole or those that are not adequate for secretion are directed to the vacuolar compartment (Graham et al., 1991). Even properly folded secreted proteins, which have gone trough the ER without activating the UPR might also be directed to vacuoles for further degradation (Rakestraw et al., 2006). The hypothesis that GFP-ROL fusion might be localized/targeted into vacuoles is consistent with the observation that daughter cells also contain fluorescence particles (figure 13A and B). It has been postulated that yeast mother cells donate approximately 50 % of its vacuolar content to the growing buds (Bryant et al. 1998).

In our analyses, we found low extracellular GFP-ROL fusion protein levels as well as an intracellular (mainly vacuolar) accumulation probably caused by the secretory apparatus, which can not cope with the fusion protein processing, consequently driving the major part of the secretory vesicles into vacuoles, either as a result of the overload of the secretion pathway or due to the recognition of a vacuolar targeting signal.

8.4. Conclusions

In this chapter, the cloning and expression of a GFP-ROL fusion protein has been achieved. A series of shake flask and batch bioreactor cultivations has been performed. The analyses of the intracellular and extracellular fluorescence and lipase activity have proved that the secretion of the soluble active GFP-fusion in *P. pastoris* is a complex task for this organism. The main conclusions extracted from this study are:

- An obvious decrease in extracellular expression levels is produced when expression ROL fused to GFP. N-terminal fusion achieved higher expression levels than the C-terminal construct; however, in both cases extracellular expression levels were significantly lower than in the control strain producing only ROL.
- The extracellular GFP fluorescence signal was overlapped by riboflavin presence in the culture medium. Riboflavin was synthesized in large quantities, masking the GFP emission signal. However, intracellular GFP signal was clearly detected either by flow cytometry and fluorimetry.

- The GFP-fusion protein was suitable for ROL subcellular localization studies. An intracellular accumulation in the cell wall/periplasm and into large subcellular, vacuolar like compartments, was observed. Limitations in secretion or vacuolar targeting recognition of the fusion protein are thought to lead the recombinant protein to degradation into the vacuolar compartments.
- The use of the GFP variant used in this work for extracellular recombinant protein expression monitoring in *P. pastoris* should be reconsidered. Other variants such as red shifted mutants or those with excitation wavelengths around 395nm, such as GFPuv, might be a better option to avoid possible interferences with riboflaving and other cellular components/products.

8.5. References

Bryant NJ and Stevens TH (1998) Vacuole biogenesis in *Saccharomyces cerevisiae*: Protein transport pathways to the yeast vacuole. Microbiology and Molecular Biology Reviews 62: 230-+.

Cha HJ, Shin HS, Lim HJ, Cho HS, Dalal NN, Pham MQ and Bentley WE (2005) Comparative production of human interleukin-2 fused with green fluorescent protein in several recombinant expression systems. Biochemical Engineering Journal 24: 225-233.

Cregg JM and Russell KA (1989) Transformation. In: Higgins DR and Cregg JM (eds) *Pichia* protocols.

d'Anjou MC and Daugulis AJ (2001) A rational approach to improving productivity in recombinant *Pichia pastoris* fermentation. Biotechnology and Bioengineering 72: 1-11.

Eiden-Plach A, Zagorc T, Heintel T, Carius Y, Breinig F and Schmitt MJ (2004) Viral preprotoxin signal sequence allows efficient secretion of green fluorescent protein by *Candida glabrata*, *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*. Applied and Environmental Microbiology 70: 961-966.

Graham TR and Emr SD (1991) Compartmental organization of golgi-specific protein modification and vacuolar protein sorting events defined in a yeast Sec18 (Nsf) mutant. Journal of Cell Biology 114: 207-218.

Hisiger S and Jolicoeur M (2005) A multiwavelength fluorescence probe: Is one probe capable for on-line monitoring of recombinant protein production and biomass activity? Journal of Biotechnology 117: 325-336.

Jones JJ, Bridges AM, Fosberry AP, Gardner S, Lowers RR, Newby RR, James PJ, Hall RM and Jenkins O (2004) Potential of real-time measurement of GFP-fusion proteins. Journal of Biotechnology 109: 201-211.

Kunze I, Hensel G, Adler K, Bernard J, Neubohn B, Nilsson C, Stoltenburg R, Kohlwein SD and Kunze G (1999) The green fluorescent protein targets secretory proteins to the yeast vacuole. Biochimica et Biophysica Acta-Bioenergetics 1410: 287-298.

Li JC, Xu HX, Bentley WE and Rao G (2002) Impediments to secretion of green fluorescent protein and its fusion from *Saccharomyces cerevisiae*. Biotechnology Progress 18: 831-838.

Passolunghi S, Brocca S, Cannizzaro L, Porro D and Lotti M (2003) Monitoring the transport of recombinant *Candida rugosa* lipase by a green fluorescent protein-lipase fusion. Biotechnology Letters 25: 1945-1948.

Patterson GH, Knobel SM, Sharif WD, Kain SR and Piston DW (1997) Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. Biophysical Journal 73: 2782-2790.

Raemaekers RJM, de Muro L, Gatehouse JA and Fordham-Skelton AP (1999) Functional phytohemagglutinin (PHA) and *Galanthus nivalis* agglutinin (GNA) expressed in *Pichia pastoris* - Correct N-terminal processing and secretion of heterologous proteins expressed using the PHA-E signal peptide. European Journal of Biochemistry 265: 394-403.

Rakestraw A and Wittrup KD (2006) Contrasting secretory processing of simultaneously expressed heterologous proteins in *Saccharomyces cerevisiae*. Biotechnology and Bioengineering 93: 896-905.

Reischer H, Schotola I, Striedner G, Potschacher F and Bayer K (2004) Evaluation of the GFP signal and its aptitude for novel on-line monitoring strategies of recombinant fermentation processes. Journal of Biotechnology 108: 115-125.

Stahmann KP, Revuelta JL and Seulberger H (2000) Three biotechnical processes using *Ashbya gossypii*, *Candida famata*, or *Bacillus subtilis* compete with chemical riboflavin production. Applied Microbiology and Biotechnology 53: 509-516.

Surribas A, Geissler D, Gierse A, Scheper T, Hitzmann B, Montesinos J and Valero F (2006) State variables monitoring by in situ multi-wavelenght fluorescence

spectroscopy in heterologous protein production by *Pichia pastoris*. Journal of Biotechnology In Press.

Thumm M (2000) Structure and function of the yeast vacuole and its role in autophagy. Microscopy Research and Technique 51: 563-572.

Waldo GS, Standish BM, Berendzen J and Terwilliger TC (1999) Rapid protein-folding assay using green fluorescent protein. Nature Biotechnology 17: 691-695.

Zimmer M (2002) Green fluorescent protein (GFP): Applications, structure, and related photophysical behavior. Chemical Reviews 102: 759-781.

CONCLUSIONS

The global conclusions extracted from this study are here highlighted:

The development of a novel (methanol-free) fed-batch cultivation strategy for recombinant protein production in *P. pastoris* based on the use of the *FLD1* promoter has been performed. Methylamine as nitrogen source and sorbitol as sole carbon source have shown to be a suitable alternative to the use of the methanol-inducible *AOX1* promoter. The use of this promoter combined with our fermentation strategy has shown to be at least as efficient as the *AOX1* promoter-based strategy for ROL production. Moreover, the control requirements of the here tested for fed-batch cultivation strategy are lower than in the methanol-based cultivation strategies.

The analysis of cell's physiological response to ROL under fed-batch process conditions has allowed implementing metabolic engineering strategies for improved ROL production. In particular, a series of strains, all expressing ROL under the control of the *FLD1* promoter have been modified to overcome the possible limitations of the *P. pastoris* expression system. The *P. pastoris* GS115H strain has been genetically modified to constitutively overexpress the UPR transcriptional factor *HAC1* of *S. cerevisiae*; such modification has provoked a 2-fold increase in the specific productivity compared to the non-modified strain. Besides, the deletion of the *GAS1* gene in *P. pastoris* X-33 resulted in the also in the 2-fold increase in specific productivity. However, the double mutant containing the *S. cerevisiae HAC1* gene and the deletion upon the *GAS1* gene was not able to grow or produce ROL.

Specific conclusions

- First, the ROL was cloned and successfully expressed in *P. pastoris* under the control of the *FLD1* promoter. Shake flask experiments combining different carbon and nitrogen sources pointed out the use of methylamine as sole nitrogen source for the induction of the *FLD1* promoter, combined with sorbitol as an alternative, non toxic and non-repressing carbon source. Bioreactor batch cultivations confirmed the preliminary results from shake flasks cultivations.

Moreover, although the *FLD1* promoter can be also induced by methanol as a sole carbon source, the results indicated that the *FLD1* promoter can not be independently induced by either an inducing carbon source or an inducing nitrogen source.

- Second, a fed-batch strategy based on the use of sorbitol as carbon source and methylamine as nitrogen source was developed. Three fed-batches were performed following this strategy at different specific growth rates, demonstrating that this parameter is critical on process productivity. In the fed-batch cultivation performed at a near-to-maximum growth rate ($0.02h^{-1}$), the productivity was 1.9-fold and 7.6 fold higher than in cultivations at $0.01h^{-1}$ and $0.05h^{-1}$, respectively.

- Third, the use of flow cytometry combined with immufluorescence-based techniques has proved to be a useful tool to monitor cell viability and intracellular proteins content. ROL overexpression under the control of the *FLD1* promoter did not have a negative effect on cell's viability, as previous studies had hypothesised. Moreover, the increase in the ER-resident chaperone BiP content during fermentation, which was concomitant with intracellular ROL accumulation, suggested that ROL overexpression probably triggered the UPR activation. Further, the results suggested that this observation might be related with the decrease in the product formation rate observed during the ROL production phase of fed-batch cultures.

- Based on the hypothesis that ROL overexpression triggers the UPR, a metabolic engineering approach has been carried out by constitutively co-expressing the *Saccharomyces cerevisiae HAC1* gene, resulting in a constitutive up regulation the UPR.. Overall, this strategy resulted in a 2-fold increase in the process specific productivity compared with the parental strain.

- Transcriptional analyses of the bioprocess-relevant genes *AOX1*, *FLD1*, *PDI*, *KAR2* and *ROL* using the bead-based sandwich hybridization assay has shown a clear relation of their overall transcriptional levels with respect to specific growth rate, observing a higher induction levels when growing at the highest

growth rate. The observed increase of *KAR2* and *PDI* mRNA levels during the induction phase of fed-batch cultivations provide additional evidence that ROL overexpression triggers the UPR in *P. pastoris*

- The deletion of the *P. pastoris GAS1* gene resulted in a 2-fold increase in the process specific productivity compared with the control strain. Enhancing the cell wall permeability and porosity and thus improving the protein secretion into the culture medium. Further, it proved that passage across the cell wall poses an additional bottleneck in extracellular ROL production.

- The fusion of ROL to GFP as protein localization marker entailed certain drawbacks. First, fusion of GFP to the ROL C-terminus resulted in undetectable levels of the fusion protein. Second, when expressing GFP-ROL, expression levels dropped 10-fold with respect to the control strain. Moreover, the extracellular GFP fluorescence signal was overlapped by a *P. pastoris* fluorescent product, probably riboflavin. In addition, the fusion product was observed to be intracellularly retained into vacuoles and the cell's periplasm and/or cell wall. GFPuv or other red shifted mutants were proposed as alternative autofluorescent proteins to overcome these difficulties.

List of publications

SURRIBAS A, **RESINA D**, FERRER P, VALERO F. Cloning and expression of a GFP-ROL fusion protein in P. pastoris under the control of the FLD1 promoter. 2006. En procés d'elaboració.

MARX H , SAUER M, **RESINA D**, VAI M, PORRO D, VALERO F, FERRER P, MATTANOVICH D. Cloning, deletion and protein secretory phenotype of the *GAS1* homologue of *Pichia pastoris.* 2006. Submitted to FEMS in Microbiology.

RESINA D, BOLLOK M, VALERO P, NEUBAUER P, FERRER P. mRNA quantification using sandwich hybridization assay of *Pichia pastoris* expressing the *Rhizopus oryzae* lipase. 2006. En procés d'elaboració.

RESINA D, COS O, GASSER B, MAUER M, VALERO F, MATTANOVICH D, FERRER P. Analysis and engineering of bottlenecks in *Rhizopus oryzae* lipase production in *Pichia pastoris* using the nitrogen source-regulated *FLD*1 promoter. Submitted to Biotechnology and Bioengineering.

RESINA D, COS O, FERRER P, VALERO F. Developing high cell density fed-batch cultivation strategies for heterologous protein production in *Pichia pastoris* using the nitrogen source-regulated FLD1 promoter. Biotechnol Bioeng. 2005 Sep 20;91(6):760-7.

COS O, **RESINA D**, FERRER P, MONTESINOS JL, VALERO. Heterologous production of Rhizopus oryzae lipase in *Pichia pastoris* using the alcohol oxidase and formaldehyde dehydrogenase promoters in batch and fed-batch cultures. Biochemical Engineering Journal. 2005 26:86–94.

RESINA D, SERRANO A, FERRER P, VALERO F: Expression of heterologous proteins in *Pichia pastoris* under the control of the nitrogen source-regulated regulated formaldehyde dehidrogenase promoter. J Biotechnol. 2004 Apr 8;109(1-2):103-13.

NOTES