COMMUNAUTÉ FRANÇAISE DE BELGIQUE

UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

Influence of heterogeneous fermentation conditions on the productive capacity of recombinant *Pichia pastoris*

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Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique et ingénierie biologique et grade de Docteur en Sciences de l'Ingénieur, mention Ingénierie Biochimique

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Année civile: 2022-2023

Abstract

Pichia pastoris is a widely used recombinant protein expression system, so much that to date, more than 500 biomolecules have been synthesized using this yeast, many of which are approved for production and commercialization by the biotechnological industry.

The pAOX1-based expression system (MUT^+) is one of the most widely used to produce rProt in both research and industry. Under productive conditions, MUT^+ shows a high consumption rate of methanol and oxygen, and the accumulation of methanol can lead to the accumulation of cytotoxic intermediates such as formaldehyde and formate. This converts methanol and oxygen critical variables in the design of an rProt production strategy.

Culture medium heterogeneity is inherent in large-scale bioreactors. The loss of mixing quality yields to the formation of concentration gradients. Consequently, cells face oscillatory culture conditions that may deeply affect their metabolism. Herein, cell response to transient perturbations, namely high methanol concentration combined with hypoxia, has been investigated using a two stirred-tank reactor compartments (STR-STR) scale-down (SD) system and a *Pichia pastoris* strain expressing the gene encoding enhanced green fluorescent protein (eGFP) under the control of the alcohol oxidase 1 (AOX1) promoter. Cell residence times under transient stressing conditions were calculated based on the typical hydraulic circulation times of bioreactors of tens and hundreds cubic meters. A significant increase in methanol and oxygen uptake rates was observed as the cell residence time was increased. Stressful culture conditions impaired biomass formation and triggered cell flocculation. More importantly, both expression levels of genes under the control of pAOX1 promoter and eGFP specific fluorescence were higher in those oscillatory culture conditions, suggesting that those a priori unfavorable culture conditions in fact benefit to recombinant protein productivity. Flocculent cells were also identified as the most productive as compared to ovoid cells.

It is known that overexpression of the pAOX1 promoter does not always leads to an improvement in rProt productivity, as many bottlenecks can impair the production ability of secreted rProt. For this reason, the production of extracellular *Candida antarctica* lipase was

analyzed using the same configurations of SD systems as with the eGFP-producer strain. Extended cell residence time in microenvironments of high methanol concentration and low oxygen availability that are typically found at the upper part of a large-scale bioreactor near the methanol feeding point, triggers the unfolded protein response (UPR) and thus impairs protein secretion. Methanol/sorbitol co-feeding was shown herein as a means to reduce the UPR response and to restore productivity of the secreted protein.

Résumé

Les protéines recombinantes (rProt) ont de nombreux domaines d'application (pharmaceutique, agroalimentaire, chimie verte, environnement). Industriellement, ells peuvent être produites par culture de microorganismes en bioréacteur, le plus souvent sous une forme sécrétées. L'inefficacité de mélanges des bioréacteurs de grands volumes conduit à la formation de gradients de concentration, principalement lorsque les cultures sont conduites en mode fed-batch. La levure méthylotrophe *Pichia pastoris* est couramment utilisée pour la production de rProt en utilisant des systèmes d'expression basés sur le promoteur pAOX1 inductible au méthanol. Dans ces procédés, du méthanol est ajouté en continu dans le milieu de culture durant la phase de production des rProts, ce qui conduit à la formation d'une zone près du point d'alimentation ou la concentration en méthanol est élevée mais ou la concentration en oxygène est faible. La réponse cellulaire à des perturbations transitoires, à savoir une forte concentration en méthanol combinée à une hypoxie, a été étudiée dans le cadre de cette thèse en cultivant *P. pastoris* dans un bioréacteur de type scale-down (SD) à deux compartiments (STR-STR). Les souches utilisées produisent des rProt intracellulaires (eGFP) ou sécrétées (lipase CalB) sous le contrôle du promoteur pAOX1. Les temps de séjour des cellules dans ces conditions de stress transitoires ont été calculés sur la base des temps de circulation hydraulique moyens des bioréacteurs de dizaines et de centaines de mètres cubes. Nos résultats démontrent clairement que dans ces conditions de cultures oscillantes la capacité des cellules à métaboliser le méthanol et à consommer l'oxygène est accrue, bien que la croissance cellulaire soit plus faible. D'une manière surprenante, les niveaux d'induction du promoteur AOX1 et de l'expression des gènes qu'il contrôle sont également plus importants. Ces conditions de culture induisent aussi la floculation d'une partie de la population cellulaire. Ces cellules floculées ont également été identifiées comme les plus induites par rapport aux cellules ovoïdes. Cependant ces conditions de culture oscillantes ont un effet néfaste sur la capacité des cellules à secréter des rProt, notamment la lipase CalB de *Candida antarctica* et ce suite à l'activation de l'unfold protein response (UPR). Une stratégie de co-alimentation méthanol/sorbitol permet cependant de réduire la réponse UPR et par conséquent d'augmenter la productivité des protéines sécrétées.

Acknowledgements

My life in the doctoral program has been a transcendental stage in my life, full of difficulties and sacrifices, and at the same time with many satisfactions both at a personal and professional level. I must mention that I was never alone, and I always had people who supported me and made this an excellent experience. For this reason, I would like to deeply thank all the people who have been part of this process, either temporarily or sporadically.
To my parents Carmen and Edgar, to whom I owe everything I am, and who, without their support and unconditional love, this achievement would not have been possible, so this achievement is entirely dedicated to them. To my siblings Belén and José Luis, I am grateful for their love and support during all these years despite the distance.
I had the opportunity to live in Chile and Belgium, and besides getting to know different places and very interesting cultures, I highlight having met excellent people during these years. Above all, I want to thank my friends Hector, Jimmy and Eduardo, with whom I have shared all this time, and who have always been present, for their unconditional support and affection. Life is better accompanied, and I thank life for putting in my way my girlfriend, Camila, who is one of the most important people, my support and my happiness.
Visiting Belgium was an adventure that brought me excellent moments and people that I remember fondly, special mention to Sofija, Jelena, Luisa, Tamara, Simon, Diem and Marie for their kindness and good vibes.
I also thank professors Patrick Fickers and Julio Berríos for their guidance and support for the achievement of this work, as well as for the good disposition they had during these years for me. I would also like to thank professors Claudia Altamirano and Frank Delvigne for their time and availability to solve my doubts at key moments of my work. Special thanks to professors and staff who extended a helping hand when I needed it most, especially Lorena Wilson, David Jeison, Stephanie Braun, Alvaro Diaz and Isabel Rojas.
Special thanks to Agencia Nacional de Investigación y Desarrollo (ANID) for the "Beca Doctorado Nacional 2017" granted, to the Pontificia Universidad Católica de Valparaíso who through the School of Biochemical Engineering supported me with maintenance, internship and fee scholarships and to the Wallonie-Bruxelles International (WBI) for the funds granted for my stay in Belgium for 8 months.

Finally, I want to thank to the academic staff and workers of the School of Biochemical Engineering of the Pontificia Universidad Católica de Valparaíso, as well as TERRA of the University of Liege for their support and good disposition during these years.

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List of Publications

1. **Article 1 (Chapter 2)**

Velastegui, E., Quezada, J., Guerrero, K., Altamirano, C., Martinez, J. A., Berrios, J., & Fickers, P. (2023). Is heterogeneity in large-scale bioreactors a real problem in recombinant protein synthesis by *Pichia pastoris*? *Applied Microbiology and Biotechnology*. https://doi.org/10.1007/s00253-023-12434-2

2. **Article 2 (Chapter 3) Accepted**

Velastegui, E., Quezada, J. Altamirano, C. Berrios, J. Fickers, P. (2023). Co-feeding strategy as a means of reducing hypoxic stress in large-scale bioreactor to produce secretory recombinant proteins in *P. pastoris*. Journal of Biotechnology

3. **Article 3 (Chapter 4)**

***Velastegui, E.**, *Theron, C., Berrios, J., & Fickers, P. (2019). Downregulation by organic nitrogen of AOX1 promoter used for controlled expression of foreign genes in the yeast *Pichia pastoris*. *Yeast*, *36*(5), 297–304. https://doi.org/10.1002/yea.3381

The first two authors contributed equally to this work.

List of communications in scientific events

Oral communication:

«Challenges in Bioprocess Scale-Up: The case of *Pichia pastoris* and recombinant protein production at industrial scale» (2nd Biotechnology International Congress, Universidad de las Américas. Quito-Ecuador. 26/11/2021)

Oral communication:

«Bioingeniería: Escalado de bioprocesos con enfoque en parámetros de operación para biorreactores» (Centro de Capacitación Biotecnológica CEBIO. Quito-Ecuador. 27 and 28/02/2021)

Poster:

«Metabolic engineering of the methanol utilization pathway of *Pichia pastoris* for increased tolerance to formaldehyde toxicity» (European Biotechnology Congress. Prague, Czech Republic. 24-26/09/2020)

Poster:

«Downregulation by organic nitrogen of AOX1 promoter used for controlled expression of foreign genes in the yeast *Pichia pastoris*». (BioProNET2, 7th Annual Scientific Meeting. Manchester, UK. 19 and 20/11/2020)

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List of abbreviations

AHRT	Average hydraulic residence time (min)
AOX1	Alcohol oxidase 1
CA	Casamino acids
CalB	*Candida antarctica* Lipase B
C_L	Dissolved oxygen concentration in the reactor (mg/L)
C_0	Dissolved oxygen concentration at equilibrium (mg/L)
CRT	Cell residence time (min)
D	Dilution rate (h^{-1})
DAS	Dihydroxyacetone synthase
DCW	Dry cell weight
DOT	Dissolved oxygen tension (%)
eGFP	Enhanced green fluorescent protein
FLD	Formaldehyde dehydrogenase
FSC	Forward scatter
HRT	Hydraulic residence time (min)
HRTD	Hydraulic residence time distribution (min)
k_La	Volumetric oxygen transfer coefficient (h^{-1})
OUR	Oxygen uptake rate (mg/L h)
OTR	Oxygen transfer rate (mg/L h)
PFR	Plug flow (bio)reactor
q_{MetOH}	Specific methanol consumption rate (g/gDCW h)
q_{O2}	Specific oxygen consumption rate (mg/gDCW h)
q_{sor}	Specific sorbitol consumption rate (g/gDCW h)
RC	Reference culture condition
RC*	Co-feeding culture conditions
rProt	Recombinant proteins
S	Substrate concentration (g/L)
SD	Scale down systems
sFU	Specific fluorescence per cell

STR	Stirred tank (bio)reactor
SRV	Standardized residual value
μ	Specific growth rate (1/h)
X	Biomass concentration (gDCW)
$Y_{x/i}$	Yield (g/g)

Preface

Pichia pastoris is a recombinant protein expression system widely used in the biotechnological industry to produce high demand products. This yeast has the ability to metabolize methanol and use it as a source of carbon and energy. *P. pastoris* has two alcohol oxidases (AOX1 and AOX2), responsible for catalyzing the oxidation of methanol to formaldehyde in the first reaction of the MUT pathway. The pAOX1 promoter is inducible, and strongly regulated by methanol at the transcriptional level, characteristics that have been exploited under productive conditions.

MUT^+ strain (pAOX1-based expression system) evidence high methanol requirements which can lead to the accumulation of cytotoxic intermediary metabolites such as formaldehyde and formate. In addition, high methanol requirements imply high oxygen requirements, since oxygen is necessary for methanol oxidation, and also it is the last electron acceptor in the oxidative phosphorylation pathway.

The production of rProt using *P. pastoris* MUT^+ is normally carried out in fed-batch mode operation, where 3 phases can be distinguished: biomass production (glycerol fed batch), transition (glycerol fed batch) and induction (methanol fed batch alone, or cofeeding with sorbitol or glycerol). Of these, the induction phase, is a challenge, because it is necessary to apply a strategy that favors rProt productivity, avoiding the accumulation of methanol (and the subsequent accumulation of formaldehyde and formate) and providing adequate oxygenation to avoid limitation.

Scale-up of rProt production adds complexity to the above, since in large-scale bioreactors, methanol and dissolved oxygen concentration gradients can occur as a consequence of loss of mixing quality due to mass transfer issues. Therefore, the generation of *a priori* negative microenvironments (high methanol concentration and low dissolved oxygen tension at the top of the tank, where the methanol feed is carried out) is possible, and the cell in its transit is subjected to oscillatory conditions between *a priori* unfavorable microenvironments and more favorable microenvironments (low methanol concentration, high dissolved oxygen tension).

To mimic conditions propitiated in large-scale bioreactors (10-100 m^3), a 2-compartment scale-down (SD) system was assembled, were cells oscillated between an *a priori* unfavorable microenvironment and a more favorable microenvironment. The pAOX1 promoter expression level was measured in the SD behavior and compared with that obtained under reference conditions (a single compartment representing a homogeneous fermentation), for which, we used pAOX1-based *P. pastoris*, producer of intracellular eGFP as a reporter protein. The fluorescent nature of eGFP also allowed us to elucidate whether the stressful conditions in SD had an impact at the population level.

The main bottlenecks that affect rProt productivity are found both at the level of protein processing (post-translational modifications and trafficking in the ER) and its subsequent secretion. As most of rProt synthesized by *P. pastoris* are released extracellularly to facilitate the downstream process, the productivity of extracellular CALB was evaluated in SD behavior, with special emphasis on the triggering of the unfolded protein response (UPR) as a response to the stress conditions propitiated in the SD systems.

SD systems propitiate conditions that can trigger ER stress which negatively affects rProt's productivity. Considering the above, methanol/sorbitol co-feeding strategy was used under SD behavior to reduce methanol and oxygen requirements without compromising the level of pAOX1 induction as an alternative to alleviate metabolic burdens on rProt production.

Regarding culture media formulation, organic nitrogen supplementation is a widely used strategy because it considerably improves the biomass yield as well as the specific growth rate of P. pastoris. During the formulation of culture media for the development of this thesis, the effect of organic nitrogen supplementation on the induction of pAOX1 was characterized.

The final section of this document outlines the most important findings of this thesis project, also future perspectives in relation to this topic are presented.

CHAPTER 1

Introduction

In this chapter, general information on the use of *Pichia pastoris* as a recombinant protein (rProt) expression system is addressed. Relevant information on fundamental aspects of rProt production such as pAOX promoter regulation, methanol metabolism, factors that modulate rProt productivity, rProt production strategies and main bottlenecks that negatively affects the production of rProt are described. Finally, the *P. pastoris* in industrial-scale bioprocesses point of view was addressed, and how the cell performance could be affected by the loss of mixing quality in large-scale bioreactors.

1) *Pichia pastoris* and methanol metabolism

Pichia pastoris is a Cabtree-negative yeast, which has been used as a recombinant protein (rProt) expression system as it shows high biomass yields in defined culture media, its genetic manipulation is relatively simple and unlike *Sacharomyches cerevisiae*, *P.pastoris* carries out glycosylation patterns suitable for the production of proteins for therapeutic purposes (Carly et al., 2016).

The success of *P. pastoris* as a rProt expression system is based on the existence of strong and tightly regulated promoters of some genes of the methanol utilization pathway (MUT pathway), the most used of these being the promoter of the gene encoding alcohol oxidase (AOX1). MUT pathway expression is tightly regulated at transcriptional level, and an important portion of this pathway is carried out in the peroxisome.

As shown in Figure 1, the first reaction of the MUT pathway is the oxidation of methanol, leading to the formation of hydrogen peroxide and formaldehyde,which generate cytotoxic effects for the cell (Berrios et al., 2022; Wakayama et al., 2016). Formaldehyde has two alternatives: assimilation in cell metabolism through the assimilative pathway, whereby this carbon is destined for the formation of biomass and energy, and oxidation through dissimilative pathway, which is related to detoxification activities (Berrios et al., 2022) and energy production (Yu et al., 2022), where CO_2 is generated as a respiration by-product.

Methanol metabolism is catalyzed by alcohol oxidase enzymes. The *AOX1* and *AOX2* genes are responsible for alcohol oxidase activity, but *AOX1* enzyme contributes with most of the alcohol oxidase expressed. The strain that maintains the expression of the *AOX1* and *AOX2* genes is the wild type, also known as MUT^+, which is one of the most used in rProt production. However, as MUT^+ exhibit high methanol and oxygen consumption rate in productive conditions, this could be a difficult technical challenge to overcome.

Through genetic engineering, other strains altered in their methanol consumption capacity have been developed. In this context, the MUT^s phenotype results from the silencing of the AOX1, considerably reducing methanol and oxygen consumption rate (Daly & Hearn, 2005). The MUT^- strain results from a double knockout, leaving the cell with no alcohol oxidase activity and supplementation with co-substrates is necessary to support growth and energy

generation. (Chiruvolu et al., 1997, 1998; Zavec et al., 2020). Other interesting genetic modifications have been made to improve the capabilities of *P. pastoris* (Kang et al., 2017), although those mentioned above are the most popular.

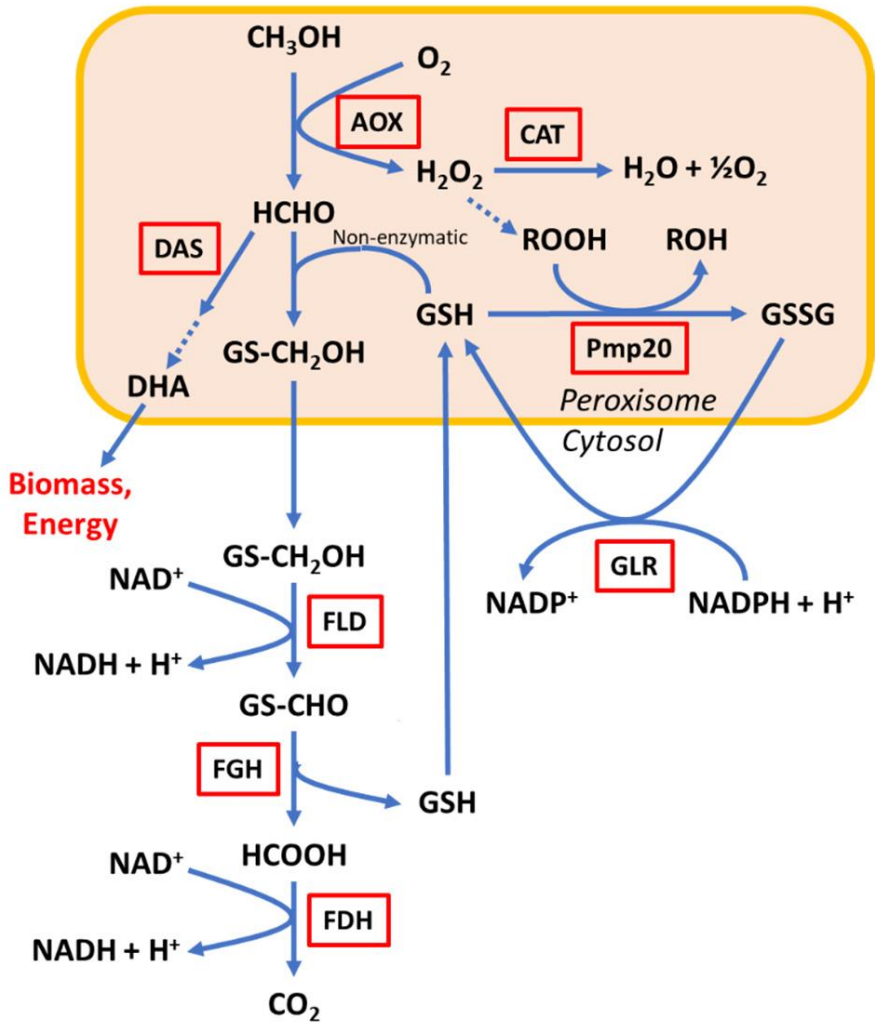

Figure 1: Main steps of the methanol utilization (Mut) pathway in P. pastoris. Relevant enzymes involved are shown in red boxes. (Adapted from (Yano et al., 2009)). AOX: alcohol oxidase; CAT: catalase; DAS: dihydroxyacetone synthase; FLD: formaldehyde dehydrogenase; FGH: S-formylglutathione hydrolase; FDH; formate dehydrogenase; GS(H): glutathione; Pmp20: peroxisomal glutathione peroxidase; GLR: glutathione reductase (Berrios et al., 2022).

2) pAOX1 promoter regulation

Methanol can be used by *P. pastoris* as a carbon and energy source, which is catabolized in the MUT pathway by the action of alcohol oxidase enzyme. The promoter of alcohol oxidase enzyme is regulated by *cis* and *trans* regulatory elements (Gidijala et al., 2018; Vogl & Glieder, 2013). In general, promoters regulate transcription as they act as specific DNA-binding sites for transcription factors, as well as all the machinery that carries out the transcription.

For the production of rProt, the fermentation process is divided into 3 phases in the context of the use of pAOX-based systems (see next section). For biomass production, highly metabolizable carbon sources such as glucose and glycerol are used, while for rProt production, methanol is used as an inducer. Both glucose and glycerol exert a catabolite repression on the pAOX1 promoter., so a de-repression phase is necessary (Liu et al., 2019).

The mechanism of repression of the pAOX1 promoter by some carbon sources is still not totally clear. Some evidence suggests that the repression is at the level of both glucose and glycerol hexose transporters. Moreover, repression of these transporters directly influences the transcription of MRX1 transcription factor, the latter identified as a critical regulator of genes involved in the methanol utilization pathway. (G. P. Lin-Cereghino et al., 2006; Zhan et al., 2017; Zhang et al., 2010).

Nitrogen sources also influence in the expression of the main genes of the MUT pathway (*AOX*, *CAT*), as well as in the expression of genes responsible of peroxisome biogenesis (*PEX*). Evidence suggest that a catabolite repression can be found with the use of certain nitrogen sources. For example, using aminoacids like glutamate, proline and asparagine results in a decrease in pAOX1 induction level compared with the use of ammonium sulphate and other aminoacids like glutamine. (Rumjantsev et al., 2013, 2014). The mechanism of pAOX1 promoter repression exerted by some nitrogen sources is not yet clear, but evidence indicates that repression depends on the nitrogen source used, so there may be specific repression mechanisms for certain amino acids..

3) *Pichia pastoris* as a recombinant protein expression system

P. pastoris is perhaps the most widely used recombinant protein (rProt) expression yeast system today, which emerges in the middle of the genomic era with the isolation and characterization of the *AOX* genes, thus constituting the first description of the use of genetic edition in the frame of bioprocesses engineering industry (Cregg et al., 1985, 1989; Tschopp et al., 1987).

To date, more than 500 recombinant proteins have been expressed with *P. pastoris*, many of them approved by major regulatory agencies such as the Food and Drug Association (FDA), the European Medicine Agency (EMA) and the European Food Safety Authority (EFSA), transforming this expression system into a key player in the development of the biotechnology industry in recent years (Ergün et al., 2022).

In industrial applications, two promoters are mainly used to produce rProt: the constitutive promoter pGAP and the inducible pAOX1.At technical level, pGAP-based expression systems have the advantage of being a methanol-free expression system, but to date, there is no clear consensus on the superiority of one of the mentioned expression systems as far as rProt productivity is concerned.

The selection of a promoter will determine the fermentation strategy to be employed (García-Ortega et al., 2019), although in general, rProt production is carried out in fed-batch mode in high cell density fermentations (HCDF).When using pAOX1-based expression, it is convenient to divide the process into three phases for better understanding: 1) Generation of high amounts of biomass by using glycerol batch (Above 100 g DCW/L in the majority of cases (Hamaker et al., 2011; Jiang et al., 2013; Schilling et al., 2001)), 2) Transition phase through glycerol fed-batch, necessary for propitiate cell metabolism adaptation for methanol consumption and, since glycerol exerts a repressor effect at a transcriptional level of the pAOX1 promoter (X. Wang et al., 2016), it is necessary to de-repress it; and finally 3) Induction phase, in which the methanol alone, or with co-substrates such as sorbitol or glycerol are added to the system in a specific fashion.

The induction phase is the most critical stage of the process, and some strategies proposed to obtain the best results in terms of rProt production can be found in the literature (Liu et al.,

2019). The choice of an induction strategy is a fundamental step towards the achievement of the objective, and regarding pAOX1-based expression systems, two conditions must be ensured: avoid oxygen limitation and methanol accumulation.

The latter then justifies the need to control the dissolved oxygen tension (DO-stat strategy) above the critical dissolved oxygen level, considered to be 20-30% of DOT (Jia et al., 2022; Lee et al., 2003; Woo et al., 2005). There are also some reports which demonstrate that specific growth rate governs the induction of the pAOX1 promoter (Garrigós-Martínez et al., 2019), and from this basis emerges the μ-stat strategy, which consists in propitiate a pseudo-steady state by keeping μ constant by means of an exponential feeding function, thus avoiding transient accumulation of methanol (i.e. formaldehyde accumulation). Despite this, accumulation of very small concentrations of methanol has proven to be a suitable induction strategy, since a strong limitation condition could be harmful for the cell (Barrigón et al., 2013).

Due to the high oxygen requirements that cells exhibit because of methanol consumption, it is usual that reaching high cell densities likely to be an important technical challenge since the system cannot adequately cover the oxygen demand, which results in oxygen limitation and consequently methanol accumulation. To decrease oxygen demand, the cofeeding strategy has been successfully employed, without compromising the induction levels of the pAOX1 promoter as in the case of the use of sorbitol (Carly et al., 2016) and glycerol (Berrios et al., 2017) as co-substrates.

4) Main bottlenecks that affect protein production

The main objective of *P. pastoris* is to generate high-level protein expression in reasonable quantities to make the process economically profitable, so the efforts are directed towards improving the expression of the pAOX1 promoter and the production of rProt. Unfortunately, improving production capabilities is not an easy task, as the rProt production process has many bottlenecks related to the pAOX1 promoter transcription, protein translation (and post translation modifications), and protein secretion.

Increase gene dosage has been used as an improvement strategy, which in many cases has not been a successful strategy (Hohenblum et al., 2004; Inan et al., 2006). At the

transcriptional level, transformed *P. pastoris* has a high stability, since the instability generated by segregation is avoided by chromosomal integration of the plasmid. However, the existence of structural instability (caused by insertion, deletion, or recombination events) has been demonstrated, and that this depends on the number of copies of the foreign gene, where the higher the number of copies, the greater the instability and negative effects on pAOX1 promotor expression is plausible (Zhu et al., 2009).

The increase in protein synthesis also requires an increase in the resource's availability, such as an adequate enzyme pool, enough quantity of amino acids and precursors, and a suitable energetic state of the cell (i.e. enough energy resources such as ATP, NADH) to meet the new demands. The latter means that the with the upregulation of the pAOX1 promoter, the metabolism should be readjusted to face a new condition, which can lead to loss of homeostasis and a drop in productivity level. In a promoter upregulation scenario, normally the rate-limiting steps are those related with protein processing in endoplasmic reticulum (ER) and protein secretion, both considered important checkpoints of protein metabolism (Shuster, 1991; Young et al., 2011).

5) ER stress and Unfolded Protein Response (UPR)

Protein secretion is a complex process carried out in various organelles of the cell. Thus, transcription of the recombinant gene takes place in the nucleus, the protein is synthesized, the folding and general processing (undergoing most post-translational modifications) occur in the endoplasmic reticulum (ER), then the protein is translocated in COPII vesicles to the Golgi apparatus for final processing, and at the end, the functional protein is disposed via vesicle transport intracellularly or extracellularly (Antonny & Schekman, 2001).

ER stress is considered the main bottleneck in protein secretion process, which appears when the processing capacity of the endoplasmic reticulum (ER) has been overwhelmed, leading to the accumulation of unfolded or misfolded proteins. This accumulation induces the expression of the unfolded protein response (UPR), which is a signaling cascade that aims to increase the processing capacity of the ER, thereby reducing the level of misfolded proteins in the organelle (Graf et al., 2008).

In summary, the UPR comprises the overexpression of genes encoding chaperones and foldases, proteins generally involved in post-translational modifications, and genes encoding proteins involved in protein translocation and quality control of the ER, although if this is not enough and the system fails to synthesize correctly folded proteins, these are translocated back to the cytosol, ubiquitinated and degraded through the ER-associated pathway (ERAD) (Gasser et al., 2007; Raschmanová et al., 2021a; Vogl et al., 2014; Zahrl et al., 2017).

Overexpression of UPR has been considered as a possible strategy for rProt productivity enhancement, but this has been a good alternative only in the case where protein folding is the rate limiting step and not protein trafficking through the secretory pathway. In this context, the chaperone Kar2, protein disulfide isomerase Pdi1, enzymes involved in the control of ER redox state such as Ero1, and the UPR transcription factor Hac1p have been co-expressed in *P. pastoris* to achieve this objective (Raschmanová et al., 2021a).

Harsh culture conditions can also trigger the UPR mechanism as observed in the case of *P. pastoris* under hypoxic conditions, where lipid stress (i.e. change in membrane lipid composition to avoid damage by hypoxic conditions) is sensed by the UPR genes, which are overexpressed in this condition (Adelantado et al., 2017). As mentioned above, cofeeding with sorbitol is capable of considerably reducing the oxygen requirements of *P. pastoris* under productive conditions (Niu, Jost, et al., 2013), In addition, this strategy has been shown to alleviate the stress caused by *Rhizopus oryzae* lipase overproduction (ROL) by means of a MUT^{s} strain (Ramón et al., 2007).

6) Scale-up of bioprocesses and "scale-up effect"

Scale-up process is the fundamental step to convert a biotechnological process into an economically profitable one. At the technical level, the main objective of bioprocesses scale-up is to reproduce at large scale the main response variables of a fermentation (i.e. productivities, yields, q-rates) obtained at a small scale. The latter is far from trivial, and the reason lies in how the transport phenomena (mass, energy, and momentum) vary with the change of size (Acevedo et al., 2002; Simpson & Sastry, 2013).

The condition of homogeneity of fermentations is fundamental, considering that all relationships and equations in bioprocesses have been derived assuming this condition.

Considering agitation as the limiting unit operation of aerobic cultures, a good quality of mixing is expected in laboratory scale bioreactors (0.5-10 L), but with the increase in bioreactor size and the consequent increase in transport resistance (e.g. increase of oxygen transfer resistance), the same agitation efficiencies as those founded in laboratory scale are impossible to expect in large-scale bioreactors (10-100 m3), resulting in a significant drop in mixing quality (Lara et al., 2006a). To better understand the mixing quality, it is useful in practice the mixing time and circulation time, which provide a quantitative measure of the quality of mixing. (Acevedo et al., 2002; Lara et al., 2006a).

Because of the loss of mixing quality, the formation of gradients. in all fermentation variables (DOT, substrate concentration, pH, temperature, etc.) can be observed (Bylund et al., 1998b; Larsson & Enfors, 1988; Oosterhuis & Kossen, 1983). The problem with gradient formation is that in their transit through the bioreactor, cells may face microenvironments that are negative for their growth capacity or their ability to produce products of interest, therefore, cells fluctuate between unfavorable and more favorable microenvironments. Moreover, if the volume increases, the cells remain longer in these negative microenvironments, which has been determined to be an important variable for the metabolism of some microorganisms (Bylund et al., 1999; Enfors et al., 2001). The problem that arises from this complex dynamic between the cell and its transit through an large-scale bioreactor with poor quality of mixing is called the "scale-up effect".

As mentioned above, the induction phase in high cell density fermentations is the most important step in the production of rProt, and when pAOX1-based expression systems are used, methanol and oxygen are critical players. In large-scale behavior then, the formation of an *a priori* unfavorable microenvironment characterized by high methanol concentrations and low dissolved oxygen concentrations in the feed zone (i.e. top of the tank) would be the result of the loss of quality of mixing.

In consequence, the “scale-up effect” problem of *P. pastoris* lies in that cells in their transit through the bioreactor undergo fluctuations between unfavorable and more favorable microenvironments for both the induction of the pAOX1 promoter and the production of intra- and extracellular recombinant proteins.

To address this complex dynamic between cells and defined microenvironments, SD systems have been proposed, which are so far the most suitable tool to investigate the scale-up effect. Several configurations of SD systems can be found in the literature, although the two-compartment systems (STR-STR and STR-PFR) are the most commonly used, since it is possible to emulate cell fluctuations between unfavorable and more favorable environments through recirculation between two small-scale bioreactors (Olughu et al., 2019).

7) Hypothesis

As previously stated, methanol concentration and oxygen availability are fundamental variables in the production of rProt, and the formation of a priori unfavorable microenvironments in relation to these variables in large-scale bioreactors could negatively affect the overall rProt production process. Therefore, the hypothesis proposed for the present work is as follows: In large-scale bioreactors, *P. pastoris* cells face oscillatory culture conditions and remain for periods in the range of circulation time in *a priori* unfavorable conditions of high methanol concentration and low oxygen availability (hypoxia) that may impair cell growth and secretory rProt productivity.

To demonstrate the hypothesis, the following objectives were proposed:

8) General Objective

To analyze the effect of cell residence in an *a priori* unfavorable microenvironment under oscillatory culture conditions between an *a prior* unfavorable and a more favorable microenvironment on cell growth ability, pAOX1 induction level, and intra- and extracellular recombinant protein productivity.

9) Specific objectives

- To study the effect cell residence time in an *a priori* unfavorable microenvironment under oscillating behavior between an *a priori* unfavorable and a more favorable microenvironments using scale-down (SD) systems on the induction level of pAOX1 promoter.
- To study the effect of cell residence time in an *a priori* unfavorable microenvironment under oscillating behavior between an *a priori* unfavorable

and a more favorable microenvironment using SD systems on the secretion of *Candida antarctica* lipase B (CALB).

- To evaluate the performance of methanol/sorbitol co-feeding strategy as a mean to improve pAOX1 promoter induction levels and CALB productivity in a situation of an extended cell residence time in an *a priori* unfavorable microenvironment under oscillating conditions between an *a prior* unfavorable and a more favorable microenvironment using SD systems.

CHAPTER 2:

Heterogeneity in large-scale bioreactors and recombinant protein synthesis

1) Introduction

The methylotrophic yeast *Pichia pastoris* is used in industry as a cell factory to produce recombinant proteins (rProt) based on processes operated in large-scale stirred tank reactor (STR). Those production processes are, most of the time, first operated in glycerol batch and fed-batch mode to yield a high cell density, followed by an rProt production phase. Different efficient expression systems have been developed to produce those rProt, but those based on the promoter of the AOX1 gene encoding alcohol oxidase 1 are by far the most employed (Ata et al., 2021). As this promoter is strongly induced in the presence of methanol, the rProt production phase is initiated by feeding methanol in a specific fashion with the aim to maximize rProt productivity while avoiding oxygen limitation (hypoxia) and accumulation of methanol catabolic products such as formaldehyde that are toxic for cells (Berrios et al., 2022). In those processes, oxygen availability to cells is a key parameter as it is used for both methanol oxidation in peroxisomes (Ata et al., 2021) but also for cellular respiration.

In lab-scale STR, the culture medium is considered as homogenous. However, increasing the scale of operation (i.e. 10 to 500 m^3) leads to the loss of mixing quality due to mass transfer concerns (Neubauer & Junne, 2010). Thus, different microenvironments can be found depending on their spatial situation as compared to the impeller discharge zones, where greater turbulence and better mixing efficiency operate. Moreover, when methanol is fed from the top of the reactor during the induction phase, this loss of medium homogeneity also yields to the formation of a zone at the top of the STR where the concentration of methanol is considerably high while that of dissolved oxygen is low as compared to the remaining culture medium. Indeed, that feeding zone is out from the zone of influence of the agitation and aeration systems (Bylund et al., 1998b; Vrábel et al., 1999); and this effect is more pronounced as the size of the STR increases. In those conditions, cells are subjected to culture conditions that oscillate between unfavorable (high methanol, low dissolved oxygen) to more favorable culture conditions (lower methanol, higher dissolved oxygen). In a large-scale STR, cells remain in that feeding zone for periods comparable to circulation time distribution of the reactor (Bylund et al., 1999; Oosterhuis et al., 1985). According to some studies (Lara et al., 2006b; Oldshue, 1966), circulation time of few tens of seconds can be found in STR

of less than 10 m^3, while circulation time of hundreds of seconds occurs in bioreactor of one hundred of cubic meters (Priyadi et al., 2019).

To characterize the influence of those oscillating culture conditions on the cell physiology, scale-down (SD) systems have been used with success for bacteria (Bylund et al., 1998b, 1999), yeasts (Delvigne et al., 2006; Lejeune et al., 2013) and animal cells (Nienow et al., 2013; Osman et al., 2002). In most cases, a negative effect on both the growth capacity and metabolite productivity has been observed, and this effect was found directly related to the increase of the hydraulic residence time (HRT) in the compartment where stressing conditions occurred. Previous studies based on the culture of *P. pastoris* grown in glycerol batch operated in a SD system composed of a STR and a plug flow reactor (PFR), which mimics the inhomogeneous regimes of the large-scale fermenters, highlighted that the growth rate was significantly reduced as the exposure time to oxygen limitation increased. Moreover, more by-products were produced in those conditions (Lorantfy et al., 2013).

Herein, a two-compartment STR-STR scale down system was set up to simulate large-scale culture conditions for *P. pastoris* grown during the induction phase (i.e. during methanol feeding). For this purpose, a *P. pastoris* MUT^+ strain expressing the enhanced green fluorescent protein (eGFP) under the control of the pAOX1 promoter was used. With such a set-up, the effects of those oscillating culture conditions on the cell physiology, rProt titre and productivity were deciphered.

2) Materials and methods

2.1) Strain, inoculum and preparation media

The *P. pastoris* strain RIY230, a prototroph derivative of strain GS115 expressing the gene encoding enhanced green fluorescence protein (eGFP) under the control of the AOX1 promoter, was used (Velastegui et al., 2019a). Pre-cultures were performed in 500-mL shake flask containing 200 mL of YNBG culture medium composed of 1.7 g/L yeast nitrogen base (Difco), 5 g/L of ammonium sulphate, 15 g/L glycerol and 50 mM phosphate buffer pH 5.5. For batch cultures, YNBGM medium (the same as YNBG but with 20 g/l glycerol and 10 g/L of methanol (Berrios et al., 2017)) was used during the batch phase while for continuous

cultures, the medium contained 20 g/L of methanol. All cultures were seeded at an initial OD_{600nm} of 0.5 with cells grown in YNBG during 24 h and washed with PBS buffer.

2.2) Culture conditions

For scale-down (SD) experiments, a two-compartment system was used while the reference culture condition (RC, non-oscillatory culture condition) was operated in a one compartment STR (Figure 2). All the cultures were operated in continuous mode at a dilution rate of 0.03 h^{-1}, and in all cases, steady state was reached after five hydraulic residence times (i.e. 166 h of culture). For the RC, an STR (New Brunswick Bioflo Celligen 115) of 2 L with 1.5 L working volume was used. The temperature was maintained at 30 °C, and pH was regulated at 5.5 by the addition of NaOH 3N. Agitation rate was set at 500 rpm while the aeration flow was maintained at 1.5 L/min of air. At the end of the batch phase, two sudden fluctuations of the dissolved oxygen tension (DOT) can be observed. Since glycerol and methanol are not consumed at the same time due to the repressive effect of glycerol, the increase in DOT indicates that glycerol has been completely consumed. Next, a decrease in DOT indicated the initiation of methanol consumption. Before the DOT rose again due to total methanol consumption, feeding was started to initiate the continuous culture (Jungo et al., 2007).

The (SD) system consisted of two interconnected reactors (R1 and R2) operated as a continuous system. The inlet line was connected to the top of STR R2 while the outlet line to that of STR R1 (Figure 2). STR R1 and R2 consisted of New Brunswick Bioflo Celligen 115 Benchtop Bioreactors with total volume of 2 L and 1 L, respectively. The total working volume (WV) of the SD system ($WV_{SD}= WV_{R1} + WV_{R2}$) was 1.5 L, similarly to the RC. The working volumes of R1 and R2 were set at WV_{R1} = 1.2 L and WV_{R2} = 0.3 L, respectively, to maintain the relationship between a zone of poor and high degree of mixing reported previously (Noorman et al., 1993). STR R1 and R2 were interconnected by a Masterflex L/S25 pipe of 1 m length. Two SD systems were employed, differentiated by the recirculation flow rates between R1 and R2. SD1 was operated at 236 mL/min while SD2 was operated at 122 mL/min. The SD cultures were carried at 30 °C, and the pH was maintained at 5.5 by the addition of NaOH 3N in both R1 and R2. In STR R1, an aeration flow rate was 1.5 L/min while no air was injected in STR R2. For STR R1, the agitation was 500 rpm, while for R2,

it was 150 rpm to avoid cell sedimentation. All cultures were performed in duplicate, and samples were collected after 5, 6 and 7 residence times (after 166, 200 and 233 h of culture).

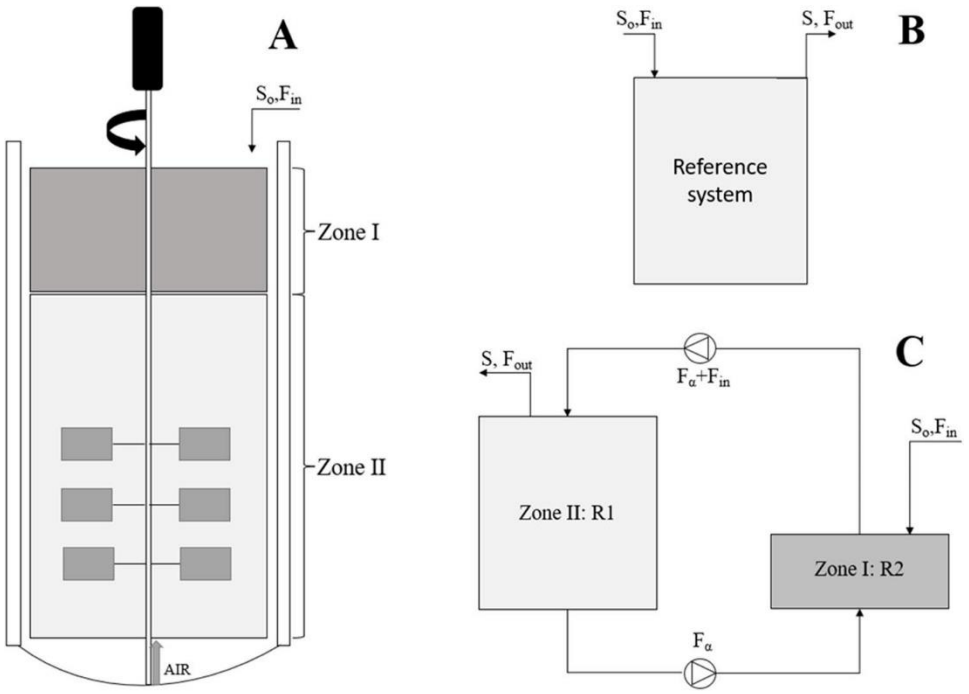

Figure 2: Schematic representation of a large-scale reactor (A), the RC reference system (B) and the STR-STR SD system used (C). The zone I depicts the feeding zone found in a large-scale bio- reactor while zone II represents the bulk of the liquid phase. F_{in} is the flow rate with which the culture medium enters the reactor, F_{out} is the flow rate with which culture leaves the reactor, S_o is the concentration of substrate in feed and S is the concentration of substrate in the output line.

2.3) Flow cytometry measurements and data treatment

Flow cytometry (FC) analysis was performed on a FACSCANTO II flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with an excitation laser at 488 nm and a 530/30 nm bandpass filter for eGFP fluorescence quantification (FITC). Prior measurements, cell samples were sonicated for 60 s at 40 kHz to break down cell aggregates (Falcioni et al., 2006) and diluted to an OD_{600nm} of 0.05. For each sample, at least 20,000 events were analyzed using the FL1 channel to quantify the fluorescence associated with the green fluorescent protein, and the median value was calculated and expressed as specific

fluorescence per cell (sFU). The cell background fluorescence was determined based on reported methodology.(C. W. Theron et al., 2019). For cell morphology discrimination, FC data sets were first cleaned of electronic instrument noise by eliminating all events with negative, infinite, or non-numerical values in the front and side scattering and fluorescent channel signals. A maximum value of 1.7% and a mean of 0.22% of noisy events were found for all individual FC data sets. After, all doublet events were eliminated by evaluating the standardized residual value (SRV) of the expected linear ratio between the area and the height of the front scattering signal. SRVs above 2 were defined as doublets and were eliminated from samples. A maximum of 2.2% and a mean of 0.96% of events were found to be doublets for all individual FC data sets. After data cleaning treatments, all experimental sets had at least 20,000 events. For quantification of cell morphological differentiation, the sample set for the strain RIY230 grown in RC (non-oscillatory condition) was used as a control for ovoid morphology. A histogram for the forward scattering (FSC) data was constructed, and the mean and standard deviation were calculated (Figure S1). Events below the mean value summed with three standard deviations were considered as ovoid cells. This value was selected as it accounts for 99.8% of the cellular events as calculated with the data approximated to a normal probability density function. The latter suggests that in the case of observing higher percentages of cells above the three standard deviation threshold, the events may belong to another population (i.e. not single ovoid cells), with other mean and standard deviation values, with a high probability (p-values < 0.022). Identification and quantification of the number of events above this threshold were performed for all samples resulting in the percentage values of flocculent cells. Means and other statistical values were then calculated for the whole population, the ovoid and flocculent cells, revealing significant differences in the forward scattering (FSC) and fluorescence (FITC) signals. The total arbitrary fluorescence (i.e. the sum of FITCA signal) and the total volume (i.e. sum of FSCA signal) were calculated for the whole cell population and the two morphotypes. From these, eGFP fluorescence volumetric and biomass yield were calculated and presented in Figure S2.

2.4) Fluorescence microscopy

Microscopy was performed with a Nikon Eclipse 50i fluorescence microscope provided with a super high pressure mercury lamp as a source of light and a blue filter B2 E/C (excitation

465–495 nm; emission 515–555 nm), using a Plan Fluor 100x/1.30 oil immersion objective. Prior observation, cells were washed with PBS and diluted at a cell concentration of 1 gDCW/L. For image processing, NIS Elements F 2.20 software was used.

2.5) RNA extraction and real-time quantitative PCR

Transcriptional analyses were performed by quantitative PCR (qPCR) using the primers listed in Table S1. Total RNA extraction was performed using TRIzol reagent (Invitrogen) (Huang et al., 2019) and converted into cDNA using the PrimeScript first strand cDNA Synthesis Kit (TAKARA- BIO) according to the manufacturer's recommendations. qPCR assays were performed on an AriaMX Real Time PCR System (Agilent Technologies®) using 2 x Brilliant II SYBR® green QPCR Master Mix (Agilent Technologies®), with 0.2 µM of each forward and reverse primers and 1 µL of cDNA (600 ng/µL) in 25 µL total reaction volume. The cycler program was as follows: initial denaturation step of 10 min at 95 °C, 40 cycles of denaturation at 95 °C for 30 s, annealing at 55–60 °C for 60 s and extension time at 72°C for 30 s. Relative expression was calculated according to the $-2^{\Delta\Delta Cq}$ method (Pfaffl, 2001). Expression level of β-actin gene was used for normalization of the target gene expression level (C. Theron et al., 2020). Gene expression values from RC culture were considered as the control group for the $-2^{\Delta\Delta Cq}$ determination.

2.6) Analytical methods

Biomass concentration was monitored by optical density at 600 nm (OD_{600nm}) and by dry cell weight (DCW) measurements. Extracellular formaldehyde concentration was determined as described elsewhere (Werringloer, 1978). Briefly, 500 µL of culture supernatant was added to 750 µL of 0.76 M trichloroacetic acid solution. The reaction mixture was centrifuged at 40,000 g for 15 min. One mL of supernatant was added to 500 µL of Nash's reagent, incubated in a wet bath at 60 °C for 10 min and allowed to cool to room temperature, and the absorbance of the resulting reaction was measured at 412 nm. Methanol concentration was measured using a gas chromatograph (Clarus 600, PerkinElmer) equipped with an FID detector and an Equity-1 Supelco column. The temperature of the injector and detector was 200 °C, the oven was maintained at 80 °C, the injection volume was 0.1 µL, nitrogen was used at 5 mL/min as carrier gas and the measurement was carried out for 3 min. Arabitol was quantified by HPLC (Jasco Series 4000) using an Aminex HPX-87H column as previously

described (Baumann et al., 2010) using an RID as detector. The total sugar content in the cells was determined by the phenol-sulfuric acid method (Dubois et al., 1956), using mannose standard solution to establish the calibration curve.

2.7) Calculations at steady state of cultures

The volumetric oxygen transfer coefficient (k_La) was calculated using the dynamic method of desorption-absorption of oxygen (Garcia-Ochoa & Gomez, 2009). Oxygen transfer rate (OTR) was calculated using the equation derived from oxygen mass balance.

$$OTR = k_L a * (C_0 - C_L) \qquad (1)$$

Where C_o is the concentration at equilibrium, which according to Henry's law is equal to 7.6 mg/l at 30°C (Berrios et al., 2017), and C_L is de dissolved oxygen concentration (DOT) in mg/l. At steady-state, OTR is equivalent to oxygen uptake rate (OUR). Therefore, the equation for specific oxygen consumption rate (q_{O2}) is as follows:

$$q_{O2} = \frac{OUR}{X} = \frac{k_L a*(C_0-C_L)}{X} \qquad (2)$$

Where X is biomass concentration (g DCW/L).

For the calculation of yields and specific consumption/production rate of methanol, formaldehyde, and total sugars:

$$q_i = Y_{\frac{X}{i}} * D = \frac{(S_0-S_F)}{(X_F-X_0)} * D \qquad (3)$$

Where q_i is the specific consumption/production rate of any metabolite (i), $Y_{\frac{X}{i}}$ is the yield of any metabolite with respect to biomass concentration, S_0 is the concentration of the metabolite in the feeding, S_F is the concentration of any metabolite in stationary state, X_0 is the biomass concentration in the feeding ($X_0 = 0$), X_F is the biomass concentration in stationary state and D is the dilution rate. In the case of the SD systems, all measurements were carried out in both R1 and R2, applying the principle of mass conservation.

2.8) Hydraulic residence time distribution analysis

For hydraulic residence time distribution (HRTD) analysis of R2, a tracer pulse experiment was performed (Levenspiel, 2012). A 100-mL beaker with 50 mL of Mili-Q water was connected to the inlet and outlet of R2. Five mL of 5 M NaOH was injected into the inlet beaker, and the conductivity was measured in the outlet beaker using a CON200 conductivity

meter (clean water analysis solutions). Data were collected until the conductivity value reached 95% of the initial value. This procedure was employed using the recirculation flows of SD1 and SD2. The average hydraulic residence time (AHRT) of R2 was calculated, as well as the residence time distribution using the equations reported previously (Levenspiel, 2012). Cell residence time (CRT) was defined equal to AHRT.

2.9) Statistical analysis

Homoscedasticity of all the output variables was determined using Levene's test. Subsequently, the student t-test was used to determine the significance of the variation. Statistical significance was accepted at $p < 0.05$.

3) Results

3.1) Experimental set-up

To monitor the cellular response of *P. pastoris* strain RIY230 subjected to periodic fluctuations in methanol concentration and oxygen availability, an SD system constituted of two STR compartments was used (Figure 2). Compartment R2 simulates the feeding zone of large-scale bioreactor (i.e. high methanol concentration and low oxygen availability) while compartment R1 represents the culture conditions in the bulk of the liquid phase. Two configurations were considered (namely SD1, SD2), differentiated by the average hydraulic retention time (AHRT) of the cells in the compartment R2 to simulate a 10 m^3 and a 100 m^3 bioreactor scale. For that purpose, an AHRT in R2 was calculated based on a hydraulic residence time distribution (HRTD) analysis. For the SD1 configuration, an AHRT of 60 s was obtained from a HRTD ranging between 0 and 180 s while for SD2 configuration, an AHRT of 180 s was obtained from an HRTD ranging between 0 and 390 s. Based on this, CRT of 60 and 180 s were used in SD1 and SD2 configurations, respectively. In our system, methanol was fed in R2 compartment while spent medium was withdrawn in R1. Moreover, values of dissolved oxygen tension (DOT) in compartment R2 were always close to 0%, whereas in R1, it was never below 30% (data is not shown). In those conditions, the cells experienced hypoxic conditions while remaining in R2, along with a high methanol concentration. The reference culture (RC) consisted in a single compartment STR operated in chemostat mode and thus without any oscillatory culture condition. RC was considered as

homogenous, and culture samples were collected at steady state after 5, 6 and 7 residence times (after 166, 200 and 233 h of culture, respectively).

3.2) Cell growth and substrate uptake under oscillating conditions

Biomass concentration, specific methanol uptake rate (q_{MetOH}) and specific oxygen consumption rate (q_{O2}) were determined for samples collected at steady state for RC, SD1 and SD2 configurations. As compared to the reference culture condition (RC, non-oscillatory culture condition), biomass concentration decreased by 10% and 18% for SD1 and SD2, respectively (Figure 3). By contrast, in those conditions q_{MetOH} increased by 27% and 43 % while q_{O2} increased by 12% and 21% for SD1 and SD2, respectively as compared to the RC (Figure 4).

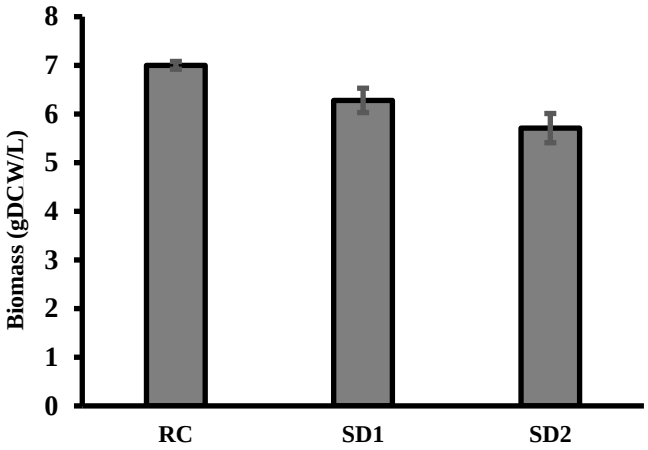

Figure 3: Biomass values obtained at steady state for *P. pastoris* strain RIY230 grown in bioreactor systems in RC, SD1 and SD2 configurations. Values are mean and standard deviation of measurements from duplicate cultures and three sampling times (i.e. after 5, 6 and 7 residence times). All values are statistically different considering $p< 0.05$. Biomass is expressed per gram of dry cell weight per liter (gDCW/L).

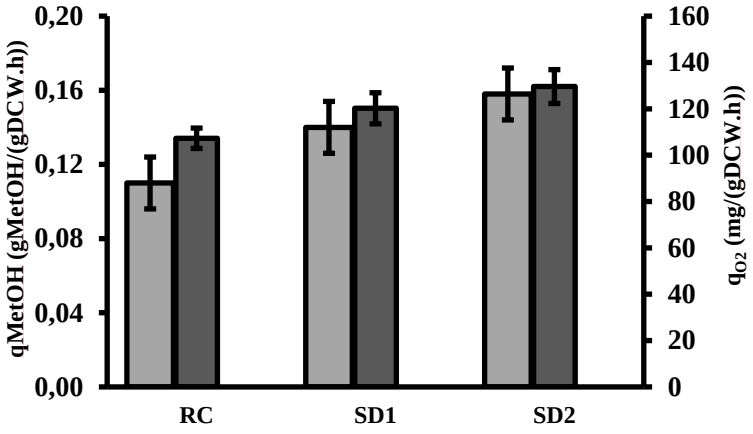

Figure 4: Specific methanol uptake rate ($q_{MetOH,}$ light grey) and oxygen consumption rate ($q_{O2,}$ dark grey) obtained at steady state for *P. pastoris* strain RIY230 grown in bioreactor systems in RC, SD1 and SD2 configurations. Values are mean and standard deviation of measurements from duplicate cultures and three sampling times (i.e. after 5, 6 and 7 residence times). All values are statistically different considering $p < 0.05$. q_{MetOH} is expressed in gram of methanol consumed per gram of dry cell weight produced and per hour while q_{O2} is expressed per gram of oxygen per gram of cell dry weight produced and per hour.

Those effects were more pronounced as the CRT in R2 was increased. Although, the oscillatory culture conditions of SD systems negatively affect the biomass concentration obtained at steady state; cells showed a higher capacity of methanol consumption. In *P. pastoris*, methanol enters the cells only by diffusion (Singh & Narang, 2020) before being oxidized into formaldehyde by alcohol oxidase AOX1 in the presence of oxygen. Therefore, it can be assumed that the higher methanol concentration in R2 yielded a higher q_{MetOH} and thus a higher oxygen consumption rate. A higher value of q_{MetOH} also implies a higher rate of formaldehyde formation. Although the concentration of this toxic compound increased in the culture medium from 0.2 mM in RC configuration to 0.5 mM in SD2, they were lower than those reported previously in chemostat culture (close to 25 mM, (Jungo et al., 2007)). Moreover, the stable value of biomass at steady state in SD systems ruled out any toxic effect of this metabolite to explain the lower biomass values obtained (data not shown).

3.3) Cell morphology under oscillating culture conditions

During cultures in SD systems, cells formed aggregates as compared to the non-oscillatory culture conditions (Figure 5), suggesting that they tended to flocculate in those experimental conditions. Although those cell aggregates can be disrupted upon ultrasound treatment, cells with ovoid morphology remain associated in small clusters (Figure 5). In *P. pastoris*, the *FLO* gene family, especially *FLO5-1* and *FLO11* are known to be involved in the transition from yeast to pseudohyphal morphology and flocculation (De et al., 2020b). Therefore, the gene expression level of those two genes was quantified for cells grown in SD systems and compared to that of the RC condition. As shown in Figure 5, both gene expressions were found up-regulated under oscillatory culture conditions and the level of gene expression can be correlated to the CRT. Therefore, the cell morphology seems to be modulated by these transient perturbations of high methanol concentration and hypoxia.

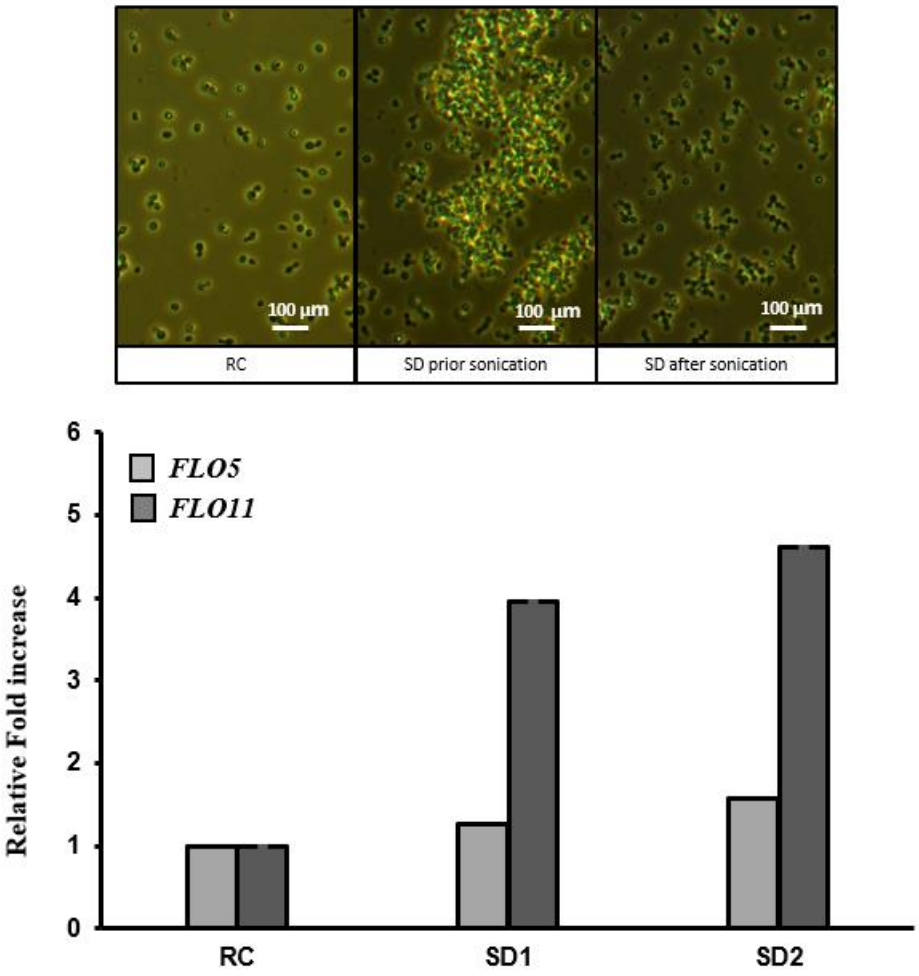

Figure 5: Cell morphology and relative *FLO* gene expression. Upper panel: Fluorescence microscopy of cells from RC and SD culture conditions. A representative sample from SD culture is shown prior and after sonication as described in material and methods. Lower panel: Relative fold increase in *FLO5* (light grey) and *FLO11* (dark grey) genes expression levels obtained at steady state for *P. pastoris* strain RIY230 grown in bioreactor systems in RC, SD1 and SD2 configurations. Measured samples were collected from duplicate cultures after 5, 6 and 7 residence times. All values are statistically different considering $p < 0.05$. Gene expression was normalized to that obtained in RC. Standard deviations on Ct values were less than 10% and 20% of the *FLO5* and *FLO11* average value, respectively.

3.4) Cell metabolism under oscillatory culture conditions

In SD systems, cells periodically face oxygen limitation with frequency related to the CRT in R2 reactor. Hypoxia is known to trigger significant modifications in lipid metabolism,

notably in ergosterol synthesis. For instance, in *P. pastoris* genes encoding enzymes that catalyze oxygen-consuming reactions of the ergosterol pathway, namely *ERG1*, *ERG3*, *ERG5*, *ERG11* and *ERG25*, are upregulated at low oxygen availability (Baumann et al., 2010). To assess a similar cell behavior during culture in SD systems, expression level of *ERG11* and *ERG25* encoding, respectively, sterol 14-demethylase and methylsterol monooxygenase was quantified. As shown in Figure 6, both genes *ERG11* and *ERG25* were upregulated during SD cultures as compared to the non-oscillatory culture condition. The overexpression was higher in SD2 than in SD1 system (61% and 33%, respectively), suggesting a direct influence of the level of hypoxia in this phenomenon. As alcohol oxidase activity also requires oxygen as a substrate, the expression level of the *AOX* genes were also quantified. As shown in Figure 6, the expression levels were 1.6 and 2.2-fold increase in SD1 and SD2 culture conditions, respectively, as compared to RC.

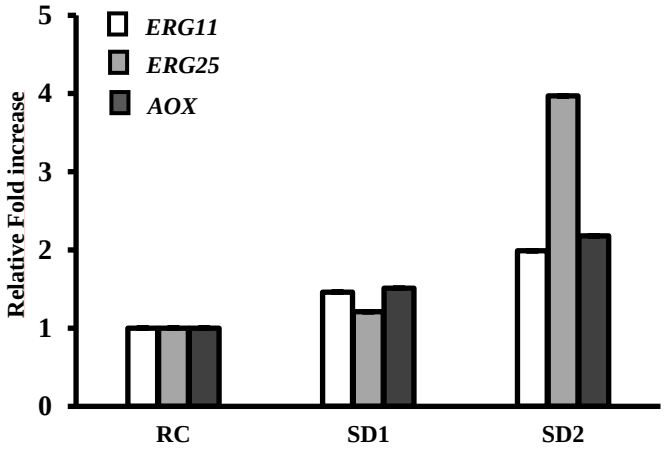

Figure 6: Relative expression level of genes *ERG11* (withe), *ERG25* (light grey) and *AOX* (dark grey) for *P. pastoris* strain RIY230 grown in bioreactor systems in RC, SD1 and SD2 configurations. Measured samples were collected from duplicate cultures after 5, 6 and 7 residence times. All values are statistically different considering $p < 0.05$. Gene expression was normalized to that obtained in RC. Standard deviations on Ct values were less than 13%, 18% and 15% of the *ERG11*, *ERG25* and *AOX* average value, respectively.

Carbohydrate metabolism is also modulated when yeasts are facing cellular stress (Francois & Parrou, 2001). Yeasts, including *P. pastoris*, are known to increase their intracellular

trehalose concentration under stressing conditions (Jordà et al., 2013). Beside this, cell wall mannose is an important player in the flocculation mechanism by interacting with flocculins encoded by the *FLO* genes, and it has been shown that mannose/glucose molar ratio is higher in flocculent cells (Vallejo & Villa, 2013). Therefore, total sugar content was quantified for cells grown in SD systems and the conversion yield from methanol was calculated. Carbohydrate production and accumulation were significantly higher for cells grown in oscillatory culture conditions, especially in the SD2 system as compared to the RC (0.15 ± 0.02 and 0.08 ± 0.01 g total carbohydrates/g methanol, respectively). Arabitol production has also been reported under hypoxic culture conditions (Baumann et al., 2010). However, that metabolite could not be detected in samples from RC and SD culture conditions (data not shown).

3.5) pAOX1 induction in cells grown under oscillating conditions.

As demonstrated above, oscillating culture conditions deeply affect the cell physiology, more specifically methanol metabolism. The significant increase of *AOX* genes expression level suggests that pAOX1 promoter might be more induced in those culture conditions. This correlates with the higher value of q_{MethOH} observed in SD systems (Figure 4). In *P. pastoris* strain RIY230, the gene encoding the extended green fluorescent protein (*EGFP*) was cloned under the control of the pAOX1 promoter and integrated at the *HIS4* locus. The quantification of *EGFP* expression level used as a proxy to monitor pAOX1 induction together with the specific fluorescence of the eGFP protein are shown in Figure 7. The relative gene expression was increased by 1.5 and 2-fold respectively in SD1 and SD2 while the mean fluorescence level was increased by 1.4 and 1.6-fold in the same conditions. This clearly demonstrates that in oscillating culture conditions expression of genes under the pAOX1 promoter is significantly higher than in homogenous culture conditions (*i.e.*, RC system).

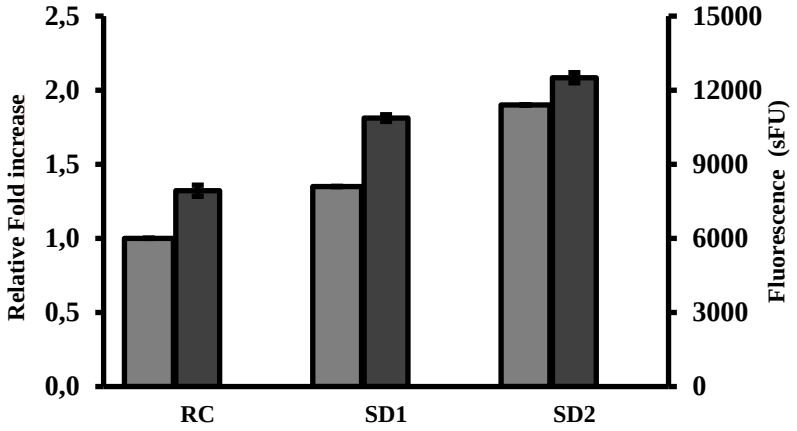

Figure 7: Relative expression level of *EGFP* gene (light grey) and mean eGFP fluorescence (dark grey) for *P. pastoris* strain RIY230 grown in bioreactor systems in RC, SD1 and SD2 configurations. Values are mean and standard deviation of measurements from duplicate cultures and three sampling times (*i.e.* after 5, 6 and 7 residence times). All values are statistically different considering $p < 0.05$. eGFP fluorescence is expressed in fluorescence unit per cells while gene expression was normalized to that obtained in RC. Standard deviations on Ct values were less than 15% of *EGFP* average value.

3.6) Phenotypic heterogeneities in the cell population

As oscillating culture conditions trigger both cell differentiation and increased expression level of genes under the control of pAOX1 promoter, cells were analyzed by flow cytometry to get insight whether flocculent cells are also those with the highest level of eGFP fluorescence. For that purpose, forward scattering data (FSCA) were used to discriminate between ovoid and flocculent cells The mean value of FSCA signal from RC condition summed with three standard deviations was used as a threshold to discriminate the flocculent cell population with a high probability (p-values < 0.022). Based on this, the fractions of flocculent cells in the whole population were calculated (Figures S1 and S2) and they were plotted according to their corresponding FITC-A signal used for eGFP fluorescence quantification. As shown in Figure 8, in all experimental conditions, flocculent cells were those with the highest fluorescence signal. More interestingly, while this proportion is low in the RC system (1.23%), it increased significantly in the SD1 and SD2 systems (8.9 and 11.6%, respectively).

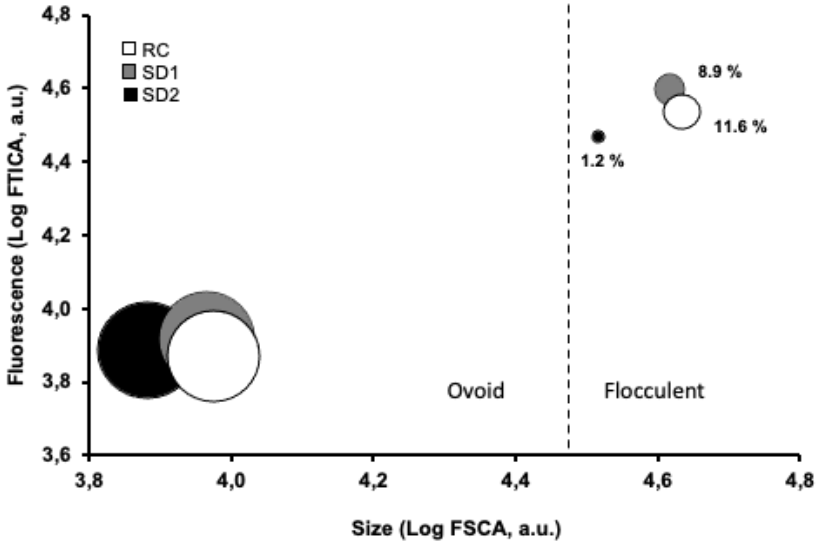

Figure 8: Flow cytometry analysis of cells grown in RC, SD1 and SD2 systems. Segregation of the cell population (ovoid, flocculent cells) was based on a threshold defined as the mean value summed with three standard deviations of FSCA data obtained in RC (see materials and methods for details). The fluorescence of intracellular eGFP was given in the FITC-A data. Circle areas and corresponding values traduce the percentage of cells in the two subpopulations (ovoid vs flocculent cells). The dotted line represents the threshold value to discriminate between the two morphotypes. Cells were collected from duplicate cultures and in three sampling times (after 5, 6 and 7 residence times).

4) Discussion

The methylotrophic yeast *P. pastoris* has become over the years one of the most used cell factories to produce rProt. Although baker yeast *Saccharomyces cerevisiae* has been used at first as a eukaryotic host system for this purpose, *P. pastoris* presents many technological advantages: it is a Crabtree-negative yeast, it can grow to high cell densities and can produce at high productivity correctly folded secretory proteins with less hypermannosylation issues as compared to *S. cerevisiae*.

The production of rProt at industrial scale relies on large-scale bioreactor that can range from several tens of cubic meters to hundred cubic meters. Although medium homogeneity can be reached in lab-scale bioreactor, heterogeneity in process variables such as dissolved oxygen and substrate concentration arise at industrial scale, due to limitations in mass transfer

efficiency (Neubauer & Junne, 2010). This phenomenon is enlarged in processes operated with feeding from the top of the bioreactor, since oxygen and fed of concentrated substrates are provided in zone of low mixing efficiency, namely the bottom and top of the STR, respectively. For instance, for *Escherichia coli* grown in a 12 m^3 bioreactor operated in glucose fed-batch, the glucose concentration was reported as 400 times higher close to the fed point as compared to the mean value of the bulk of the medium (Bylund et al., 1998a). The inefficiency of mass transfer in industrial scale reactors also results in oxygen concentration gradients, leading to hypoxic microenvironments where cells remain for times comparable to the circulation time (Bylund et al., 1999; Oosterhuis et al., 1985; Oosterhuis & Kossen, 1983).

Most of the rProt production process using *P. pastoris* are based on *AOX1* promoters and most of the time they are operated with methanol feeding. Due to the low mixing efficiency, a feeding zone appears at the top of the STR where methanol concentration is high while oxygen is scarce as compared to the bulk of the culture medium. Consequently, cells grown in those conditions face oscillatory culture conditions in which transient variations in substrate concentration and oxygen availability occur.

Yeasts are known to rapidly respond to fluctuation in environmental changes, as it is the essential condition for their survival. For instance, *S. cerevisiae* modulates directly or indirectly the expression pattern of thousands of genes upon stresses such as nutrient starvation, oxidative stress, or heat shock (Ronen & Botstein, 2006). Such adaptations to stress have been also observed for *P. pastoris*, namely hypoxia (Adelantado et al., 2017; Baumann et al., 2010) or oxidative stress generated by methanol metabolism (Zepeda et al., 2014).

With the aim to better characterize *P. pastoris* behavior during methanol feeding in large-scale bioreactor, a scale down approach was used to mimic the oscillatory culture conditions faced by cells in those systems. In our experimental set up, the compartment R2 represents the environment of the feeding zone (high methanol, hypoxia) while compartment R1 is representative of the bulk of the culture medium; with cell passing from R1 to R2 with a frequency related to circulation time characteristic of bioreactors of 10 and 100 m^3.

Under adverse culture conditions, *P. pastoris* is known to switch from unicellular to multicellular lifestyle, leading to the emergence of subpopulations (Ata et al., 2021). This phenotypic diversification provides the cell population advantages to adapt to environmental changes (Delvigne et al., 2018). The morphology transition is triggered by the *FLO* gene family. Members of this family encode GPI-anchored proteins that allow cell-to-cell adhesion, leading to the formation of flocculent cells and pseudohyphae. So far, pseudohyphal growth has been only observed when *P. pastoris* is grown at a slow growth rate (< 0.075 h^{-1}) in glucose limited lab-scale chemostats (Rebnegger et al., 2014, 2016). In our experimental conditions, a cell floc formation was observed. In SD systems, up to 11.6% of the cell population formed flocs as demonstrated by flow cytometry analysis and those flocculent cells could not be separated by ultrasound treatment, suggesting a strong interaction between them. Cells were also able to form flocs that can be disrupted by sonication. So far, this ability has been only observed in *P. pastoris* with modified rhamnose metabolism grown in specific conditions (Yan et al., 2018). Our observations correlate with an increase of *FLO5* and *FLO11* gene expression. More importantly, flow cytometry clearly showed that only a part of the cell population undergoes a morphological switch, most probably to ensure a faster adaptation of the cell population to the oscillatory culture conditions.

Although, specific methanol uptake rate and oxygen consumption rate were higher during growth in oscillating culture conditions, the biomass obtained at steady state was lower as compared to homogenous culture condition (*i.e*. RC system), suggesting thus that part of the substrate energy is derived to side metabolisms. Intracellular carbohydrate accumulation is known as a metabolic response to environmental stress. The significant increases in the carbohydrate conversion yield from consumed methanol, especially in SD2 correlate with the decrease in biomass, as more energy is needed for carbohydrate synthesis. Polyols such as arabitol are also known to accumulate under hypoxic culture conditions (Baumann et al., 2010). However, it could not be detected in samples from both RC and SD culture conditions. In SD systems, rProt synthesis and methanol consumption rate are also significantly higher. This might request more energy for methanol metabolism (i.e. peroxisome biogenesis, enzymes for methanol catabolic pathway) and for rProt synthesis (gene expression, protein

synthesis). Therefore, the higher rProt synthesis and methanol metabolism as well as side metabolisms may explain the lower biomass obtained in oscillating culture conditions.

Oxygen availability is critical for many biochemical reactions, notably for methanol oxidation. Surprisingly, both *AOX1* gene expression and specific methanol uptake rate was significantly higher in SD systems as compared to RC. This suggests that the hypoxic culture conditions faced transiently by cells in R2 compartment together with the higher methanol concentration triggered a higher AOX1 activity. Beside this, both expression levels of gene encoding *EGFP* under the control of pAOX1 and eGFP intracellular fluorescence were also significantly increased in SD systems. It has been previously highlighted that gene encoding oxygen consuming reactions are upregulated in hypoxic conditions. For instance, in *P. pastoris* transcript level of *ERG* genes coding for enzymes that catalyze oxygen-consuming reactions of the ergosterol pathway are upregulated upon hypoxic conditions most probably for compensation of oxygen scarcity (Baumann et al., 2010). In SD systems where cells face transient hypoxia, both genes *ERG11* and *ERG25* were also significantly up-regulated. Knowing that oxygen is a substrate of alcohol oxidase, a similar regulation is likely to occur. Therefore, transient hypoxic conditions seem also beneficial for expression of genes under the control of pAOX1 promoter and thus the production of rProt synthesis as demonstrated with eGFP.

In conclusion, this study demonstrated that these *a priori* unfavorable culture conditions that cannot be circumvented in industrial bioreactor are in fact profitable for the process productivity. Indeed, rProt synthesis is increased and part of the cell population tends to flocculate, which is advantageous in industrial bioprocesses for the separation of the biomass from the culture supernatant that contains the secretory rProt. Flocculent cells were also those with the highest ability of rProt synthesis.

CHAPTER 3:

Co-feeding as a means of reducing hypoxic stress

1) Abstract

The loss of mixing efficiency inherent to bioreactor process operated at large-scale yields to the formation of concentration gradient and thus to heterogeneous culture conditions. For processes operated with methanol feeding, *P. pastoris* faces oscillatory culture conditions that significantly affect the cell ability to produce secretory recombinant proteins at high yield. Extended cell residence time in microenvironments of high methanol concentration and low oxygen availability that are typically found in the upper part of the bioreactor near the feeding point, triggers the unfolded protein response (UPR) and thus impairs proper protein secretion. Methanol co-feeding with sorbitol was shown herein to reduce the UPR response and to restore productivity of secreted protein.

2) Materials and Methods

2.1) Strains

The *P. pastoris* RIY308 (GS115, pAOX1-αMF-CalB) is a prototroph derivative of strain GS115 expressing the gene encoding *Candida antarctica* lipase B (CalB) under the control of the AOX1 promoter (Velastegui et al., 2019a). CalB is a 317 amino acid protein with molecular weight of 33 kDa that belongs to the alpha/beta hydrolase-fold family. The protein is glycosylated at Asn74 and the six cysteine residues of the CalB sequence are involved in disulphide bonds (Uppenberg et al., 1994) . The αMF signal peptide was used for CalB secretion.

2.2) Culture medium

YNBG: 1.7 g L^{-1} yeast nitrogen base (Difco), 5 g L^{-1} $(NH_4)_2SO_4$, 15 g L^{-1} glycerol, 50 mM phosphate buffer pH 5.5.

YNBGM: 1.7 g L^{-1} yeast nitrogen base (Difco), 5 gL^{-1} $(NH_4)_2SO_4$, 20 g L^{-1} glycerol, 10 g L^{-1} methanol, 50 mM phosphate buffer pH 5.5.

YNBM: 1.7 g L^{-1} yeast nitrogen base (Difco), 5 g L^{-1} $(NH_4)_2SO_4$, 20 g L^{-1} methanol, 50 mM phosphate buffer pH 5.5.

YNBMS: 1.7 g L^{-1} yeast nitrogen base (Difco), 5 g L^{-1} $(NH_4)_2SO_4$, 12 g L^{-1} methanol, 7.5 g L^{-1} sorbitol, 50 mM phosphate buffer pH 5.5.

2.3) Culture conditions

Shake flask culture were operated in 500 mL shake flask containing 200 mL YNBG medium at 30 °C and with agitation (200 rpm). The scale-down system (SD) consisted of two STR (R1 and R2 with total volume of 2 L and 1 L, respectively, New Brunswick Bioflo Celligen 115 benchtop bioreactor) interconnected with a culture medium recirculation loop (Figure 2). The working volume of R1 and R2 were respectively of 1.2 L and 0.3 L to maintain the relationship between the zone of poor and high degree of mixing reported previously (Noorman et al., 1993). The system was operated at a dilution rate of 0.03 h^{-1} which correspond to the typical growth rate observed for *P. pastoris* during the rProt production phase (Barrigón et al., 2013; Çelik et al., 2009a). Three SD systems were employed, differentiated by the culture medium used and the recirculation flow rates between R1 and R2. SD1 was operated at recirculation flow rates between R1 and R2 of 236 mL.min-1 while SD2 and SD3 were operated at recirculation flow rates of 122 mL.min-1. Medium YNBM was used for SD1 and SD2 whereas YNBMS was used for SD3. The reference culture condition (RC and RC*) were operated in chemostat mode at a dilutions rate of 0.03 h^{-1} in 1.5 L working volume (2 L total volume) with medium YNBM (RC) and YNBMS (RC*). The batch phase was conducted in YNBGM and the feeding with medium YNBM or YNBMS started after the second increased in dissolved oxygen tension (DOT). All culture were performed at 30°C and pH was regulated at 5.5 by the addition of NaOH 3N (in R1 and R2 in case of SD system). Aeration flow rate was set at 1.5 $L.min^{-1}$ and 1 $L.min^{-1}$ for RC and RC*, respectively, while agitation rate was set at 500 rpm in both reference conditions (RC and RC*). In SD system, agitation was set at 500 rpm and 150 rpm in R1 and R2, respectively, while air was injected only in R2 with flow rate of 1.5 L.min-1 for SD1 and SD2, and 1 $L.min^{-1}$ for SD3. In those conditions, DOT values of dissolved in compartment R2 were always close to 0%, whereas it was never below 30%. in R1, RC and RC*. All cultures were performed in duplicate, and samples were collected after 5, 6 and 7 residence times (after 166, 200 and 233 h of culture).

3) Results and Discussions

Recombinant protein (rProt) production by yeast cell factories such as *Pichia pastoris* (aka *Komagataella phaffii*) are operated at industrial scale in large-scale stirred tank reactors

(STR). As the size of the STR increases (10-100 m^3), the mass transfer efficiency is impaired, leading thus to a culture medium heterogeneity (Neubauer & Junne, 2010). Moreover, when substrates are fed from the top of the STR, a feeding zone appears where nutrient concentration is high while that of dissolved oxygen (DO) is low comparatively to the remaining part of the bioreactor. Therefore, cells face oscillatory culture conditions and remain in the feeding zone for period comparable to average circulation time of the STR (Oosterhuis et al., 1985). When gene expression systems based on the methanol regulated *AOX1* promoter (pAOX1) are used for rProt synthesis by *P. pastoris*, methanol feeding is of common practice (Ergun et al., 2021). In those conditions, cells oscillate between unfavourable (high methanol, low DO) to more favourable culture conditions (lower methanol, higher DO). Recently, the cell responses to those transient culture conditions were characterised using a two-compartment STR-STR scale-down system (SD) and the *P. pastoris* strain RIY230 that produce the intracellular eGFP expressed under the control of pAOX1 (Velastegui et al., 2023). The SD system was composed of two interconnected STR (R1, R2), with R2 mimicking the methanol feeding zone of large-scale STR. Two specific recirculation flow rates between R1 and R2 were used to simulate the oscillatory culture conditions of bioreactor of 10 m^3 (SD1, average cell residence time in R2 of 60 sec) and 100 m^3 (SD2, average cell residence time in R2 of 180 sec) (see chapter 2 (Velastegui et al., 2023). Although those stressful culture conditions were found to impair cell growth and to trigger cell flocculation, both the recombinant gene expression (*EGFP*) and the intracellular rProt productivity (eGFP) were increased significantly under oscillatory culture conditions as compared to the corresponding homogenous counterpart used as a reference culture condition (RC).

Herein, the effect of those oscillatory culture conditions on the synthesis of a secretory recombinant protein (srProt) was investigated. For that purpose, the strain RIY308 producing the secreted CalB lipase from *Candida antarctica* under the control of pAOX1 (Velastegui et al., 2019a) was used. Cultures were performed at a dilution rate of 0.03 h^{-1} in SD1, SD2 and RC systems in YNBM medium, and samples were collected at steady-state after 5, 6 and 7 retention times. The same analytical procedures and calculations described in Chapter 2 was used, with the exception that instead of flow cytometry measurements, lipase activity was quantified as described elsewhere (Fickers et al., 2003). As compared to the RC, the

biomass decreased by 16% and 28% for SD1 and SD2 cultures, respectively, while an increase in both methanol uptake rate (*i.e.*, q_{MetOH}, 21% and 45%, respectively) and oxygen consumption rate (*i.e.*, q_{O2}, 21% and 45%, respectively) was observed (Table 1). The expression of *FLO* and *ERG* genes, used as reporters for cell flocculation and hypoxia stress, respectively (Velastegui et al., 2023), were upregulated in SD systems as compared to RC (Table 1). All these results are in line with those obtained with strain RIY230 grown in the same conditions (Velastegui et al., 2023).

Table 1: Parameters determined at steady state for *P. pastoris* strain RIY308 grown in bioreactor systems in RC, RC*, SD1, SD2 and SD3 configurations. Values are mean and standard deviation of measurements on duplicate cultures and three sampling times (i.e. after 5, 6 and 7 residence times). Medium YNBM (SD1, SD2, RC) or YNBMS (SD3, RC*) were used for continuous cultures. Gene expressions were normalized to that obtained in RC or RC* and expressed as fold change with RC or RC* conditions as a reference. MetOH: methanol, Sorb: sorbitol

Culture condition Carbon source	RC MetOH	SD1 MetOH	SD2 MetOH	RC* MetOH/Sorb	SD3 MetOH/Sorb
Biomass ($gDCW \cdot L^{-1}$)	6.6 □ 0.3	5.5 □ 0.2	4.7 □ 0.1	5.9 □ 0.1	5.2 □ 0.1
q_{MetOH} ($gMetOH \cdot (gDCW \cdot h)^{-1}$)	0.12 □ 0.05	0.15 □ 0.01	0.18 □ 0.02	0.06 □□□□□□	0.07 □□□□□□
q_{O2} ($mgO_2 \cdot (gDCW \cdot h)^{-1}$)	117 □ 5	139 □ 6	166 □ 7	90 □ 1	114 □ 2
FLO5	1	6	27	1	0.8
FLO11	1	11	33	1	1.7
ERG11	1	6	25	1	1
ERG25	1	1.7	3.8	1	0.7

For strain RIY230 (p*AOX1-EGFP*), the genes under the control of pAOX1, namely *AOX1* and *EGFP*, were upregulated in SD system as compared to RC system. Moreover, a significant increase in cell specific eGFP fluorescence was observed between SD1 and SD2 configurations (*i.e.*, 37% and 57 %, respectively) (Velastegui et al., 2023). Herein, a similar behavior was observed for strain RIY308, with the increase in *CALB* expression level by 30 % and 240 % in SD1 and SD2, as compared to RC, respectively (Figure 9).

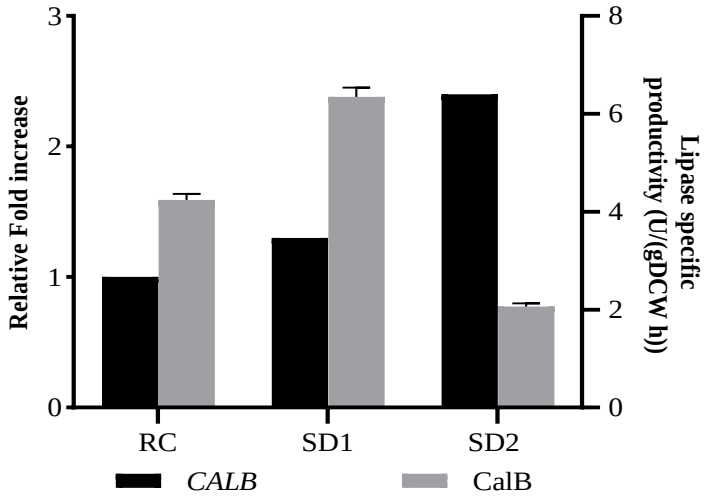

Figure 9: Relative expression level of genes *CALB* (black) and specific CalB lipase activity (grey) for *P. pastoris* strain RIY308 grown in bioreactor system in RC, SD1 and SD2 configurations. Values are mean and standard deviation on measurements from duplicate cultures and three sampling times. All values are statistically different considering $p<0.05$. Gene expression was normalised to that obtained for RC. Standard deviations on Ct values were less than 14 % of the *CALB* average value. Lipase activity is expressed in unit per g of dry cell weight ($U \cdot gDCW^{-1}$).

Regarding the extracellular enzyme activity, although a significant increase in the lipase productivity (*i.e.*, 50%) was observed in SD1 as compared to RC, the value was reduced by 42 % in SD2 (*i.e.* 1.8 $U \cdot (gDCW \cdot h)^{-1}$) where cells faced hypoxic culture conditions for extended period (180 sec., Figure 9).

Under stressful culture conditions such as hypoxic shock, misfolded or unfolded srProt may accumulate in the endoplasmic reticulum (ER), creating thus an ER-stress that activates the unfolded protein response (UPR). Considering this, genes encoding chaperones (Kar2), folding assisting proteins (Pdi1, Ero1) and transcriptional activator (Hac1) (Raschmanová et al., 2021b) were used as UPR-marker genes, and their relative expression in cells grown SD1 and SD2 systems was quantified and compared to that obtained in the RC configuration. As shown in Figure 10, there was a significant difference in the gene expression level between SD1 and SD2 culture configurations. Although gene expression levels were slightly increased in SD1 as compared to RC, the value in SD2 were increased by 3.2-fold for gene *HAC1* to 4.6 -fold for Kar2 encoding gene as compared to RC condition. This suggests that

an extended average cell residence time in hypoxic conditions triggered the UPR. This may also explain the lower CalB productivity found in SD2 (*i.e.* 1.8 $U \cdot (gDCW \cdot h)^{-1}$) although the CalB gene expression was significantly higher than that obtained for SD1 configuration (0.3 and 2.4 fold increased as compared to RC, respectively) (Figure 9).

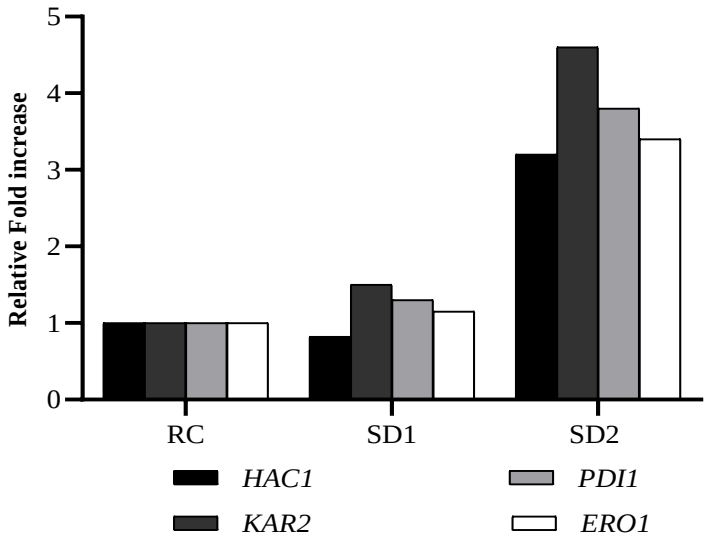

Figure 10: Fold change in relative gene expression for genes *HAC1* (black), *KAR2* (dark grey), *PDI1* (light grey) and *ERO1* (black) for *P. pastoris* strain RIY308 grown in bioreactor system in RC, SD1 and SD2 configurations. Values are mean and standard deviation on measurements from duplicate cultures and three sampling times. All values are statistically different considering $p<0.05$. Gene expression was normalized to that obtain in RC. Standard deviations on Ct values were less than 15 %, 12 %, 10 % and 15 % of the *HAC1*, *KAR2*, *PDI1* and *ERO1* average value, respectively.

Hypoxia is known as a stress factor, especially when *P. pastoris* is grown on methanol-based medium, as it requires oxygen for its oxidation in peroxisomes. Cofeeding methanol with sorbitol has been demonstrated as a beneficial strategy to reduce q_{O2} without any loss of rProt productivity ((Berrios et al., 2017; Carly et al., 2016; Niu, Daukandt, et al., 2013). Therefore, an SD3 culture configuration (methanol/sorbitol co-feeding, cell retention time in R2 of 180 s) was tested and compared to the homogenous co-feeding culture condition (RC*), the same conditions were used, except that air was supplied at a flow rate of 1 $L.min^{-1}$, instead of 1.5 $L.min^{-1}$. In those conditions, the expression level of *ERG11* and *ERG25* used here as a hypoxia reporter system were equal and lower (*i.e.*, 30 %) as compared to RC* system (Table

1), suggesting thus, that sorbitol cofeeding can contribute to reduce the hypoxic stress in cells. Regarding the cell performances, the biomass obtained at steady state in SD3 (*i.e*. 5.2 ± 0.1) was lower than in RC* (*i.e*. 5.9 ± 0.1) but slightly higher than culture operated in SD2 configuration (*i.e*. 4.7 ± 0.1, methanol feeding only, Table 1). This suggests that methanol/sorbitol cofeeding has a slight positive impact on cell growth. Both q_{MetOH} and q_{O2} were only slightly increased in SD3 configuration (*i.e*. 12% and 26 %, respectively) as compared to the corresponding increase observed in SD2 configuration (45% and 40% respectively, Figure 11, Table 1). Beside this, sorbitol uptake rate in SD3 and RC* were in the same range (Figure 11).

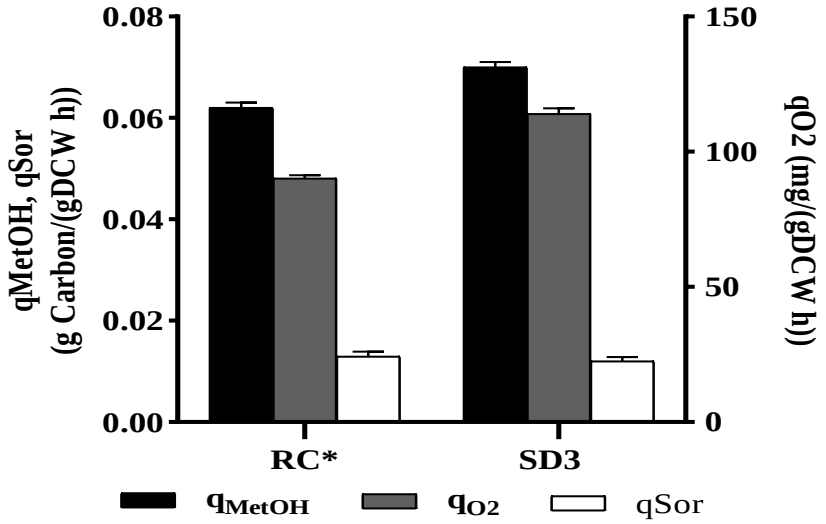

Figure 11: Specific methanol uptake rate ($q_{MetOH,}$ black), specific sorbitol uptake rate ($q_{Sor,}$ white) and oxygen consumption rate ($q_{O2,}$ grey) obtained at steady state for *P. pastoris* strain RIY308 grown in bioreactor systems in RC* and SD3 configurations. Values are mean and standard deviation on measurements from duplicate cultures and three sampling times. All values are statistically different considering $p<0.05$. q_{MetOH} and q_{Sor} is expressed in gram of carbon consumed per gram of dry cell weight produced and per hour while q_{O2} is expressed in mg of oxygen per gram of cell dry weight produced and per hour.

Except for Kar2 encoding an ER chaperone, the UPR-marker genes were down regulated in SD3 configuration as compared to RC* (Figure 12). This suggests that the cells were less under UPR stress in those culture conditions. Regarding the CalB lipase, the expression level of the corresponding encoding gene was reduced by 20 % in SD3 configuration as compared

to RC* (Figure 12). By contrast, the extracellular lipase productivity was increased by 13 % and 41% in SD3 configuration as compared to RC* and SD2 configuration (2.6 $U\cdot(gDCW\cdot h)^{-1}$ and 1.8 $U\cdot(gDCW\cdot h)^{-1}$), respectively (Figure 13).

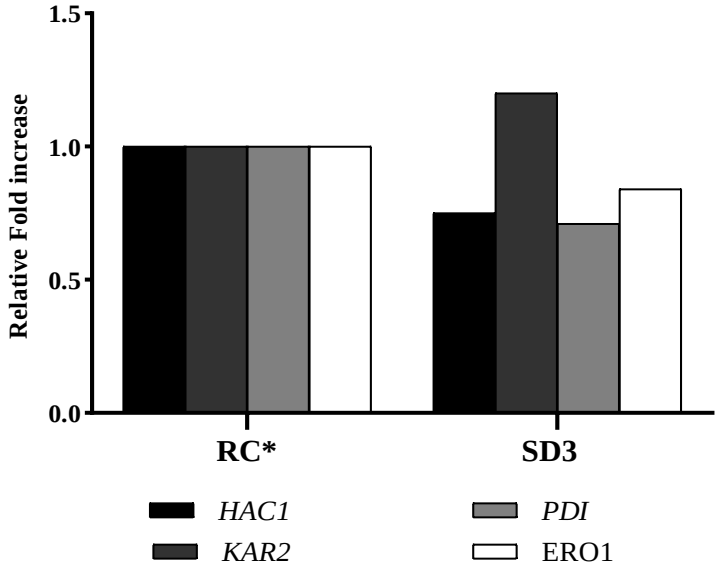

Figure 12: Fold change in relative gene expression for genes *HAC1* (black), *KAR2* (dark grey), *PDI1* (light grey) and *ERO1* (white) for *P. pastoris* strain RIY308 grown in bioreactor systems in RC* and SD3 configurations. Values are mean and standard deviation on measurements from duplicate cultures and three sampling times. All values are statistically different considering $p<0.05$. Gene expression was normalized to that obtain in RC. Standard deviations on Ct values were less than 12 %, 8 %, 20 % and 15 % of the *HAC1*, *KAR2, PDI1* and *ERO1* average value, respectively.

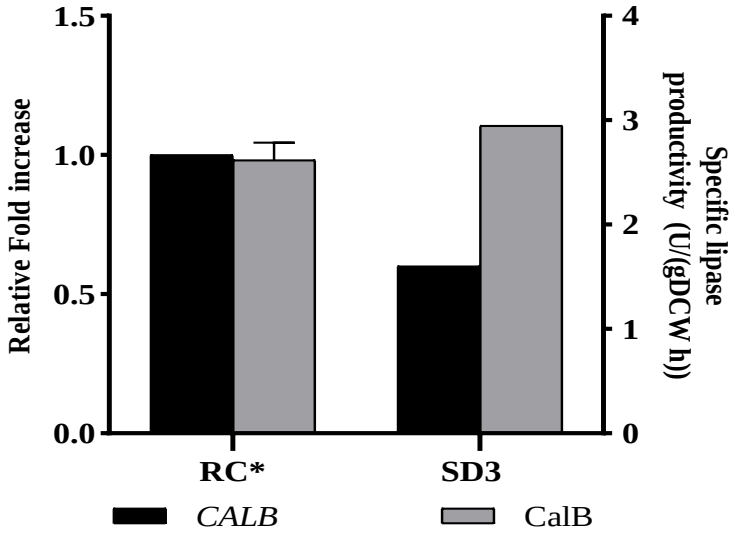

Figure 13: Fold change in relative gene expression for gene *CALB* (grey) and specific CalB lipase activity (black) for *P. pastoris* strain RIY308 grown in bioreactor systems in RC* and SD3 configurations. Values are mean and standard deviation on measurements from duplicate cultures and three sampling times. All values are statistically different considering $p<0.05$. Gene expression was normalized to that obtained in RC*. Standard deviations on Ct values were less than 14 % of the *CALB* average value. Lipase activity is expressed in unit per g of dry cell weight ($U \cdot gDCW^{-1}$).

Using a scale down approach, the oscillatory culture conditions faced by cells in large-scale bioreactor during methanol fed-batch were shown as detrimental for secretory protein production and that secretion pathway was the limiting step. Indeed, hypoxia and high methanol concentration were shown to trigger the UPR response that negatively affected the srProt productivity. Nevertheless, a methanol/sorbitol co-feeding strategy allowed the reduction of the UPR and the increase in srProt productivity.

CHAPTER 4:

Downregulation of pAOX1 by organic nitrogen sources

1) Introduction

The methylotrophic yeast *Pichia pastoris (Komagataella spp.*) has become over the years one of the most extensively used cell factory for the production of heterologous proteins. More recently, this yeast has also emerged as a host for metabolite synthesis (Gasser & Mattanovich, 2018; Prabhu & Dasu, 2017). *P. pastoris* presents the advantage over other yeast systems (i.e., *Saccharomyces cerevisiae*), to produce properly folded proteins with a very high productivity. This could be related to the ability of *P. pastoris* to grow at high cell density as well as on the availability of strong regulated promoters that drive recombinant gene expression. Among them, the promoter of the *AOX1* gene (encoding methanol oxidase, the first step of methanol utilization) is the most widely used. It is tightly regulated by methanol, and feeding of methanol during the protein production phase is the most used strategy to achieve high pAOX1 induction levels. However, the utilization of methanol as carbon source and inducer poses the major drawback of the requirement of high amounts of oxygen for cell respiration and methanol oxidation. We previously reported the benefits of methanol/sorbitol co-feeding (both methanol/sorbitol ratio and specific feeding rate) in regard to reducing oxygen demand without loss of pAOX1 induction level and thus protein productivity (Carly et al., 2016; Niu, Jost, et al., 2013). Additionally, the reduction of methanol consumption also contributes to reducing the formation of toxic degradation intermediates such as hydrogen peroxide and formaldehyde (Jungo et al., 2007).

Nitrogen sources also play a key role in yeast physiology, including cell growth and recombinant protein synthesis. For example, when cod skin was used as the co-nitrogen source, (G. Y. Li et al., 2018) reported a 21% higher cell density and 18.3% higher human cathepsin S yield. Different protein sources can however also alter regulation of metabolic pathways. For instance, (Shen et al., 1998), using β-lactamase as a reporter system, demonstrated that the promoter of glutathione-dependent formaldehyde dehydrogenase (FLD), a key enzyme of methanol catabolism in *P. pastoris*, is strongly induced by methylamine. By contrast, in medium containing ammonium sulfate, expression levels of methanol utilization genes and PEX genes were significantly higher than in medium containing proline as a nitrogen source (Rumjantsev et al., 2014).

Casamino acids (CA) constitute a mixture of amino acids obtained from the acid hydrolysis of casein (Mueller & Johnson, 1941). It contains all of the amino acids except tryptophan, which is degraded during sulfuric acid hydrolysis. For pAOX1-based expression system, several authors have reported a positive effect of CA on protein productivity. (Cha et al., 2005) reported that addition of CA caused a three-fold increase of the amount of secreted human interleukin-2; (Shi et al., 2003) reported a positive effect of CA (several fold increase) on the productivity of single-chain antibody; and (J. Wang et al., 2005) reported a 3.5-fold increase in the production yield of merozoite surface protein 3. A positive effect of CA was also found on the secretion of recombinant dengue virus serotype-3 envelope domain III (Kaushik et al., 2016) and on the stability of mouse epidermal growth factor in minimal medium (Clare et al., 1991). It also prevented the partial deglycosylation or proteolysis of the haloalkane dehalogenase LinB (Nakamura et al., 2006). Moreover, the presence of CA in the culture media does not interfere in the protein purification methods, unlike components of yeast extract and peptones (Nakamura et al., 2006).

Although the positive effect of CA on process productivity has been demonstrated, it concerned only the final product (the recombinant protein itself), and little is known about the regulatory effect of CA at gene expression level and less in the context of methanol-sorbitol-based medium. Here, we report the regulatory effect of CA on pAOX1 induction using enhanced green fluorescent protein (EGFP) as reported system monitored by flow cytometry.

2) Materials and Methods

2.1) Strains, media and culture conditions

Strains used in this study are listed in Table S1(see appendix section). *Escherichia coli* was grown at 37°C in Luria-Bertani medium supplemented with appropriate antibiotic (ampicillin, 100 µg/ml; zeocin 50 µg/ml; chloramphenicol 25 µg/ml). The yeast media used were YPD and MS media (3.6 g/L of Yeast nitrogen broth [Sigma Aldrich]; 0.4 mg/L of biotin; 2.3 g/L of sorbitol; 5 ml/L of methanol; 2.5 g/L of $(NH_4)_2SO_4$) supplemented with CA (BD Difco) as stipulated in the text. MS medium was buffered with 100 mM of Na/K phosphate buffer at pH 5.8. M medium composition was the same as MS medium except that it did not contain sorbitol. Precultures were performed at 30°C for 12 hr in YPD medium.

MS medium was inoculated at an initial optical density at 600 nm of 0.1. Yeast transformants were selected from selective medium agar plates (6.8 g/L of YNB without amino acids; 10 g/L of glucose, 0.4 mg/L of biotin; 0.1 M of potassium phosphate buffer, pH 6; 20 g/L of agar).

2.2) General molecular biology techniques

Standard media and techniques were used for *E. coli* (Sambrook et al., 1989), and the media and techniques used for *P. pastoris* have been described elsewhere (Cregg et al., 2010). The restriction enzymes and ligase were supplied from New England Biolabs. Q5 DNA polymerase (New England Biolabs) was used for cloning, and ExTaq (Takara, France) was used for verifying genomic structure. Polymerase chain reaction (PCR) was performed using the primers listed in Table S1, appendix section. The amplicons were purified from agarose gels using Monarch DNA purification kit (NEB). DNA sequencing was performed by GATC Biotech (https://www.gatc-biotech.com), and the primers were synthetized by Eurogentec (https://secure. eurogentec.com/). Transcriptional analyses were performed as described in (Sassi et al., 2016) with primer pair listed in Table S1. The reference actin gene (ACT) was amplified using primers Act-F and Act-R. Prior to yeast transformation as described in (J. Lin-Cereghino et al., 2005), expression vectors were linearized by StuI restriction. Correctness of the vector sequence was verified by DNA sequencing using primer AOX-int.

2.3) Vectors and yeast strain construction

To construct vector RIP171 (hosted in *E. coli* strain RIE171), enhanced green fluorescent protein (EGFP) encoding gene was PCR amplified from vector PP246 (Table S1, appendix) using primers pair PAE01/PAE02. The resulting 800 BP fragment was digested by EcoRI and BamHI and cloned at corresponding sites of pIB4 vector (Table S3, appendix section). *P. pastoris* strain GS115 was then transformed with the resulting construct to give rise to strain RIY230. To construct plasmid RIP257 (hosted in *E. coli* strain RIE257), CalB gene from *Candida antarctica* lipase B was PCR amplified from plasmid RIE191 using the primers CalB-F and CalB-R (Table S3, appendix section). The resulting 1 kb fragment was digested by EcoRI and BamHI and cloned at corresponding site of pIB4 vector to give rise to vector RIE255. The full-length *S. cerevisiae* secretion signal for α-mating factor (αMF) was amplified from plasmid pPTK005-3a-αMF (Table S3, appendix section) using the

primers αMF-F and αMF-R. The resulting 267 BP fragment was digested with EcoRI and cloned in RIE255 at corresponding site. Correctness of the construct was verified by analytical PCR and DNA sequencing using primers αMF-F and GFP-R. The final construct was then digested by StuI prior transformation of *P. pastoris* strain GS115 to give yield strain RIY308. Strain RIY230 was obtained by transforming strain *P. pastoris* GS115 with RIE171 vector digested by StuI,

2.4) Analytical methods

Cell growth was monitored either by optical density at 600 nm or dry cell weight (DCW) as previously described (Carly et al., 2016). Methanol and sorbitol were analyzed by isocratic RID-HPLC (Agilent 1100 series, Agilent Technologies) using an Aminex HPX-87H ion-exclusion column (300 × 7.8 mm Bio-Rad, Hercules, USA) with 5-mM H_2SO_4 as mobile phase at a flow rate of 0.5 ml/min at 30°C. Cell fluorescence was quantified using a BD Accuri C6 flow cytometer (excitation was performed with a 20-mW, 488-nm solid-state blue laser, and the emission wavelength was 533 nm). For each sample, 40,000 cells were analyzed using the FL1 channel to identify the fluorescence associated with the synthesis of the green fluorescent protein. For both the FL1 and FSC, the area of the global signal was recorded (FL1-A, FSC-A). The flow cytometry dotplots (FL1/FSC) were analyzed using CFlowPlus software (Accuri, BD Bioscience). Median fluorescence was calculated, and the gates were defined as follows. Gate Q2-LR correspond to the fluorescence not related to EGFP (i.e., fluorescence of strain RIY231), and gate Q2-UR correspond to the fluorescence of EGFP. The percentages represent the proportion of cells in each gate. Induction rate was calculated as the increase of cell fluorescence between 8 and 14 hr of growth and expressed as fluorescence unit (FU) per hour (hr). The lipase activity in the culture supernatant was determined by monitoring the hydrolysis of p-nitrophenyl butyrate (p-NPB), as described by (Fickers et al., 2003). Briefly, p-NPB was dissolved in acetonitrile (20% v/v) and added to a final concentration of 1 mM in 100 mM phosphate buffer, pH 7.2, containing 100-mM NaCl. The resulting solution was sonicated for 2 min. Then, the reaction of 20 µl of supernatant with 1 ml of p-NPB solution was monitored by measuring absorbance at 405 nm (A405) for 3 min. Supernatant samples were diluted to obtain initial velocities below A405 of 0.3 U/min. All lipase activity assays were performed at least in duplicate on two independent cultures.

One unit of lipase activity was defined as the amount of enzyme releasing 1 μmol of p-nitrophenol per minute at 25°C and pH 7.2 (εPNP = 0.0148 μM^{-1}/cm).

3) Results and Discussions

3.1) Cellular metabolism in regard to CA concentration

The effect of CA addition in the culture medium containing sorbitol and methanol as carbon sources was investigated. For that purpose, cell growth and carbon uptake rate were monitored for 28 hr in MS medium containing 0.1% and 1% of CA and compared with that obtained for the nonsupplemented media. Addition of CA positively affects cell growth. Biomass obtained at the end of the exponential growth phase was increased 3.4-fold in the presence of 1% CA as compared with the nonsupplemented medium (1.65 and 0.48 gDCW/L, respectively). The corresponding specific growth rates were equal to 0.03, 0.04 and 0.07 hr^{-1} in medium containing 0%, 0.1%, and 1% of CA, respectively (data not shown). These values are in the range of the maximum specific growth rate observed in optimal growth conditions on either methanol or sorbitol medium (i.e., 0.10 and 0.04 hr^{-1}; (Canales et al., 2018)).

To assess the influence of CA on sorbitol and methanol uptake capacity, q_{sorb} and q_{met} were calculated during the exponential growth phase (between 8 and 14 hr of culture). As shown in Figure 14, a slight increase of q_{sorb} could be observed in the presence of CA; however, this effect is not significant. By contrast, q_{met} was significantly reduced (i.e., 6.6-fold) in the presence of 1% CA as compared with the nonsupplemented MS medium (1.66 and 0.25 mmol/gDCW.h, respectively).

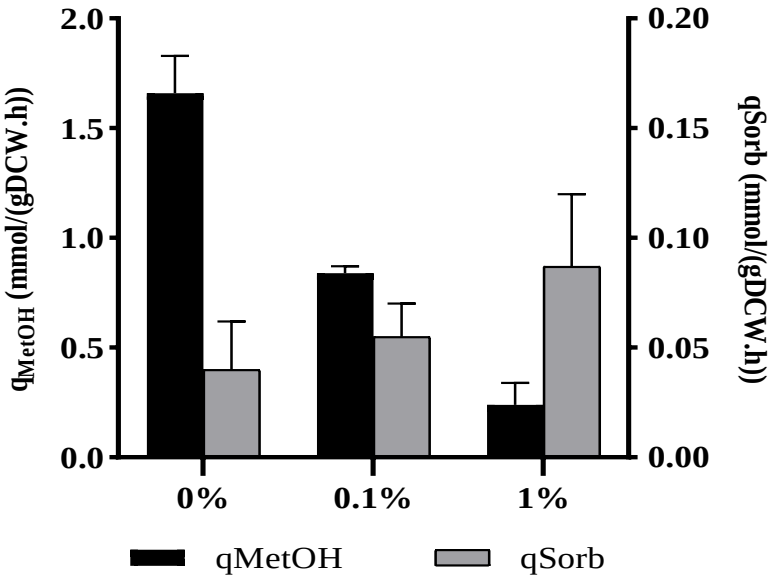

Figure 14: Specific uptake rate for methanol (black) and sorbitol (light grey) during culture of *Pichia pastoris* in MS medium supplemented with 0%, 0.1%, and 1% casamino acids. DCW: dry cell weight,

In addition to nitrogen source, amino acids from CA could also serve as carbon and energy sources. They could be easily converted into pyruvate, acetyl-CoA, and different intermediates of the Krebs cycle and thus feed the glucose metabolism pathway. Recently, (Celińska et al., 2017) demonstrated that in *Yarrowia lipolytica*, bactopeptone could be used as a carbon source to build biomass. Therefore, to assess the contribution of CA in biomass formation, uptake rate of CA was estimated by flux balance analysis (FBA). For this estimation, a metabolic model of 58 reactions depicting sorbitol and methanol catabolism, amino acid synthesis and catabolism, energy production as ATP, and biomass synthesis was built (see Appendix section). The measured values of both q_{met} and q_{sorb} and biomass formation rate (q_X) were considered as FBA inputs (see Supplementary Data, Appendix section). Because oxygen uptake rate (q_{O2}) did not show any important variations in previous cultures performed in bioreactor under either fully aerobic or limited oxygen conditions (Carly et al., 2016), the average value (4.07 mmol/gDWC.h) was used as an additional input to the calculation. The FBA was performed by minimizing the ATP flux for cell maintenance,

considering thus all the possible reaction in which ATP might be consumed, excluding the biomass formation. CA uptake rates estimated by the model for cells growing in MS medium supplemented with 0.1% and 1% were respectively equal to 6.79 and 8.72 C-mol/ gDCW.h. This value demonstrated that an important part of energy required for cell growth was derived from CA catabolism. This observation could, thus, explain why a higher biomass was obtained when less methanol is consumed.

3.2) Effect of CA on pAOX1 induction

In order to assess the effect of CA on pAOX1 promoter induction, a reporter system based on the green fluorescent protein from the jelly fish *Aequorea victoria* was constructed. For that purpose, the EGFP-encoding sequence was cloned into IB4 vector; and the resulting construct RIE171 was integrated into the genome of strain GS115. The genotype of the resulting strain RIY230 was verified by analytical PCR, and expression of the EGFP encoding gene in the presence of methanol in the culture medium was verified by qPCR (data not shown). This reporter system enables the quantification of pAOX1 induction at the single-cell level when combined with flow cytometry analysis.

Strain RIY230 was grown in MS medium supplemented either with CA or not, and EGFP fluorescence was analyzed by flow cytometry at various time points for 30 hr. As shown in Figure 15, addition of CA negatively affects the induction level of pAOX1. Indeed, after 14 hr of growth, the median fluorescence values were equal to 49299 FU/cell, 22803 FU/cell, and 2185 FU/cell in the presence of 0%, 0.1%, and 1% CA, respectively. These differences were also observed at the gene expression level as at the early exponential phase (i.e., after 3 hr) EGFP transcription was reduced by 2.5 and 12-fold in the presence of 0.1% and 1% CA, respectively, compared with in the absence of CA (Figure 16).

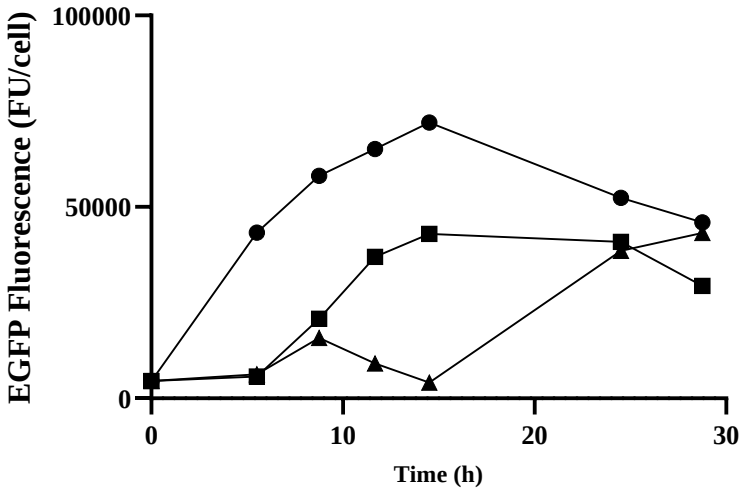

Figure 15**:** Time course of EGFP fluorescence per cell of strain RIY258 (pAOX1-GFP, (b)) during culture in MS medium supplemented with 0% (circle), 0.1% (square), and 1% (triangle) of casamino acids. The values are mean fluorescence of 40,000 cells from two independent experiments. EGFP: enhanced green fluorescent protein.

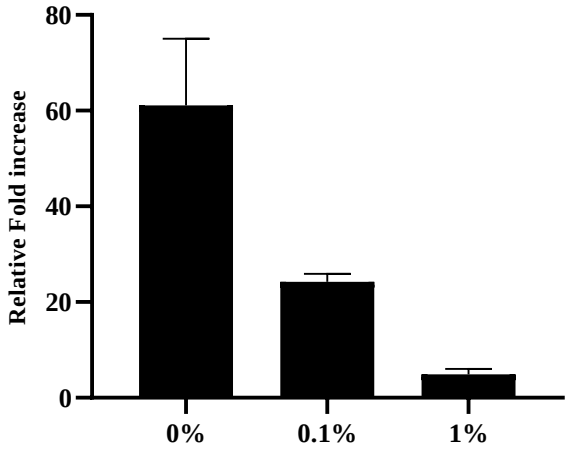

Figure 16: Transcription of mRNA from enhanced green fluorescent protein encoding gene in strain RIY258 after 5 hr of growth in MS medium supplemented with 0%, 1%, and 1% of casamino acids. The data were normalized to that of actin gene. The values are the means.

To further characterize this phenomenon, the flow cytometry dotplots (FL1/FSC) of strain RIY230 growing in MS medium supplemented with CA were analyzed after 8 hr (early

growth phase), 14 hr (midgrowth phase), and 24 hr (end of growth phase; Figure 17). On the dotplots, the area delimited by Q1-LR corresponds to a noninduced phenotype (i.e., EGFP non-producing cells), whereas the area delimited by gate Q1-UR corresponds to an induced phenotype (i.e., EGFP producing cells). The flow cytometry analysis showed that CA influences the kinetic of pAOX1 induction. Indeed, in the control medium (CA nonsupplemented), 96.2% of the cell population is in an induced state after 8 hr of growth, and this value drops to 51.3% and 8.3% in medium containing 0.1% and 1% CA, respectively. At the end of the exponential growth phase (i.e., after 24 hr), the proportion of the cell population in an induced state is equal to 97.1%, 91.6%, and 74.2% for culture supplemented with 0%, 0.1%, and 1% CA, respectively. After 14 hr of growth, an intermediate situation could be observed. Therefore, the decrease of mean fluorescence value observed upon addition of CA into the culture medium seems be a consequence of a lower proportion of the cell population that are in an induced state rather than a lower level of pAOX1 induction. It is worth mentioning that even in the absence of CA, a proportion of the cell population (between 2% and 4%) remained in a noninduced state regardless of the growth phase considered. Moreover, the transition rate from a noninduced state to an induced state seems also to be directly correlated to the growth rate (Figure 18; $R^2 = 0.92$), this growth rate being modulated by the cell capacity to metabolize methanol through the action of the AOX1 oxidase.

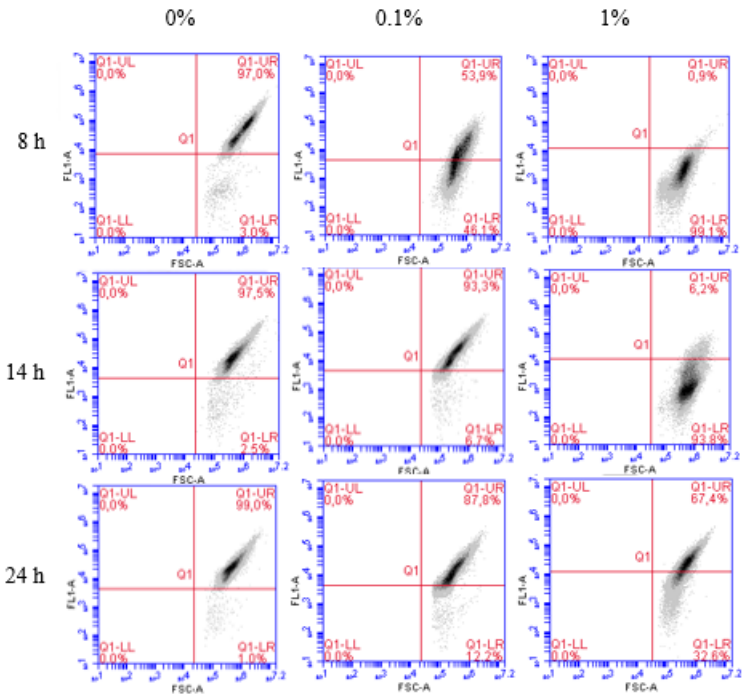

Figure 17: Flow cytometry dotplots (FL1/FSC) of strain RIY258 growing in MS medium supplemented with casamino acids (0%, 0.1%, and 1%) were analyzed after 8 hr (early growth phase), 14 hr (midgrowth phase), and 24 hr (end of growth phase).

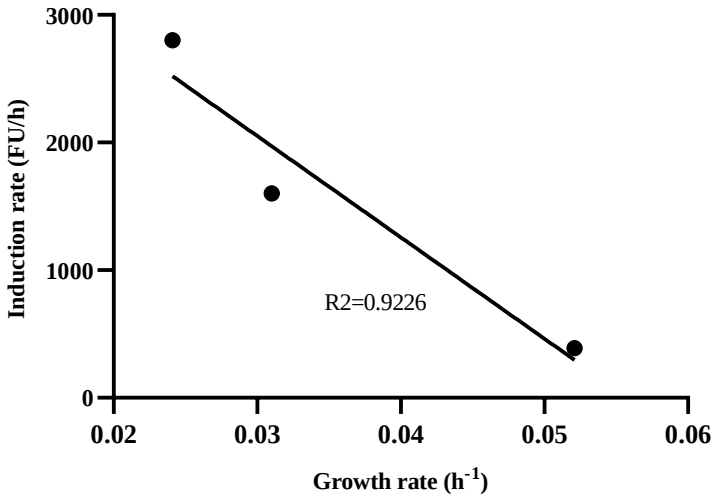

Figure 18: Evolution of the induction rate as a function of the growth rate. The induction rate was calculated as the difference of median fluorescence measured during the exponential

growth phase (i.e., between 8 and 14 hr of culture) of *Pichia pastoris* strain RIY230. The growth rate was calculated for the same time period. FU: fluorescence unit.

To confirm that the effect of CA on pAOX1 induction is not related to the presence of sorbitol in the medium, strain RIY230 was grown in medium containing methanol as sole carbon source (M medium). In the midgrowth phase (i.e., after 14 hr), mean fluorescence values were equal to 142,065 FU and 74,442 FU, in the presence of 0% and 1% of CA (data not shown), demonstrating thus that the observed downregulation of pAOX1 is indeed related to CA content and not related to sorbitol.

In bioprocesses, soypeptone, a papaic digestion of soya flour, is increasingly used as an alternative to animal nitrogen source such as casein peptone or tryptone. In order to investigate a possible effect of this nitrogen source on pAOX1 regulation, strain RIY230 was grown in MS medium containing 0%, 0.1%, and 1% of soytone, and samples were collected during the exponential growth phase (i.e., after 18 hr, data not shown). In the presence of 0.1% and 1% of soypeptone, the median fluorescence value was reduced by 29% and 32%, respectively, as compared with the nonsupplemented medium (that yielded to 48594 FU; data not shown). In the same conditions, q_{Met} was reduced by 20% (i.e., 0.52 and 0.42 mmol/gDCW.h, respectively; data not shown).

We previously reported in *Y. lipolytica* that the constitutive peptides from casein hydrolysate regulate the expression of some genes and that the regulatory effect could be related to the method of casein hydrolysis (Fickers et al., 2003). Here, the downregulation effect could be observed for two drastically different protein hydrolysates (casein and soya flour) obtained by hydrolysis with two different proteases (papain and pepsin). Therefore, it is unlikely that the observed downregulation effect was triggered by a similar peptide contained in these protein hydrolysates. The downregulation effect is more likely to link with the lower ability of cell to metabolize methanol in the presence of these protein hydrolysates (described in Section 3.4).

3.3) Effect of CA on methanol metabolism

In *P. pastoris*, the formaldehyde produced by oxidation of methanol by AOX enzymes in the peroxisome could be further metabolized by two different routes, known as the assimilatory

pathway (that leads to biomass and energy production) and the dissimilatory pathway (that ends in the formation of carbon dioxide). To investigate the effect of CA on methanol metabolism, key genes in those pathways, namely, AOX1 (encoding alcohol oxidase 1, first step of methanol catabolism), DAS (encoding dihydroxyacetone synthase, assimilatory pathway), and FLD (encoding FLD, dissimilatory pathway), were monitored in relation to CA content. As shown in Figure 19, the AOX1 expression level was reduced by 1.9- and 4.3-fold in the presence of 0.1% and 1% of CA, respectively, compared with in its absence. Similarly, the presence of 0.1% and 1% of CA reduced DAS and FLD levels by 1.3- and 1.9-fold and by 1.2- and 1.1-fold, respectively. Therefore, CA seems to affect methanol metabolism at different key node.

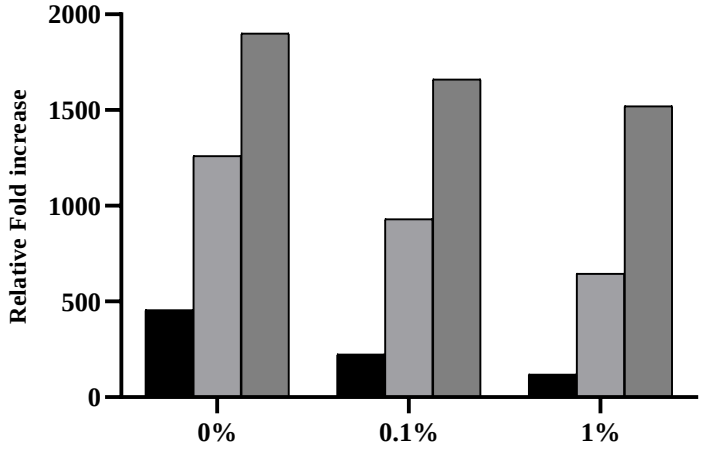

Figure 19: Transcription of mRNA from AOX1 (encodes alcohol oxidases, black), DAS (encodes dihydroxyacetone synthase, light grey), and FLD (encodes formaldehyde dehydrogenase, medium grey) in strain RIY258 after 5 hr of growth in MS medium supplemented with 0%, 1%, and 1% of casamino acids. The data were normalized to that of actin gene. The values are the means calculated from two independent replicates. In all cases, standard deviation was less than 30%.

3.4) Effect of CA on protein productivity

The effect of CA was investigated for a protein of interest. Lipases are serine hydrolase that in nature catalyze the hydrolysis of ester bonds of long-chain triacylglycerol into fatty acid and glycerol. In addition, in favorable thermodynamic conditions, they are also able to

catalyze reactions of synthesis such as esterification and amidation. Panoply of applications for lipase has been developed (Sarmah et al., 2018). The gene encoding the CalB lipase from *Candida antartica* was fused to αMF signal peptide from *S. cerevisiae* and cloned under the pAOX1 promoter. The final construct was then introduced in the genome of GS115 strain. The resulting strain RIY308 was grown in MS medium containing 0%, 0.1%, or 1% CA, and the lipase productivity was determined after 24 hr. As shown in Figure 20, addition of CA led to a reduced lipase productivity. For 1% CA, a 43% reduction of lipase productivity could be observed.

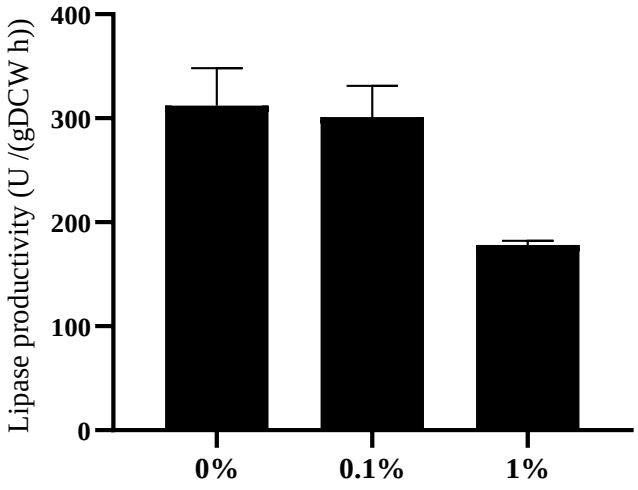

Figure 20: Lipase productivity in culture supernatant after 24 hr of growth of strain RIY308 grown in MS medium containing 0%, 0.1%, and 1% casamino acids. The values are mean values from three independent experiments. The bars represent the standard deviations. DCW: dry cell weight.

2) Conclusions

The nitrogenous composition of a culture medium has a significant impact on cell physiology, including growth rate and the capacity to produce recombinant protein at high yield. Here, we focus on the effect of organic nitrogen sources on the regulation of the promoter of the *AOX1* gene encoding alcohol oxidase used to drive the expression of recombinant genes. Our investigations were performed using methanol and sorbitol as main

carbon sources because we previously demonstrated the beneficial effect of this dual carbon source-based medium to enhance the yield and productivity of recombinant protein using *P. pastoris* as a cell factory (Carly et al., 2016; Niu, Jost, et al., 2013). Although addition of CA to the culture medium enhances both the cell growth rate and the final biomass, it significantly lowers the cell capacity to metabolize methanol. This effect was not observed for sorbitol, whose conversion into fructose in the presence of NAD^+ is catalyzed by a sorbitol dehydrogenase constitutively produced within the cell (Periyasamy et al., 2013). From this, we could hypothesize that the effect of CA could be more gene-specific than pleotropic (i.e., inhibition of carbon source catabolism). Single-cell analysis by means of flow cytometry and of EGFP reporter system demonstrated that nitrogenous compounds, either free amino acid or small peptide, downregulate the expression level of a gene under the control of the pAOX1 promoter (i.e., EGFP). This could also explain the lower methanol consumption rate observed in the presence of CA because AOX1 is the first enzyme involved in methanol catabolism. Our data clearly demonstrated that CA does affect not only the induction level of pAOX1 promoter but also the kinetics of induction. The transition of cells from a noninduced state to an induced state was shown related to the CA concentration in the medium. Moreover, the transition rate to an induced state was found to be growth rate dependent. Despite reports on the positive effects of organic nitrogen source on protein productivity, here, we provide evidence that CA in fact downregulates AOX1 expression as a consequence of a lower ability to catabolize methanol and a lower capacity to synthetize recombinant protein at the cell level.

CHAPTER 5:

General conclusions and perspectives

1) General conclusions

In the present work, the effect of loss of mixing quality with respect to methanol concentration and oxygen availability on the general performance of *P. pastoris* (i.e. growth capacity, pAOX1 promoter induction level and extracellular rProt secretion) was elucidated, also a strategy to decrease the metabolic burdens that emerge in large-scale behavior was proposed. For this, scale down (SD) systems were developed to mimic the medium heterogeneity that occurs in large-scale bioreactors. In such system, cells face oscillatory culture conditions (between unfavorable to more favorable conditions) as those expected in large-scale bioreactors (i.e. 10 and 100 m^3).

Despite the evidence of negative effects on other expression systems of oscillatory behavior, in **chapter 2** we demonstrated that these *a priori* unfavorable conditions that cannot be circumvented in large-scale bioreactors are in fact beneficial for the pAOX1 induction level, and at this point, for the expression of intracellular rProt such as eGFP, which in this case was also used as a reporter protein for the expression of the *AOX1* gene. It is worth mentioning that the improvement of productive capacities is accentuated with increasing retention time in the *a priori* unfavorable microenvironment, thus demonstrating that scale-up in this case would benefit *P. pastoris* performance as an rProt expression system.

In scale-down behavior, flocculation could be observed, which has been reported as a mechanism that cells triggers to survive harsh culture conditions (Bony et al., 1997; Hope et al., 2017). Through flow cytometry, a diversification of the population was observed, being the cells that tend to flocculate those that showed a higher eGFP productive capacity with respect to non-flocculated cells, which is a clear advantage.

It should be considered that the vast majority of rProt are produced in *P. pastoris* extracellularly to facilitate the downstream process, and as mentioned in chapter 1, many bottlenecks at traslational, post-traslational and secretion level have been documented. In **chapter 3**, we demonstrated that the increase in retention time in unfavorable microenvironments (i.e 180 s) under oscillatory behavior, contrary to what happened regarding the expression of pAOX1 induction level, is detrimental for secretory protein production, as lipase activity value decrease 42% in comparison to the reference condition.

Some UPR-related genes (i.e. *HAC1, PDI, ERO1, KAR2*) expression level was measured, showing an over-induction in most of them, which demonstrate that in the aforementioned condition the secretory pathway become a rate limiting step.

Hypoxia and high methanol concentration are two factors that have been observed to trigger the UPR response which negatively affects the productivity of extracellular rProt. It has been demonstrated that co-feeding strategy reduce considerably, methanol and oxygen requirements of the cells. By using the methanol/sorbitol cofeeding strategy in a scale-down behavior, it was possible to decrease the expression of UPR, which resulted in improved productivity of extracellular CALB.

Regarding culture media formulation, an adequate supplementation with organic nitrogen sources such as yeast extract, soybean peptone or casamino acids has been shown to benefit the growth capacity of *P. pastoris*. Despite the advantage of supplementation on cell growth, in **chapter 4** we demonstrated that the addition of casamino acids (CA) to the culture medium has an inhibitory effect on the MUT pathway, which deregulates the expression of the pAOX1 promoter because of the loss of methanol consumption ability. In this case, reduction of pAOX1 induction level is accompanied by a decrease in the ability to synthesize rProt (CALB), despite the evident improvement in terms of specific growth rate and final biomass concentration obtained.

2) Future perspectives

The study of oscillatory conditions on large-scale behavior using 2-compartment SD systems was successfully implemented. Contrary to what was expected, the propitiated *a priori* unfavorable microenvironment positively affects the level of induction of the pAOX1 promoter, as well as the production of intracellular proteins such as eGFP.

Additionally, under scale-down conditions, the cells flocculate, and prove to be more productive than non-flocculated cells. We were able to confirm the relation between cell flocculation to the expression of *FLO* gene family (De et al., 2020b). From the above then, the study of the expression of *FLO* gene family can be exploited. For this purpose, optimization of operational conditions and culture medium, as well as genetic manipulation

can be the first steps in this direction, to induce improvement in methanol consumption capacity as well as rProt productivity.

Induction of cell flocculation could require the arrest of cells in this morphotype, as cells tend to return to a non-flocculent morphotype (Rebnegger et al., 2014). This strategy has been suggested to improve the production of fatty acids with *Yarrowia lipolytica* (Xie, 2017). Nevertheless, at technical level it would result a considerable challenge, so alternatives to the traditional stirred tank bioreactor could be explored, such as fixed bed and fluidized bed bioreactors, which would open a new and interesting perspective to the production of rProt using *P. pastoris*.

Following the same line as the above mentioned, the proposed SD configuration already provides a significant improvement in the performance of *P. pastoris*, which means that the proposed SD system as a rProt production modality could be an alternative to the use of large-scale bioreactors (i.e. larger than 10 m^3). Although the implementation of 2-compartment SD systems is time consuming and technologically challenging, the use of 1-compartment SD systems could be a useful alternative in this context.

We found that under SD conditions, there is an overexpression of some UPR genes (*HAC1*, *PDI, ERO1, KAR2*), which has been related to the drop in extracellular CalB productivity as a consequence of ER stress triggering. We have described cofeeding with methanol/sorbitol mixture to restore homeostasis, reducing UPR and recovering CalB productivity levels. Considering the presence of population diversification with respect to the level of pAOX1 induction, the existence of the same phenomenon at the level of UPR expression is possible.

Recently, some UPR reporter systems have been determined that allow easy and rapid monitoring at the single-cell level by flow cytometry (Raschmanová et al., 2019), which in tune with the advances in the understanding of the relationship between UPR and rProt productivity, will allow new ways of bioprocess control. Recently, segregostat has been proposed as a new and ground-breaking concept (Sassi et al., 2019), which consists of controlling phenotypic diversification for the benefit of bioprocess performance.

It is worth mentioning that other fundamental culture conditions such as pH and temperature modulate growth rate, the pAOX1 promoter induction level as well as rProt productivity

(Berrios et al., 2017; Çalik et al., 2010), so it is necessary to design experiments based on SD systems to elucidate the effect of the formation of concentration gradients of this variables as a way to describe in a more comprehensive way the complex problem of rProt with *P. pastoris* in large scale bioreactors.

Finally, we highlight nitrogen source selection as an important consideration for AOX1-based protein production. In this context, screening in mini bioreactors has become popular tool for high throughput optimization, although the challenge of size change is also applicable in this case. Therefore, it is necessary to determine how culture media rheology in the mini bioreactors affects cell metabolism (C. Li et al., 2019).

3) Main outputs of the thesis

1. Using Scale-Down (SD) systems, we could mimic in some extent cell oscillating conditions between an *a priori* unfavorable microenvironments and a more favorable microenvironment regarding methanol concentration and dissolved oxygen tension propitiated in large-scale bioreactors. Our results showed that SD behavior are in fact profitable for the induction of pAOX1 promoter and intracellular eGFP specific productivity. We were also able to describe diversification of the population, where flocculent cells shows a higher level of pAOX1 induction with respect to non-flocculated cells.
2. Despite the pAOX1 improvement under SD behavior, ER stress emerges when cells stayed longer in an *a priori* unfavorable microenvironment. This situation triggers UPR overexpression, and a drop in extracellular CALB specific productivity was observed. Fortunately, methanol/sorbitol co-feeding in SD behavior reduces UPR signaling, thus restoring (even slight improving) productive capacities in comparison with the reference condition.
3. We found that organic nitrogen supplementation (i.e. Casamino acids), although it improves biomass yields and specific growth rate of *P. pastoris*, has a negative impact on pAOX1 promoter induction level, as well as CalB productivity.

APPENDIX

Chapter 2

Table S1: Primers used in chapter 2

Primer name	Sequence (5'→ 3')	Reference
β-Actin_Fo	AGATGGCTCCGAGAAGTTCA	(C. Theron et al., 2020)
β-Actin_Rev	GTTGCTCAGAGGGCTTCAAC	(C. Theron et al., 2020)
eGFP_Fo	ACGTAAACGGCCACAAGTTC	(Velastegui et al., 2019b)
eGFP_Rev	AAGTCGTGCTGCTTCATGTG	(Velastegui et al., 2019b)
FLO5_Fo	CAGGCAAGTGATGACAATTCAATACAGGAC	(De et al., 2020a)
FLO5_Rev	CGTTTGGTATCCCATAAAGTGTTGTGGTG	(De et al., 2020a)
FLO11_Fo	AGTCCACACACCATTGACAACTGC	(De et al., 2020a)
FLO11_Rev	ACACCAGTAACTATTGTAGCAACTGAGC	(De et al., 2020a)
ERG11_Fo	GATGGTAGCAAGATGACCG	(Adelantado et al., 2017)
ERG11_Rev	TCCTGCAGCTCTGGTTTC	(Adelantado et al., 2017)
ERG25_Fo	CAATGACACCCTTGCCAC	(Adelantado et al., 2017)
ERG25_Rev	CCCACTGCTCCTTGTTG	(Adelantado et al., 2017)
AOX1_Fo	GTGCCCAACTTGAACTGAGG	(Velastegui et al., 2019b)
AOX1_Rev	AGATCGTCAAATGGGGTGGT	(Velastegui et al., 2019b)

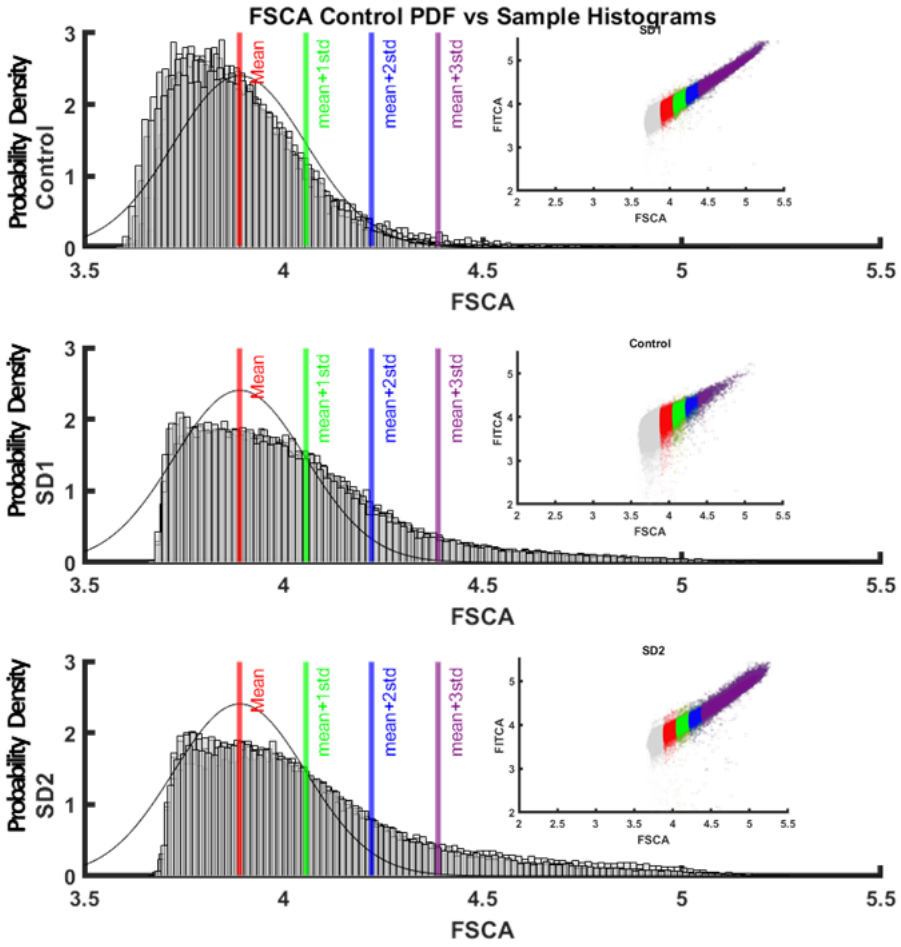

Figure S1:Flow cytometry analysis of cells grown in RC, SD1 and SD2 systems. Histograms represent the cell distribution according to front scattering data (FSCA). Inserts represent dotplots according to FSCA versus FITCA values. Colours discriminate cells with FSCA below the mean value (red), below the mean valued summed with one (green), two (blue) or three (purple) standard deviation.

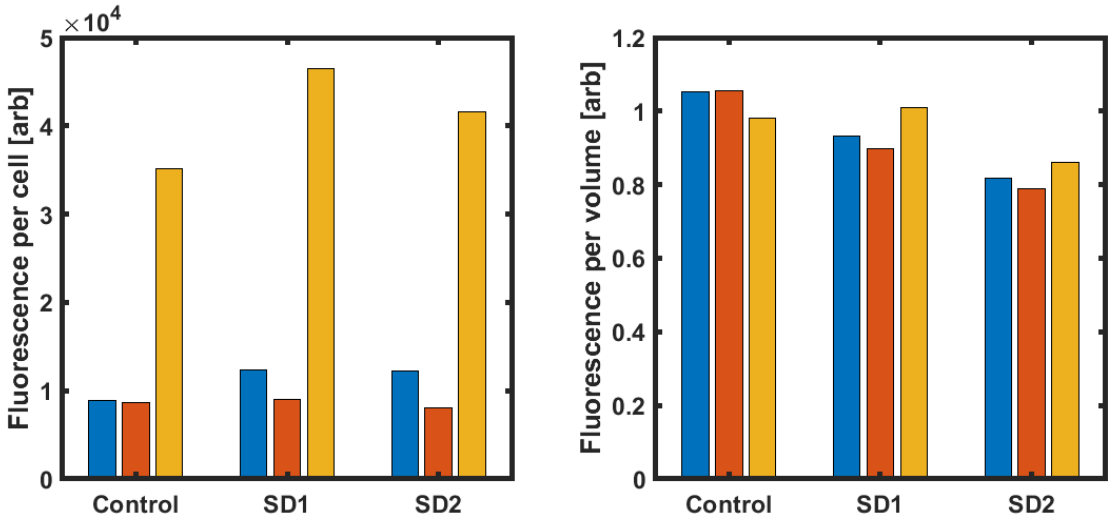

Figure S2: Evolution of the eGFP fluorescence normalised to cell number and volume for control RC and oscillatory culture conditions SD1 and SD2. The whole cell population is in blue; non-aggregates/ovoid cells in red and aggregates/flocculated cells are in yellow. Fluorescence was monitored by flow cytometry and data treated as described in materials and methods. Total arbitrary fluorescence was obtained as the sum of FTICA signals while the cell volume was obtained by the sum of FSCA signals.

Chapter 3

Table S 2: Primers used in chapter 3.

Primer name	Sequence (5'→ 3')	Reference
β-Actin_Fo	AGATGGCTCCGAGAAGTTCA	(C. Theron et al., 2020)
β-Actin_Rev	GTTGCTCAGAGGGCTTCAAC	(C. Theron et al., 2020)
CalB_Fo	TCTCTGCTCCTTCTGTGTGG	(C. Theron et al., 2020)
CalB_Rev	GTCGAACAGAGGTCCACAGA	(C. Theron et al., 2020)
FLO5_Fo	CAGGCAAGTGATGACAATTCAATACAGGAC	(De et al., 2020b)
FLO5_Rev	CGTTTGGTATCCCATAAAGTGTTGTGGTG	(De et al., 2020b)
FLO11_Fo	AGTCCACACACCATTGACAACTGC	(De et al., 2020b)
FLO11_Rev	ACACCAGTAACTATTGTAGCAACTGAGC	(De et al., 2020b)
ERG11_Fo	GATGGTAGCAAGATGACCG	(Adelantado et al., 2017)
ERG11_Rev	TCCTGCAGCTCTGGTTTC	(Adelantado et al., 2017)
ERG25_Fo	CAATGACACCCTTGCCAC	(Adelantado et al., 2017)
ERG25_Rev	CCCACTGCTCCTTGTTG	(Adelantado et al., 2017)
Kar2_Fo	CCTACTTCAACGACGCTCAA	(Huang et al., 2019)
Kar2_Rev	CCACCCTCAATAGAAAGCAGA	(Huang et al., 2019)
Pdi1_Fo	GAGCAACAAGAAGTTTGGAGTTCC	(Huang et al., 2019)
Pdi1_Rev	CCTCATAAGCAGGAGCCATTC	(Huang et al., 2019)
Ero1_Fo	CGTTAGCAAACCCTCAAATCC	(Huang et al., 2019)
Ero1_Rev	GCAGAATCCCTCATCACCATT	(Huang et al., 2019)
Hca1_Fo	CAAGAATCAGCCAAAGCC	(Huang et al., 2019)
Hca1_Rev	TGCGAGTGGATGTAGATGC	(Huang et al., 2019)

Chapter 4

Supplementary Data: Flux balance analysis of *Pichia pastoris*

In order to estimate the flux distribution under the experimental conditions described in this article, a flux balance analysis of *Pichia pastoris* was performed. Thus, a stoichiometric model was developed based on a set of reactions that represented the main metabolic processes in the cell (see the reaction list below). Glycolysis, gluconeogenesis, amino acids synthesis and catabolism, TCA cycle, pentose phosphate pathway and phosphorylative oxidation were included in the model. The metabolic pathway information was taken from previous works in P. pastoris (Çelik et al., 2009b; Solà et al., 2007; Unrean, 2014) and lumped when possible, in order to reduce the size of the stoichiometric matrix. The reaction describing the biomass formation was built by lumping the biosynthesis of proteins, lipids, polysaccharides, DNA and RNA. Their contribution to the macromolecular composition of the cell was considered and balanced to obtain one C-mol of biomass (cell empiric formula: $CH_{1.89}N_{0.137}O_{0.785}$) as it is defined from *P. pastoris* elementary composition (Jordà et al., 2013; Zepeda et al., 2014b). For methanol consumption, both dissimilative (R3) and assimilative (R4) pathways were included in order to estimate their role at the different experimental conditions. The stoichiometric model was solved using MetaFluxNet software (J. Y. Lee et al., 2017) minimizing the flux to ATP for maintenance (R57) under the assumption that for a given specific growth rate, cells will produce new biomass at the lowest energy consumption. It has been established that, under unlimited substrate cell growth condition, the minimization of energy production is among the best objective functions (García Sánchez & Torres Sáez, 2014), together with biomass production. In this work, biomass production was used as an input to the model and then cannot be used as objective function. The energy for maintenance has been previously used in several modelling approaches in yeast, including stoichiometric models (Bideaux et al., 2016; Çelik et al., 2010) as a way to represent any metabolic process that is occurring in the cell that requires energy (e.g. intracellular pH homeostasis), in addition to the biomass formation. All fluxes were expressed in mmol/gDWC h.

Reaction used for the stoichiometric model:

1 Sorb + ATP -> F6P + NADH

2 MetOH + 0.5 O2 -> FAH

3 FAH -> 2 NADH + CO2

4 FAH + ATP -> 0.33334 G3P

5 G3P <-> PEP + ATP + NADH

6 PEP -> Pyr + ATP

7 F6P <-> G6P

8 2 G3P <-> F6P

9 G6P -> CO2 + R5P + 2 NADPH

10 2 R5P <-> F6P + E4P

11 E4P + R5P <-> F6P + G3P

12 Pyr -> AcCoA + CO2 + NADH

13 AcCoA + OA -> aKG + CO2 + NADH

14 aKG -> CO2 + SucCoA + NADH

15 SucCoA <-> Mal + ATP + FADH2

16 Mal <-> OA + NADH

17 Glu + NH3 + ATP -> Gln

18 aKG + NADPH + NH3 -> Glu

19 Glu + ATP + 2 NADPH -> Pro

20 AcCoA + 2 ATP + 2 Glu + NADPH -> Arg + Mal + aKG

21 AcCoA + ATP + 2 Glu + NADPH -> aKG + CO2 + Lys

22 Glu + OA <-> aKG + Asp

23 Asp + 2 ATP + NH3 -> Asn

24 Asp + 2 ATP + 2 NADPH -> Thr

25 Asp + ATP + 2 NADPH + SucCoA + Cys -> FADH2 + Mal + NH3 + Pyr + Met

26 Thr + NADPH + Glu + Pyr -> Ile + CO2 + NH3 + aKG

27 2 Pyr + NADPH + Glu -> Val + aKG + CO2

28 AcCoA + Glu + 2 Pyr + NADPH -> Leu + aKG + 2 CO2

29 Ser <-> Gly

30 PEP + Glu -> NADH + Ser + aKG

31 Ser + AcCoA + 2 ATP + 2 NADPH -> Cys

32 Glu + Pyr <-> aKG + Ala

33 E4P + 2 PEP + Ser + ATP + R5P + NADPH -> Trp + CO2 + G3P + Glu + Pyr

34 2 PEP + ATP + E4P + Glu + NADPH -> aKG + Tyr + CO2

35 ATP + E4P + Glu + 2 PEP + NADPH -> aKG + CO2 + Phe

36 Glu + R5P -> His + aKG

37 Gln -> NH3 + Glu

38 Glu -> NADH + NH3 + aKG

39 Ser -> NH3 + Pyr

40 Asn -> NH3 + Asp

41 aKG + Arg -> CO2 + 2 Glu + NADH + 2 NH3

42 aKG + ATP + Ile -> AcCoA + Glu + 2.6 NADH + SucCoA

43 ATP + Leu + SucCoA + aKG -> 3 AcCoA + CO2 + Glu + Mal + 2.2 NADH

44 2 aKG + Lys -> 2 AcCoA + 2 Glu + 3 NADH

45 Pro -> Glu + 2 NADH

46 aKG + O2 + Val -> CO2 + Glu + 3 NADH + SucCoA

47 Cys + O2 -> NH3 + Pyr

48 Met + O2 + Ser -> NADH + 2 NH3 + SucCoA

49 ATP + Thr -> AcCoA + Gly + NADH

50 NADH + O2 + Phe -> Tyr

51 aKG + 2 O2 + Tyr -> 2 AcCoA + CO2 + Glu + Mal

52 His -> CO2 + Glu + NADH + 2 NH3

53 2 Gly -> CO2 + NADH + NH3 + Ser

54 CO2 + PEP <-> OA

55 NADH + 0.5 O2 -> 2.5 ATP

56 FADH2 + O2 -> 1.5 ATP

57 ATP -> Maintenance

58 0.013 Ala + 0.003 Arg + 0.005 Asn + 0.023 Asp + 0.444 ATP + 0.006 Cys + 0.045 Gln + 0.007 Glu + 0.012 Gly + 0.001 His + 0.006 Ile + 0.006 Leu + 0.006 Lys + 0.006 Met + 0.003 Phe + 0.005 Pro + 0.007 Ser + 0.007 Thr + 0.000 Trp + 0.004 Tyr + 0.006 Val + 0.1476 ATP + 0.042 AcCoA + 0.0185 CO2 + 0.002392 G3P + 0.043754 G6P + 0.0241 NH3 + 0.0078 O2 + 0.0127 R5P + 0.0006 NADH + 0.0828 NADPH -> Biomass

Table S3: Primers and microorganisms used in chapter 4

Strain, primers	**Genotypes, primer sequence (5′ to 3′)**	**Reference/restriction site/gene**
E. coli		
DH5α	fhuA2 lac (del)U169 phoA glnV44 Φ80' lacZ (del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	Thermo-Fisher Scientific
RIE158	DH5α/PIB4 vector	(Sears et al., 1998)

RIE160	DH5α/PP246 vector	(Perez-Pinera et al., 2016)
RIE171	DH5α/RIP171 PAOX1-EGFP)	This work
RIE236	DH5α/RIP236pEYK3AB-CalB)	Lab collection
RIE252	DH5α/pPTK005-3a-αMF	(Obst et al., 2017)
RIE255	DH5α/RIP255 PAOX1-CalB)	This work
RIE257	DH5α/RIP257 PAOX1- αMF-CalB)	This work
P. pastoris		
GS115	His4	
RIY230	GS115, PAOX1-EGFP	This work
RIY231	GS115 prototroph	This work
RIY308	GS115- PAOX1-CalB	This work
Primers		
PAE01	GAAACGCGAATTCATGGTGAGC	EcoRI
PAE02	CCAGGATCCTCACTTGTACAGCTCGTCCATGCCG	BamHI
GFP-F	GAAACGCGAATTCATGGTGAGC	EcoRI
GFP-R	CCAGGATCCGCAAGACCGGTCAATGATGATG	BamHI
CalB-F	GCGGCCGCTGAATTCATGCTGCCTTCTGGATCTGACCCTGC	NotI, EcoRI
CalB-R	GGATCCTTAAGCGGCCGCAGGGGTGACAATACCAGAACAGG	BamHI, NotI
αMF-F	GACTGGAATTCATGAGATTTCCTTCAATTTTTACTGCTG	EcoRI
αMF-R	GAATTCAGCTTCAGCCTCTCTTTTCTCGAG	EcoRI
AOX-int	ACGCAAATGGGGAAACAC	
Act-F	AGATGGCTCCGAGAAGTTCA	Actin
Act-R	GTTGCTCAGAGGGCTTCAAC	Actin
qGFP-F	ACGTAAACGGCCACAAGTTC	EGFP
qGFP-R	AAGTCGTGCTGCTTCATGTG	EGFP
qAOX-F	GTGCCCAACTTGAACTGAGG	AOX
qAOX-R	AGATCGTCAAATGGGGTGGT	AOX
qDAS-F	GACTCCTGGACACCCTGAAA	DAS
qDAS-R	CCTCTTGCAAACAAGCATCA	DAS

qFLD-F	ATCACTGACGGAGGCTTTGA	FLD
qFLD-R	TGGCATTTGAGTACGTCCCT	FLD

References

Acevedo, F., Gentina, J. C., & Illanes, A. (2002). *Fundamentos de Ingeniería Bioquímica*. Ediciones Universitarias de Valparaíso.

Adelantado, N., Tarazona, P., Grillitsch, K., García-Ortega, X., Monforte, S., Valero, F., Feussner, I., Daum, G., & Ferrer, P. (2017). The effect of hypoxia on the lipidome of recombinant *Pichia pastoris*. *Microbial Cell Factories*, *16*(1), 1–15. https://doi.org/10.1186/s12934-017-0699-4

Antonny, B., & Schekman, R. (2001). ER export: public transportation by the COPII coach. *Current Opinion in Cell Biology*, *13*(4), 438–443. https://doi.org/10.1016/S0955-0674(00)00234-9

Ata, O., Gunduz, B., Fickers, P., Heistinger, L., Mattanovich, D., Rebnegger, C., & Gasser, B. (2021). What makes *Komagataella phaffii* non-conventional ? *FEMS Yeast Research*, *21*(8), 1–15. https://doi.org/https://doi.org/10.1093/femsyr/foab059

Barrigón, J. M., Montesinos, J. L., & Valero, F. (2013). Searching the best operational strategies for *Rhizopus oryzae* lipase production in *Pichia pastoris* Mut^+ phenotype: Methanol limited or methanol non-limited fed-batch cultures? *Biochemical Engineering Journal*, *75*, 47–54. https://doi.org/10.1016/j.bej.2013.03.018

Baumann, K., Carnicer, M., Dragosits, M., Graf, A., Stadlmann, J., Jouhten, P., Maaheimo, H., Gasser, B., Albiol, J., Mattanovich, D., & Ferrer, P. (2010). A multi-level study of recombinant *Pichia pastoris* in different oxygen conditions. *BMC Systems Biology*, *4*(141), 1–22. https://doi.org/10.1186/1752-0509-4-141

Berrios, J., Flores, M., Díaz-Barrera, A., Altamirano, C., Martínez, I., & Cabrera, Z. (2017). A comparative study of glycerol and sorbitol as co-substrates in methanol-induced cultures of *Pichia pastoris*: temperature effect and scale-up simulation. *Journal of Industrial Microbiology & Biotechnology*, *44*(3), 407–411. https://doi.org/10.1007/s10295-016-1895-7

Berrios, J., Theron, C. W., Steels, S., Ponce, B., Velastegui, E., Bustos, C., Altamirano, C., & Fickers, P. (2022). Role of Dissimilative Pathway of *Komagataella phaffii* (*Pichia pastoris*): Formaldehyde Toxicity and Energy Metabolism. *Microorganisms*, *10*(7). https://doi.org/10.3390/microorganisms10071466

Bideaux, C., Montheard, J., Cameleyre, X., Molina-Jouve, C., & Alfenore, S. (2016). Metabolic flux analysis model for optimizing xylose conversion into ethanol by the natural C5-fermenting yeast *Candida shehatae*. *Applied Microbiology and Biotechnology*, *100*(3), 1489–1499. https://doi.org/10.1007/s00253-015-7085-0

Bony, M., Thines-Sempoux, D., Barre, P., & Blondin, B. (1997). Localization and cell surface anchoring of the *Saccharomyces cerevisiae* flocculation protein Flo1p. *Journal of Bacteriology*, *179*(15), 4929–4936. https://doi.org/10.1128/jb.179.15.4929-4936.1997

Bylund, F., Collet, E., Enfors, S., & Larsson, G. (1998a). Substrate gradient formation in the large-scale bioreactor lowers cell yield and increases by-product formation. *Bioprocess Engineering*, *18*(3), 171–180. https://doi.org/10.1007/s004490050427

Bylund, F., Collet, E., Enfors, S.-O., & Larsson, G. (1998b). Substrate gradient formation in the large-scale bioreactor lowers cell yield and increases by-product formation. *Bioprocess Engineering*, *18*, 171–180.

Bylund, F., Guillard, F., Enfors, S., Trägârdh, C., & Larsson, G. (1999). Scale down of recombinant protein production: A comparative study of scaling performance. *Bioprocess Engineering*, *20*(5), 377–389. https://doi.org/10.1007/s004490050606

Çalik, P., Bayraktar, E., Inankur, B., Soyaslan, E. Ş., Şahin, M., Taşpinar, H., Açik, E., Yilmaz, R., & Özdamar, T. H. (2010). Influence of pH on recombinant human growth hormone production by *Pichia pastoris*. *Journal of Chemical Technology and Biotechnology*, *85*(12), 1628–1635. https://doi.org/10.1002/jctb.2474

Canales, C., Altamirano, C., & Berrios, J. (2018). The growth of *Pichia pastoris* Mut^+ on methanol–glycerol mixtures fits to interactive dual-limited kinetics: model development and application to optimised fed-batch operation for heterologous protein production. *Bioprocess and Biosystems Engineering*, *41*(12), 1827–1838. https://doi.org/10.1007/s00449-018-2005-1

Carly, F., Niu, H., Delvigne, F., & Fickers, P. (2016). Influence of methanol/sorbitol co-feeding rate on pAOX1 induction in a Pichia pastoris Mut^+ strain in bioreactor with limited oxygen transfer rate. *Journal of Industrial Microbiology and Biotechnology*, *43*(4), 517–523. https://doi.org/10.1007/s10295-015-1722-6

Çelik, E., Çalik, P., & Oliver, S. G. (2009a). Fed-batch methanol feeding strategy for recombinant protein production by *Pichia pastoris* in the presence of co-substrate sorbitol. *Yeast*, *26*(9), 473–484. https://doi.org/10.1002/yea.1679

Çelik, E., Çalik, P., & Oliver, S. G. (2009b). Fed-batch methanol feeding strategy for recombinant protein production by *Pichia pastoris* in the presence of co-substrate sorbitol. *Yeast*, *26*(9), 473–484. https://doi.org/10.1002/yea.1679

Çelik, E., Çalik, P., & Oliver, S. G. (2010). Metabolic flux analysis for recombinant protein production by *Pichia pastoris* using dual carbon sources: Effects of methanol feeding rate. *Biotechnology and Bioengineering*, *105*(2), 317–329. https://doi.org/10.1002/bit.22543

Celińska, E., Borkowska, M., & Białas, W. (2017). Enhanced production of insect raw-starch-digesting alpha-amylase accompanied by high erythritol synthesis in

recombinant *Yarrowia lipolytica* fed-batch cultures at high-cell-densities. *Process Biochemistry*, *52*, 78–85. https://doi.org/10.1016/j.procbio.2016.10.022

Cha, H. J., Dalal, N. N., & Bentley, W. E. (2005). Secretion of human interleukin-2 fused with green fluorescent protein in recombinant *Pichia pastoris*. *Applied Biochemistry and Biotechnology*, *126*(1), 1–11. https://doi.org/10.1007/s12010-005-0001-9

Chiruvolu, V., Eskridge, K., Cregg, J., & Meagher, A. M. (1998). Effects of Glycerol Concentration and pH on Growth of Recombinant *Pichia pastoris* Yeasf. In *Applied Biochemistry and Biotechnology]* (Vol. 63).

Chiruvolu, V., Gregg, J. M., & Meagher, M. M. (1997). Recombinant protein production in an alcohol oxidase-defective strain of *Pichia pastoris* in fedbatch fermentations. *Enzyme and Microbial Technology*, *21*, 277–283.

Clare, J. J., Romanes, M. A., Rayment, F. B., Rowedder, J. E., Smith, M. A., Payne, M. M., Sreekrishna, K., & Henwood, C. A. (1991). Production of mouse epidermal growth factor in yeast: high-level secretion using Pichia pastoris strains containing multiple gene copies. *Gene*, *105*(2), 205–212. https://doi.org/10.1016/0378-1119(91)90152-2

Cregg, J. M., Barringer, K. J., Hessler, A. Y., & Madden, K. R. (1985). *Pichia pastoris* as a Host System for Transformations. In *Molecular and Cellular Biology* (Vol. 5, Issue 12).

Cregg, J. M., Madden, K. R., Barringer, K. J., Thill, G. P., & Stillman, C. A. (1989). Functional characterization of the two alcohol oxidase genes from the yeast *Pichia pastoris*. *Molecular and Cellular Biology*, *9*(3), 1316–1323. https://doi.org/10.1128/MCB.9.3.1316

Cregg, J. M., Tolstorukov, I., Kusari, A., Sunga, A. J., Madden, K., & Chappell, T. (2010). Expression of Recombinant Genes in the Yeast *Pichia pastoris*. *Current Protocols Essential Laboratory Techniques*, *4*(1). https://doi.org/10.1002/9780470089941.et1302s04

Daly, R., & Hearn, M. T. W. (2005). Expression of heterologous proteins in *Pichia pastoris*: A useful experimental tool in protein engineenring and production. *Journal of Molecular Recognition*, *18*(2), 119–138. https://doi.org/10.1002/jmr.687

De, S., Rebnegger, C., Moser, J., Tatto, N., Graf, A., Mattanovich, D., & Gasser, B. (2020). Pseudohyphal differentiation in Komagataella phaffii : Investigating the FLO gene family. *FEMS Yeast Research*, *20*(August), 1–15. https://doi.org/https://doi.org/10.1093/femsyr/foaa044

Delvigne, F., Lejeune, A., Destain, J., & Thonart, P. (2006). Stochastic models to study the impact of mixing on a Fed-Batch culture of *Saccharomyces cerevisiae*. *Biotechnology Progress*, *22*(1), 259–269. https://doi.org/10.1021/bp050255m

Delvigne, F., Zacchetti, B., Fickers, P., Fifani, B., Roulling, F., Lefebvre, C., Neubauer, P., & Junne, S. (2018). Improving control in microbial cell factories : from single-cell to

large-scale bioproduction. *FEMS Microbiology Letters*, *365*(22), 1–11. https://doi.org/10.1093/femsle/fny236

Dubois, M., Gilles, K., Hamilton, J., Rebers, P., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350–356. https://doi.org/10.1021/ac60111a017

Enfors, S. O., Jahic, M., Rozkov, A., Xu, B., Hecker, M., Jürgen, B., Krüger, E., Schweder, T., Hamer, G., O'Beirne, D., Noisommit-Rizzi, N., Reuss, M., Boone, L., Hewitt, C., McFarlane, C., Nienow, A., Kovacs, T., Trägårdh, C., Fuchs, L., … Manelius, Å. (2001). Physiological responses to mixing in large scale bioreactors. *Journal of Biotechnology*, *85*(2), 175–185. https://doi.org/10.1016/S0168-1656(00)00365-5

Ergun, B., Berrios, J., Binay, B., & Fickers, P. (2021). Recombinant protein production in *Pichia pastoris*: from transcriptionally redesigned strains to bioprocess optimization and metabolic modelling. *FEMS Yeast Research*, *21*(7), 1–14. https://doi.org/https://doi.org/10.1093/femsyr/foab057

Ergün, B. G., Laçın, K., Çaloğlu, B., & Binay, B. (2022). Second generation *Pichia pastoris* strain and bioprocess designs. *Biotechnology for Biofuels and Bioproducts*, *15*(1), 150. https://doi.org/10.1186/s13068-022-02234-7

Falcioni, T., Manti, A., Boi, P., Canonico, B., Balsamo, M., & Papa, S. (2006). Comparison of disruption procedures for enumeration of activated sludge floc bacteria by flow cytometry. *Cytometry Part B (Clinical Cytometry)*, *70B*, 149–153. https://doi.org/10.1002/cyto.b.20097

Fickers, P., Nicaud, J. M., Destain, J., & Thonart, P. (2003). Overproduction of lipase by *Yarrowia lipolytica* mutants. *Applied Microbiology and Biotechnology*, *63*(2), 136–142. https://doi.org/10.1007/s00253-003-1342-3

Francois, J., & Parrou, J. (2001). Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiology Letters*, *25*, 125.145. https://doi.org/https://doi.org/10.1111/j.1574-6976.2001.tb00574.x

García Sánchez, C. E., & Torres Sáez, R. G. (2014). Comparison and analysis of objective functions in flux balance analysis. *Biotechnology Progress*, *30*(5), 985–991. https://doi.org/10.1002/btpr.1949

Garcia-Ochoa, F., & Gomez, E. (2009). Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview. *Biotechnology Advances*, *27*(2), 153–176. https://doi.org/10.1016/j.biotechadv.2008.10.006

García-Ortega, X., Cámara, E., Ferrer, P., Albiol, J., Montesinos-Seguí, J. L., & Valero, F. (2019). Rational development of bioprocess engineering strategies for recombinant protein production in *Pichia pastoris* (*Komagataella phaffii*) using the methanol-free GAP promoter. Where do we stand? *New Biotechnology*, *53*, 24–34. https://doi.org/10.1016/j.nbt.2019.06.002

Garrigós-Martínez, J., Nieto-Taype, M. A., Gasset-Franch, A., Montesinos-Seguí, J. L., Garcia-Ortega, X., & Valero, F. (2019). Specific growth rate governs AOX1 gene expression, affecting the production kinetics of *Pichia pastoris* (*Komagataella phaffii*) P AOX1 -driven recombinant producer strains with different target gene dosage. *Microbial Cell Factories*, *18*(1). https://doi.org/10.1186/s12934-019-1240-8

Gasser, B., & Mattanovich, D. (2018). A yeast for all seasons – Is *Pichia pastoris* a suitable chassis organism for future bioproduction? *FEMS Microbiology Letters*, *365*(17), 1–4. https://doi.org/10.1093/femsle/fny181

Gasser, B., Maurer, M., Rautio, J., Sauer, M., Bhattacharyya, A., Saloheimo, M., Penttilä, M., & Mattanovich, D. (2007). Monitoring of transcriptional regulation in *Pichia pastoris* under protein production conditions. *BMC Genomics*, *8*, 1–18. https://doi.org/10.1186/1471-2164-8-179

Gidijala, L., Uthoff, S., Kampen, S. J., Steinbüchel, A., & Verhaert, R. M. D. (2018). Presence of protein production enhancers results in significantly higher methanol-induced protein production in *Pichia pastoris*. *Microbial Cell Factories*, *17*(1). https://doi.org/10.1186/s12934-018-0961-4

Graf, A., Gasser, B., Dragosits, M., Sauer, M., Leparc, G. G., Tüchler, T., Kreil, D. P., & Mattanovich, D. (2008). Novel insights into the unfolded protein response using *Pichia pastoris* specific DNA microarrays. *BMC Genomics*, *9*. https://doi.org/10.1186/1471-2164-9-390

Hamaker, K. H., Johnson, D. C., Bellucci, J. J., Apgar, K. R., Soslow, S., Gercke, J. C., Menzo, D. J., & Ton, C. (2011). Design of a novel automated methanol feed system for pilot-scale fermentation of *Pichia pastoris*. *Biotechnology Progress*, *27*(3), 657–667. https://doi.org/10.1002/btpr.560

Hohenblum, H., Gasser, B., Maurer, M., & Borth, N. (2004). Effects of Gene Dosage , Promoters , and Substrates on Unfolded Protein Stress of Recombinant *Pichia pastoris*. https://doi.org/10.1002/bit.10904

Hope, E. A., Amorosi, C. J., Miller, A. W., Dang, K., Heil, C. S., & Dunham, M. J. (2017). Experimental Evolution Reveals Favored Adaptive Routes to Cell Aggregation in Yeast. *Genetics*, *206*(2), 1153–1167. https://doi.org/10.1534/genetics.116.198895

Huang, J., Wang, Q., Bu, W., Chen, L., Yang, Z., & Zheng, W. (2019). Different construction strategies affected on the physiology of *Pichia pastoris* strains highly expressed lipase by transcriptional analysis of key genes. *Bioengineered*, *10*(1), 150–161. https://doi.org/10.1080/21655979.2019.1614422

Inan, M., Aryasomayajula, D., Sinha, J., & Meagher, M. M. (2006). Enhancement of protein secretion in *Pichia pastoris* by overexpression of protein disulfide isomerase. *Biotechnology and Bioengineering*, *93*(4), 771–778. https://doi.org/10.1002/bit.20762

Jia, L., Li, T., Yang, Z., Li, T., & Ding, J. (2022). Enhancing monellin production in *Pichia pastoris* fed-batch fermentation through an efficient on-line methanol/sorbitol co-feeding strategy. *Journal of Cleaner Production*, *369*. https://doi.org/10.1016/j.jclepro.2022.133350

Jiang, X., Lin, J., Liang, S., & Zhang, M. (2013). High-Efficient Expression and Pilot Scale Fermentation of Streptomyces Xylanase from a Constitutive *Pichia pastoris* Vector. *Food Biotechnology*, *27*(1), 54–65. https://doi.org/10.1080/08905436.2012.755693

Jordà, J., de Jesus, S., Peltier, S., Ferrer, P., & Albiol, J. (2013). Metabolic flux analysis of recombinant *Pichia pastoris* growing on different glycerol/methanol mixtures by iterative fitting of NMR-derived 13C-labelling data from proteinogenic amino acids. *New Biotechnology*, *31*(1), 120–132. https://doi.org/10.1016/j.enzmictec.2014.10.006

Jungo, C., Marison, I., & von Stockar, U. (2007). Regulation of alcohol oxidase of a recombinant *Pichia pastoris* Mut^+ strain in transient continuous cultures. *Journal of Biotechnology*, *130*(3), 236–246. https://doi.org/10.1016/j.jbiotec.2007.04.004

Kang, Z., Huang, H., Zhang, Y., Du, G., & Chen, J. (2017). Recent advances of molecular toolbox construction expand *Pichia pastoris* in synthetic biology applications. In *World Journal of Microbiology and Biotechnology* (Vol. 33, Issue 1). Springer Netherlands. https://doi.org/10.1007/s11274-016-2185-2

Kaushik, N., Rohila, D., Arora, U., Raut, R., Lamminmäki, U., & Khanna, N. (2016). Casamino acids facilitate the secretion of recombinant dengue virus serotype-3 envelope domain III in *Pichia pastoris*. *BMC Biotechnology*, 1–9. https://doi.org/10.1186/s12896-016-0243-3

Lara, A., Galindo, E., Ramírez, O., & Palomares, L. (2006). Living with Heterogeneities in Bioreactors. *Molecular Biotechnology*, *34*, 355–381. https://doi.org/10.1385/mb:34:3:355

Larsson, G., & Enfors, S. O. (1988). Studies of insufficient mixing in bioreactors: Effects of limiting oxygen concentrations and short term oxygen starvation on Penicillium chrysogenum. *Bioprocess Engineering*, *3*(3), 123–127. https://doi.org/10.1007/BF00373475

Lee, C. Y., Lee, S. J., Jung, K. H., Katoh, S., & Lee, E. K. (2003). High dissolved oxygen tension enhances heterologous protein expression by recombinant *Pichia pastoris*. *Process Biochemistry*, *38*(8), 1147–1154. https://doi.org/10.1016/S0032-9592(02)00280-7

Lee, J. Y., Chen, H., Liu, A., Alba, B. M., & Lim, A. C. (2017). Auto-induction of *Pichia pastoris* AOX1 promoter for membrane protein expression. *Protein Expression and Purification*, *137*, 7–12. https://doi.org/10.1016/j.pep.2017.06.006

Lejeune, A., Delvigne, F., & Thonart, P. (2013). Physiological response of yeast to process perturbations: A mini-bioreactor approach. *Cerevisia*, *38*(1), 15–19. https://doi.org/10.1016/j.cervis.2013.04.004

Levenspiel, O. (2012). The E and Eo Curves from Pulse and Step Tracer Experiments. In *Tracer Technology. Fluid Mechanics and Its Applications* (pp. 11–24). Springer.

Li, C., Tian, J., Wang, W., Peng, H., Zhang, M., Hang, H., Zhang, S., & Xia, J. (2019). Numerical and experimental assessment of a miniature bioreactor equipped with a mechanical agitator and non-invasive biosensors. *Journal of Chemical Technology & Biotechnology*, *94*(8), 2671–2683. https://doi.org/10.1002/jctb.6076

Li, G. Y., Fu, M., Qin, M., & Xue, L. M. (2018). High Expression of Human Cathepsin S by Recombinant *Pichia pastoris* with Cod Skin as an Organic Co-Nitrogen Source. *Journal of Molecular Microbiology and Biotechnology*, *27*(6), 363–370. https://doi.org/10.1159/000486395

Lin-Cereghino, G. P., Godfrey, L., de la Cruz, B. J., Johnson, S., Khuongsathiene, S., Tolstorukov, I., Yan, M., Lin-Cereghino, J., Veenhuis, M., Subramani, S., & Cregg, J. M. (2006). Mxr1p, a Key Regulator of the Methanol Utilization Pathway and Peroxisomal Genes in Pichia pastoris . *Molecular and Cellular Biology*, *26*(3), 883–897. https://doi.org/10.1128/mcb.26.3.883-897.2006

Lin-Cereghino, J., Wong, W. W., Xiong, S., Giang, W., Luong, L. T., Vu, J., Johnson, S. D., & Lin-Cereghino, G. P. (2005). Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast *Pichia pastoris*. *BioTechniques*, *38*(1), 44–48. https://doi.org/10.2144/05381BM04

Liu, W. C., Inwood, S., Gong, T., Sharma, A., Yu, L. Y., & Zhu, P. (2019). Fed-batch high-cell-density fermentation strategies for *Pichia pastoris* growth and production. *Critical Reviews in Biotechnology*, *39*(2), 258–271. https://doi.org/10.1080/07388551.2018.1554620

Lorantfy, B., Jazini, M., & Herwig, C. (2013). Investigation of the physiological response to oxygen limited process conditions of *Pichia pastoris* Mut+ strain using a two-compartment scale-down system. *Journal of Bioscience and Bioengineering*, *116*(3), 371–379. https://doi.org/10.1016/j.jbiosc.2013.03.021

Mueller, J. H., & Johnson, E. R. (1941). Acid Hydrolysates of Casein to Replace Peptone in the Preparation of Bacteriological Media. *The Journal of Immunology*, *40*(1), 33–38. https://doi.org/10.4049/jimmunol.40.1.33

Nakamura, T., Zámocký, M., Zdráhal, Z., Chaloupková, R., Monincová, M., Prokop, Z., Nagata, Y., & Damborský, J. (2006). Expression of glycosylated haloalkane dehalogenase LinB in *Pichia pastoris*. *Protein Expression and Purification*, *46*(1), 85–91. https://doi.org/10.1016/j.pep.2005.08.022

Neubauer, P., & Junne, S. (2010). Scale-down simulators for metabolic analysis of large-scale bioprocesses. *Current Opinion in Biotechnology*, *21*(1), 114–121. https://doi.org/10.1016/j.copbio.2010.02.001

Nienow, A., Scott, W., Hewitt, C., Thomas, C., Lewis, G., Amanullah, A., Kiss, R., & Meier, S. (2013). Scale-down studies for assessing the impact of different stress parameters on growth and product quality during animal cell culture. *Chemical Engineering Research and Design*, *91*(11), 2265–2274. https://doi.org/10.1016/j.cherd.2013.04.002

Niu, H., Daukandt, M., Rodriguez, C., Fickers, P., & Bogaerts, P. (2013). Dynamic modeling of methylotrophic *Pichia pastoris* culture with exhaust gas analysis: From cellular metabolism to process simulation. *Chemical Engineering Science*, *87*, 381–392. https://doi.org/10.1016/j.ces.2012.11.006

Niu, H., Jost, L., Pirlot, N., Sassi, H., Daukandt, M., Rodriguez, C., & Fickers, P. (2013). A quantitative study of methanol/sorbitol co-feeding process of a *Pichia pastoris* Mut+/pAOX1-lacZ strain. *Microbial Cell Factories*, *12*(1), 33. https://doi.org/10.1186/1475-2859-12-33

Noorman, H., Morud, K., Hjertager, B. H., Traegaardh, C., Larsson, G., & Enfors, S. O. (1993). CFD modeling and verification of flow and conversion in a 1 m^3 bioreactor. *BHR Group Conf. Ser. Publ.*, *5*(3rd International Conference on Bioreactor and Bioprocess Fluid Dynamics, 1993), 241–258.

Obst, U., Lu, T. K., & Sieber, V. (2017). A Modular Toolkit for Generating *Pichia pastoris* Secretion Libraries. *ACS Synthetic Biology*, *6*(6), 1016–1025. https://doi.org/10.1021/acssynbio.6b00337

Oldshue, J. (1966). Fermentation mixing scale-up techniques. *Biotechnology and Bioengineeing*, *8*(1), 3–24. https://doi.org/https://doi.org/10.1002/bit.260080103

Olughu, W., Deepika, G., Hewitt, C., & Rielly, C. (2019). Insight into the large-scale upstream fermentation environment using scaled-down models. *Journal of Chemical Technology and Biotechnology*, *94*(3), 647–657. https://doi.org/10.1002/jctb.5804

Oosterhuis, N., & Kossen, N. (1983). Oxygen transfer in a production scale bioreactor. *Chemical Engineering Research and Design*, *61*(5), 308–312.

Oosterhuis, N., Kossen, N., Olivier, A., & Schenk, E. (1985). Scale-down and optimization studies of the gluconic acid fermentation by *Gluconobacter oxydans*. *Biotechnology and Bioengineering*, *27*(5), 711–720. https://doi.org/10.1002/bit.260270521

Osman, J., Birch, J., & Varley, J. (2002). The response of GS-NS0 myeloma cells to single and multiple pH perturbations. *Biotechnology and Bioengineering*, *79*(4), 398–407. https://doi.org/10.1002/bit.10198

Perez-Pinera, P., Han, N., Cleto, S., Cao, J., Purcell, O., Shah, K. A., Lee, K., Ram, R., & Lu, T. K. (2016). Synthetic biology and microbioreactor platforms for programmable

production of biologics at the point-of-care. *Nature Communications*, *7*, 1–10. https://doi.org/10.1038/ncomms12211

Periyasamy, S., Govindappa, N., Sreenivas, S., & Sastry, K. (2013). Isolation, characterization and evaluation of the *Pichia pastoris* sorbitol dehydrogenase promoter for expression of heterologous proteins. *Protein Expression and Purification*, *92*(1), 128–133. https://doi.org/10.1016/j.pep.2013.09.008

Pfaffl, M. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, *29*(900), 2002–2007. https://doi.org/10.1111/j.1365-2966.2012.21196.x

Prabhu, A. A., & Dasu, V. V. (2017). Dual-substrate inhibition kinetic studies for recombinant human interferon gamma producing *Pichia pastoris*. *Preparative Biochemistry and Biotechnology*, *47*(10), 953–962. https://doi.org/10.1080/10826068.2017.1350977

Priyadi, K., Lu, C., & Sutanto, H. (2019). Optimization of impeller design for stirred tank using computational fluid dynamics. *IOP Conference Series: Materials Science and Engineering*, *567*(1), 1–6. https://doi.org/10.1088/1757-899X/567/1/012032

Ramón, R., Ferrer, P., & Valero, F. (2007). Sorbitol co-feeding reduces metabolic burden caused by the overexpression of a *Rhizopus oryzae* lipase in *Pichia pastoris*. *Journal of Biotechnology*, *130*(1), 39–46. https://doi.org/10.1016/j.jbiotec.2007.02.025

Raschmanová, H., Weninger, A., Knejzlík, Z., Melzoch, K., & Kovar, K. (2021). Engineering of the unfolded protein response pathway in *Pichia pastoris*: enhancing production of secreted recombinant proteins. *Applied Microbiology and Biotechnology*, *105*, 4397–4414. https://doi.org/10.1007/s00253-021-11336-5/Published

Raschmanová, H., Zamora, I., Borčinová, M., Meier, P., Weninger, A., Mächler, D., Glieder, A., Melzoch, K., Knejzlík, Z., & Kovar, K. (2019). Single-cell approach to monitor the unfolded protein response during biotechnological processes with Pichia pastoris. *Frontiers in Microbiology*, *10*(FEB), 1–18. https://doi.org/10.3389/fmicb.2019.00335

Rebnegger, C., Graf, A., Valli, M., Steiger, M., Gasser, B., Maurer, M., & Mattanovich, D. (2014). In *Pichia pastoris*, growth rate regulates protein synthesis and secretion, mating and stress response. *Biotechnology Journal*, *9*(4), 511–525. https://doi.org/10.1002/biot.201300334

Rebnegger, C., Vos, T., Graf, A., Valli, M., Pronk, J., Daran-Lapujade, P., & Mattanovich, D. (2016). *Pichia pastoris* exhibits high viability and low maintenance-energy requirement at near-zero specific growth rates. *Applied and Environmental Microbiology*, *82*(15), AEM.00638-16. https://doi.org/10.1128/AEM.00638-16

Ronen, M., & Botstein, D. (2006). Transcriptional response of steady-state yeast cultures to transient perturbations in carbon source. *PNAS*, *103*(2), 389–394. https://doi.org/10.1073/pnas.0509978103

Rumjantsev, A. M., Bondareva, O. V., Padkina, M. V., & Sambuk, E. V. (2014). Effect of nitrogen source and inorganic phosphate concentration on methanol utilization and PEX genes expression in *Pichia pastoris*. *Scientific World Journal*, *2014*. https://doi.org/10.1155/2014/743615

Rumjantsev, A. M., Padkina, M. V., & Sambuk, E. V. (2013). Effect of nitrogen source on gene expression of first steps of methanol utilization pathway in *Pichia pastoris*. *Russian Journal of Genetics*, *49*(4), 394–400. https://doi.org/10.1134/S102279541304011X

Sambrook, J., Fritsch, E., & Maniatis, T. (1989). Molecular Cloning: A laboratory Manual. *Cold Spring Harbor*, Ed.; Second edition. Cold Spring Harbor Laboratory Press.

Sarmah, N., Revathi, D., Sheelu, G., Yamuna Rani, K., Sridhar, S., Mehtab, V., & Sumana, C. (2018). Recent advances on sources and industrial applications of lipases. *Biotechnology Progress*, *34*(1), 5–28. https://doi.org/10.1002/btpr.2581

Sassi, H., Delvigne, F., Kar, T., Nicaud, J. M., Coq, A. M. C. Le, Steels, S., & Fickers, P. (2016). Deciphering how LIP2 and POX2 promoters can optimally regulate recombinant protein production in the yeast *Yarrowia lipolytica*. *Microbial Cell Factories*, *15*(1), 1–11. https://doi.org/10.1186/s12934-016-0558-8

Sassi, H., Nguyen, T. M., & Telek, S. (2019). Segregostat : a novel concept to control phenotypic diversification dynamics on the example of Gram negative bacteria. *Microbil Biotechnology*, 12(5), 1064-1075. https://doi.org/10.1111/1751-7915.13442

Schilling, B. M., Goodrick, J. C., & Wan, N. C. (2001). Scale-up of a high cell-density continuous culture with *Pichia pastoris* X-33 for the constitutive expression of rh-chitinase. *Biotechnology Progress*, *17*(4), 629–633. https://doi.org/10.1021/bp010041e

Sears, I. B., O'Connor, J., Rossanese, O. W., & Glick, B. S. (1998). A versatile set of vectors for constitutive and regulated gene expression in *Pichia pastoris*. *Yeast*, *14*(8), 783–790. https://doi.org/10.1002/(SICI)1097-0061(19980615)14:8<783::AID-YEA272>3.0.CO;2-Y

Shen, S., Sulter, G., Jeffries, T. W., & Cregg, J. M. (1998). A strong nitrogen source-regulated promoter for controlled expression of foreign genes in the yeast *Pichia pastoris*. *Gene*, *216*(1), 93–102. https://doi.org/10.1016/S0378-1119(98)00315-1

Shi, X., Karkut, T., Chamankhah, M., Alting-Mees, M., Hemmingsen, S. M., & Hegedus, D. (2003). Optimal conditions for the expression of a single-chain antibody (scFv) gene in *Pichia pastoris*. *Protein Expression and Purification*, *28*(2), 321–330. https://doi.org/10.1016/S1046-5928(02)00706-4

Shuster, J. R. (1991). Gene expression in yeast: protein secretion. In *Current Opinion in Biotechnology* (Vol. 2).

Simpson, R., & Sastry, S. K. (2013). Scale-up Chemical and Bioprocess Engineering. In *Chemical and Bioprocess Engineering* (Vol. 10). https://doi.org/10.1007/978-1-4614-9126-2

Singh, A., & Narang, A. (2020). The Mut^+ strain of *Komagataella phaffii* (*Pichia pastoris*) expresses PAOX1 5 and 10 times faster than Mut^s and Mut^- strains : evidence that formaldehyde or/and formate are true inducers of PAOX1. *Applied Microbiology and Biotechnology*, *104*(18), 7801–7814.

Solà, A., Jouhten, P., Maaheimo, H., Sánchez-Ferrando, F., Szyperski, T., & Ferrer, P. (2007). Metabolic flux profiling of *Pichia pastoris* grown on glycerol/methanol mixtures in chemostat cultures at low and high dilution rates. *Microbiology*, *153*(1), 281–290. https://doi.org/10.1099/mic.0.29263-0

Theron, C., Vandermies, M., Telek, S., Steels, S., & Fickers, P. (2020). Comprehensive comparison of *Yarrowia lipolytica* and *Pichia pastoris* for production of Candida antarctica lipase B. *Scientific Reports*, *10*(1741), 1–9. https://doi.org/10.1038/s41598-020-58683-3

Theron, C. W., Berrios, J., Steels, S., Telek, S., Lecler, R., Rodriguez, C., & Fickers, P. (2019). Expression of recombinant enhanced green fluorescent protein provides insight into foreign gene-expression differences between Mut^+ and Mut^S strains of *Pichia pastoris*. *Yeast*, *36*(5), 285–296. https://doi.org/10.1002/yea.3388

Tschopp, J. F., Brust, P. F., Cregg, J. M., Stillman, C. A., & Gingeras, T. R. (1987). Expression of the lacZ gene from two methanol-regulated promoters in *Pichia pastoris*. In *Nucleic Acids Research* (Vol. 15).

Unrean, P. (2014). Pathway analysis of *Pichia pastoris* to elucidate methanol metabolism and its regulation for production of recombinant proteins. *Biotechnology Progress*, *30*(1), 28–37. https://doi.org/10.1002/btpr.1855

Uppenberg, J., Hansen, M. T., Patkar, S., & Jones, T. A. (1994). The sequence, crystal structure determination and refinement of two crystal forms of lipase B from *Candida antarctica*. *Structure*, *2*(4), 293–308. https://doi.org/10.1016/S0969-2126(00)00031-9

Vallejo, J., & Villa, T. (2013). Cell aggregations in yeasts and their applications. *Applied Microbiology and Biotechnology*, *97*, 2305–2318. https://doi.org/10.1007/s00253-013-4735-y

Velastegui, E., Quezada, J., Guerrero, K., Altamirano, C., Martinez, J. A., Berrios, J., & Fickers, P. (2023). Is heterogeneity in large-scale bioreactors a real problem in recombinant protein synthesis by *Pichia pastoris*? *Applied Microbiology and Biotechnology*. https://doi.org/10.1007/s00253-023-12434-2

Velastegui, E., Theron, C., Berrios, J., & Fickers, P. (2019). Downregulation by organic nitrogen of AOX1 promoter used for controlled expression of foreign genes in the yeast Pichia pastoris. *Yeast*, *36*(5), 297–304. https://doi.org/10.1002/yea.3381

Vogl, T., & Glieder, A. (2013). Regulation of *Pichia pastoris* promoters and its consequences for protein production. *New Biotechnology*, *30*(4), 385–404. https://doi.org/10.1016/j.nbt.2012.11.010

Vogl, T., Thallinger, G. G., Zellnig, G., Drew, D., Cregg, J. M., Glieder, A., & Freigassner, M. (2014). Towards improved membrane protein production in *Pichia pastoris*: General and specific transcriptional response to membrane protein overexpression. *New Biotechnology*, *31*(6), 538–552. https://doi.org/10.1016/j.nbt.2014.02.009

Vrábel, P., Van der Lans, R., Cui, Y., & Luyben, K. (1999). Compartment model approach: Mixing in large scale aerated reactors with multiple impellers. *Chemical Engineering Research and Design*, *77*(June), 291–302. https://doi.org/10.1205/026387699526223

Wakayama, K., Yamaguchi, S., Takeuchi, A., Mizumura, T., Ozawa, S., Tomizuka, N., Hayakawa, T., & Nakagawa, T. (2016). Regulation of intracellular formaldehyde toxicity during methanol metabolism of the methylotrophic yeast *Pichia methanolica*. *Journal of Bioscience and Bioengineering*, *122*(5), 545–549. https://doi.org/10.1016/j.jbiosc.2016.03.022

Wang, J., Nguyen, V., Glen, J., Henderson, B., Saul, A., & Miller, L. H. (2005). Improved yield of recombinant merozoite surface protein 3 (MSP3) from *Pichia pastoris* using chemically defined media. *Biotechnology and Bioengineering*, *90*(7), 838–847. https://doi.org/10.1002/bit.20491

Wang, X., Cai, M., Shi, L., Wang, Q., Zhu, J., Wang, J., Zhou, M., Zhou, X., & Zhang, Y. (2016). PpNrg1 is a transcriptional repressor for glucose and glycerol repression of AOX1 promoter in methylotrophic yeast *Pichia pastoris*. *Biotechnology Letters*, *38*(2), 291–298. https://doi.org/10.1007/s10529-015-1972-4

Werringloer, J. (1978). Assay of formaldehyde generated during microsomal oxidation reactions. *Methods in Enzymology*, 297–302. https://doi.org/10.1016/s0076-6879(78)52031-4

Woo, S. H., Park, S. H., Lim, H. K., & Jung, K. H. (2005). Extended operation of a pressurized 75-L bioreactor for shLkn-1 production by *Pichia pastoris* using dissolved oxygen profile control. *Journal of Industrial Microbiology and Biotechnology*, *32*(10), 474–480. https://doi.org/10.1007/s10295-005-0248-8

Yan, C., Xu, X., Zhang, X., Zhang, Y., Zhang, Y., & Zhang, Z. (2018). Decreased Rhamnose Metabolic Flux Improved Production of Target Proteins and Cell Flocculation in *Pichia pastoris*. *Frontiers in Microbiology*, *9*(August), 1–11. https://doi.org/10.3389/fmicb.2018.01771

Yano, T., Takigami, E., Yurimoto, H., & Sakai, Y. (2009). Yap1-Regulated Glutathione Redox System Curtails Accumulation of Formaldehyde and Reactive Oxygen Species in Methanol Metabolism of *Pichia pastoris*. *Eukaryotic Cell*, *8*(4), 540–549. https://doi.org/10.1128/EC.00007-09

Young, C. L., Yuraszeck, T., & Robinson, A. S. (2011). Decreased secretion and unfolded protein response upregulation. In *Methods in Enzymology* (Vol. 491, pp. 235–260). Academic Press Inc. https://doi.org/10.1016/B978-0-12-385928-0.00014-6

Yu, Y. fan, Yang, J., Zhao, F., Lin, Y., & Han, S. (2022). Comparative transcriptome and metabolome analyses reveal the methanol dissimilation pathway of *Pichia pastoris*. *BMC Genomics*, *23*(1). https://doi.org/10.1186/s12864-022-08592-8

Zahrl, R. J., Peña, D. A., Mattanovich, D., & Gasser, B. (2017). Systems biotechnology for protein production in *Pichia pastoris*. In *FEMS Yeast Research* (Vol. 17, Issue 7). Oxford University Press. https://doi.org/10.1093/femsyr/fox068

Zavec, D., Gasser, B., & Mattanovich, D. (2020). Characterization of methanol utilization negative *Pichia pastoris* for secreted protein production: New cultivation strategies for current and future applications. *Biotechnology and Bioengineeing*, *117*, 1394–1405. https://doi.org/10.1002/bit.27303

Zepeda, A., Figueroa, C., Abdalla, D., Maranhão, A., Ulloa, P., Pessoa, A., & Farías, J. (2014a). Biomarkers to evaluate the effects of temperature and methanol on recombinant *Pichia pastoris*. *Brazilian Journal of Microbiology*, *45*(2), 475–483. https://doi.org/10.1590/S1517-83822014000200014

Zepeda, A., Figueroa, C., Abdalla, D., Maranhão, A., Ulloa, P., Pessoa, A., & Farías, J. (2014b). HSF-1, HIF-1 and HSP90 expression on recombinant *Pichia pastoris* under fed-batch fermentation. *Brazilian Journal of Microbiology*, *45*(2), 485–490. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4166272&tool=pmcentrez&rendertype=abstract

Zhan, C., Yang, Y., Zhang, Z., Li, X., Liu, X., & Bai, Z. (2017). Transcription factor Mxr1 promotes the expression of Aox1 by repressing glycerol transporter 1 in *Pichia pastoris*. *FEMS Yeast Research*, *17*(4). https://doi.org/10.1093/femsyr/fox015

Zhang, P., Zhang, W., Zhou, X., Bai, P., Cregg, J. M., & Zhang, Y. (2010). Catabolite Repression of Aox in *Pichia pastoris* is dependent on hexose transporter PpHxt1 and pexophagy. *Applied and Environmental Microbiology*, *76*(18), 6108–6118. https://doi.org/10.1128/AEM.00607-10

Zhu, T., Guo, M., Sun, C., Qian, J., Zhuang, Y., Chu, J., & Zhang, S. (2009). A systematical investigation on the genetic stability of multi-copy *Pichia pastoris* strains. *Biotechnology Letters*, *31*(5), 679–684. https://doi.org/10.1007/s10529-009-9917-4