Chapter 1

# Bioprocess Engineering of *Pichia pastoris*, an Exciting Host Eukaryotic Cell Expression System

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Additional information is available at the end of the chapter

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## 1. Introduction

Yeasts are the favorite alternative hosts for the expression of heterologous proteins for research, industrial or medical use [1]. As unicellulars microorganism have the advantages of bacteria as ease of manipulation and growth rate. But comparing with bacterial system, they are capable of many of the post-translational modifications performed by higher eukaryotic cells, such as proteolytic processing, folding, disulfide bond formation and glycosylation [2].

Historically *Saccharomyces cerevisiae* has been the most used yeast host due to the large amount of knowledge on genetics, molecular biology and physiology accumulated for this microorganism [3-5]. However, it was rapidly found to have certain limitations: low product yields, poor plasmid stability, hyperglycosylation and low secretion capacities. These limitations are now relieved by a battery of alternative yeast as cell factories to produce recombinant proteins.

Some of these alternative yeast cell factories are fission yeast as *Schizosaccharomyces pombe* [6], *Kluyveromyces lactis* [7], methylotrophic species as *Pichia pastoris* [8], *Candida boidinii* [9], *Pichia methanolica* [10], *Hansenula polymorpha* [11], and the dimorphic species *Yarrowia lipolytica* [12], and *Arxula adeninivorans* [13]. It is very usual that the performance of these alternative hosts frequently surpass those of *S. cerevisiae* in terms of product yield, reduced hyperglycosylation and secretion efficiency, especially for high molecular weight proteins [14].

Several reviews compare advantages and limitations of expression systems for foreign genes [15-20]. Between them *Pichia pastoris* has emerged in the last decade as the favorite yeast cell factory for the production of heterologous proteins. A search in ISI Web of knowledge (web of science) with the keywords microorganism+ heterologous protein *P. pastoris* is the preferred host (667 entrances) followed by *Candida* and *Schizosaccharomyces*

(161 and 124 entrances respectively). Specifically for heterologous lipase production *P. pastoris* is the most used host [21].

Why *P. pastoris* emerged as an excellent host system to produce recombinant products?. The story started one decade after oil crisis in the 70's when Phillips Petroleum and the Salk Institute Biotechnology/Industrial Associates Inc. (SIBIA, La Jolla, Ca, USA) used *Pichia* as a host system for heterologous protein expression [22-24]. Nowadays, more than 500 proteins have been expressed using this system [25] and it also has been selected by several protein production platforms for structural genomics programs [26]. *P. pastoris* combines the ability of growing on minimal medium at very high cell densities (higher than 100 g DCW/L), secreting the heterologous protein simplifying their recovery. Also, it performs many of the higher eukaryotic post-translational modifications such as protein folding, proteolytic processing, disulfide bond and glycosylation [24]. However, it has been shown that both, N- and O-linked oligosaccharide structures, are quite different from mammalian cells, for example, they are of a heterogeneous high-mannose type. The consequence is that high mannose type N-glycans attached to recombinant glycoproteins can be cleared rapidly from the human bloodstream, and they can cause immunogenic reactions in humans [27]. Nevertheless GlycoFi's glycoengineering technology allows the generation of yeast stains capable of replicating the most essential steps of the N-glycosylation pathway found in mammals [28].

But, probably the most important characteristic of *P. pastoris* is the existence of a strong and tightly regulated promoter from alcohol oxidase 1 gene, *PAOX1*. Thus, methanol was used as carbon source and inducer of heterologous protein production in this system [29].

Daly and Hearn [30] reviewed various aspects of the *P. pastoris* expression system and also consider the factors that need to be taken into account to achieve successful recombinant protein expression, particular when more complex systems are contemplated, such as those used in tandem gene or multiple gene copy experiments. Between them, several genetic and physiological factors such as the codon usage of the expression gene, the gene copy number, efficient transcription by using strong promoters, translation signals, translocation determined by the secretion signal peptide, processing and folding in the endoplasmatic reticulum and Golgy and, finally, secretion out of the cell, as well as protein turnovers by proteolysis, but also of the optimization of fermentation strategy [31].

The objective of this chapter is to review the classic and alternative operational strategies to maximize yield and/or productivity from an industrial point of view and also how to obtain a repetitive product from batch to batch applying process analytical technology (BioPAT)

## 2. Host strains and *PAOX1* promoter

Host strains and vectors are available as commercial kits from Invitrogen Corporation (Carlsbad, CA) [32]. *PAOX1* is the preferred promoter. Previous to design operational strategies is necessary to know the machinery to inducer this promoter and how *Pichia* metabolizes methanol.

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*PAOX1* is strongly repressed in presence of carbon sources as glucose, glycerol, ethanol and most of other carbon sources, being strongly induced by the presence of methanol [33]. Alcohol oxidase is the first enzyme of methanol assimilation pathway, which catalyzes its oxidation to formaldehyde [34]. The genome of *Pichia* contains two genes of this functional enzyme AOX1 and AOX2. Around the 85% of alcohol oxidase activity is regulated by *AOX1* gene, whereas *AOX2* gene regulates the other 15% [35]. AOX concentration can reach 30% of the total cell protein when is growing on methanol, which compensates for the low affinity of the enzyme for methanol [22].

There are three types of *P. pastoris* host strains available which vary with regards to their ability to utilize methanol. The wild-type or methanol utilization plus phenotype ($Mut^+$), and the strains resulting from deletions in the *AOX1* gene, methanol utilization slow ($Mut^s$), or both *AOX* genes, methanol utilization minus ($Mut^-$) [36].

Although AOX1 is the promoter most commonly used, it presents a serie of limitations. Oxygen supply becomes a major concern in *P. pastoris* in methanol non-limited fed-batch cultures when high cell densities are desired for the production process using $Mut^+$ phenotype, since the bioreactor oxygen transfer capacity unable to sustain the oxygen metabolic demand [24]. Another important disadvantage of *PAOX1*, especially in $Mut^+$ phenotype in large scale productions, is the necessity to storage huge amount of methanol which constitutes a potential industrial risk. On the other hand, methanol presents a high heat of combustion (-727 kJ C-$mol^{-1}$) [37]. Thus, considerable heat is generated during the bioprocess growing on this carbon source. It requires rapid and efficient cooling systems, particularly at large scale where heat losses through the bioreactor walls may be limiting due to the small surface area to volume ratio. Failure to remove this heat may result in reactor temperature increase affecting the productivity and quality of the recombinant protein [38]. Furthermore since methanol is mainly derived from petrochemical sources, may require purifications steps for the production of certain foods and additives products [39].

## 3. *Pichia* Process Analytical Technology (PAT)

It is necessary to develop bioprocess optimization and control tools in order to implement a Process Analytical Technology (PAT), BIOPAT when it is applied to bioprocesses [40]. This initiative has been promoted by regulatory agencies such as FDA and EMEA [41]. PAT is a multidisciplinary platform for designing, analyzing and controlling manufacturing through timely measurements of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality [42].The final goal is guarantee consistent product quality at the end of the process, ease the regulatory review bioprocess and increase flexibility with respect to post-approval manufacturing changes [43] [Figure 1].

Applied to *Pichia* cell factory, on-line monitoring of biomass, methanol and product are the dream of all researchers involved in the production of heterologous protein in this host.

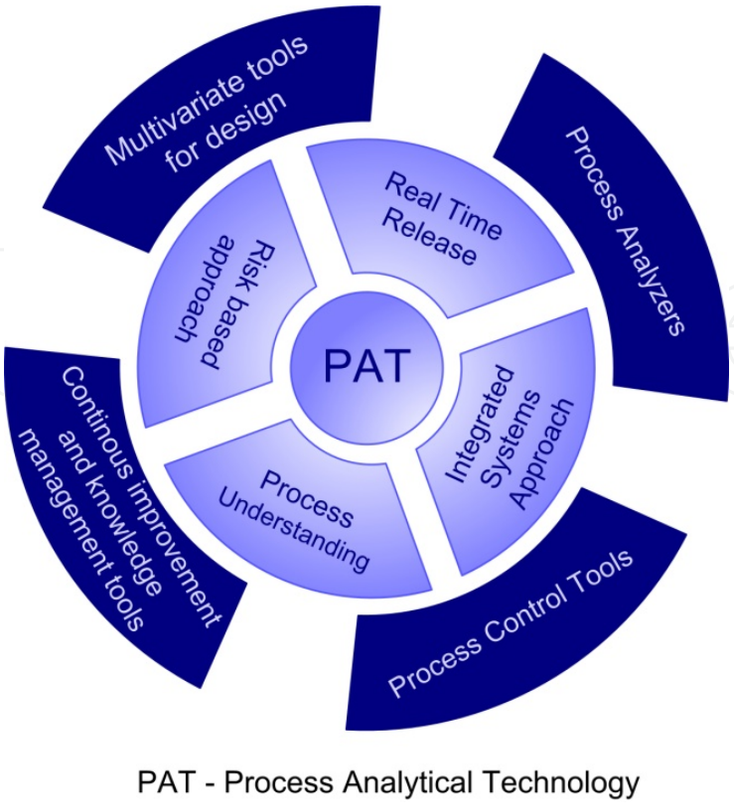


**Figure 1.** Scheme of a process analytical technology (PAT).

Different approaches have been applied for the on-line determination of biomass in *Pichia's* fermentation. Multi-wavelength fluorescence coupling with PARAFAC-PLS chemometric methodology resulting in important qualitative and quantitative bioprocess information [Figure 2; Figure 3]. Biomass and substrate (glycerol or methanol) were determined successfully. The recombinant lipase, the heterologous product, could also be on-line determined in the exponential phase. However in the stationary Phase, where proteolytic problems appears, the estimation of the product could not be estimated accurately [44-46].

Multi-wavelength fluorescence is not standard equipment used in bioprocesses. Thus, when direct biomass quantification methods are not available, biomass can be determined from indirect on-line measurements using software sensors. The estimation of biomass, substrate and specific growth rate by two non-linear observers, nonlinear observed-based estimator (NLOBE) and second-order dynamic tuning (AO-SODE) and a linear estimator, recursive least squares with variable forgetting factor (RLS-VFF) have been applied to *Pichia* bioprocess using different indirect measurements, carbon dioxide transfer rate (CTR), oxygen uptake rate (OUR) from conventional infrared and paramagnetic gas analysis, and sorbitol. The AO-SODE algorithm using OUR on-line measurement was the most efficient approach demonstrating the robustness of this methodology [47]. A comparison of the performance of the different observers is presented in table 1.

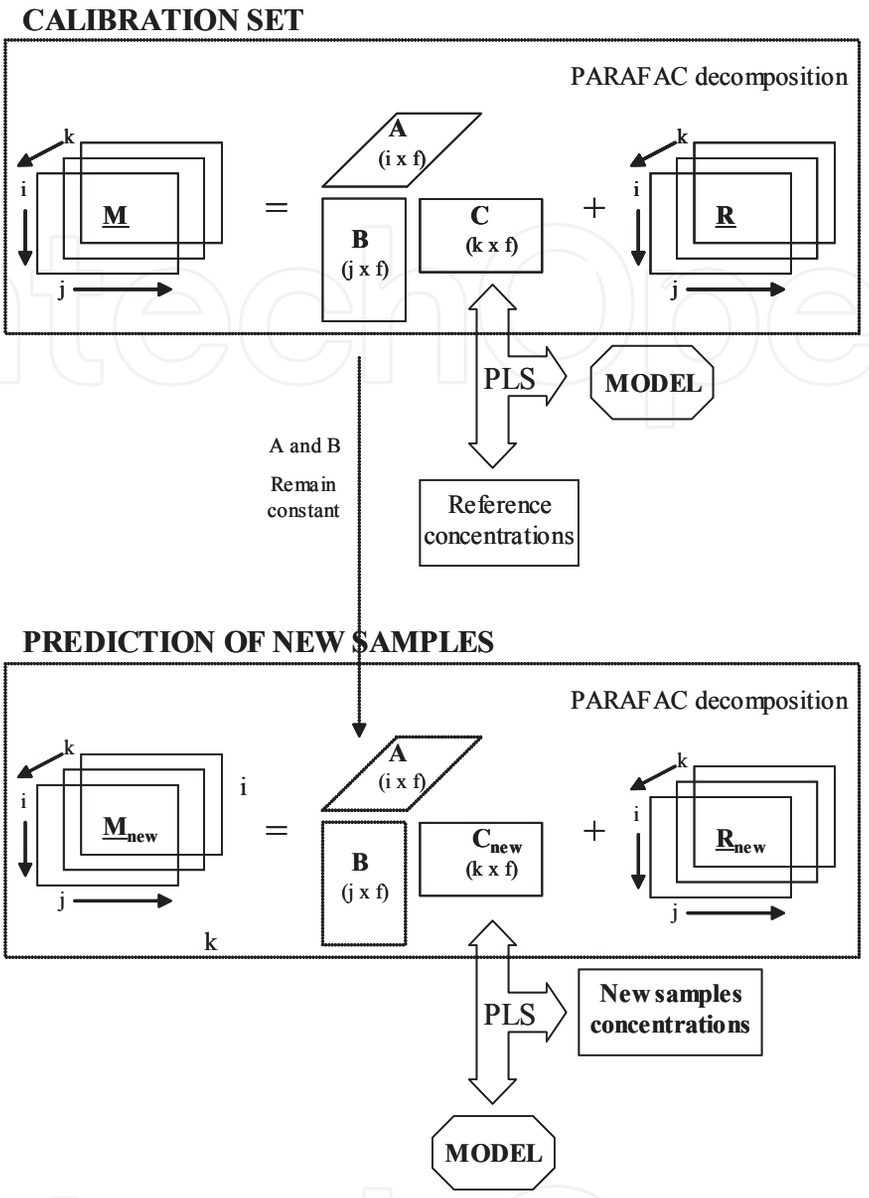


**Figure 2.** Scheme of the calibration and prediction processes for PARAFAC combined with PLS regression for state variables determination.

Methanol concentration, the inducer substrate, is the most important variable for on-line monitoring because the productivity of the bioprocess is quite related to this parameter. Concentrations between 2-3.5 g/L are referenced as optimal concentrations to maximize protein production [48,49], higher concentrations present inhibition problems and in some cases lower concentration stops recombinant protein production [50].

Although chromatographic methods such as GC and HPLC are common methods for the off-line analysis, their on-line implementation is not usual due to the low sampling frequency [49].

On-line methods are generally based on liquid-gas equilibrium by analyzing the fermenter exhaust gas [51]. Nowadays, commercial equipments based in this principle are available from

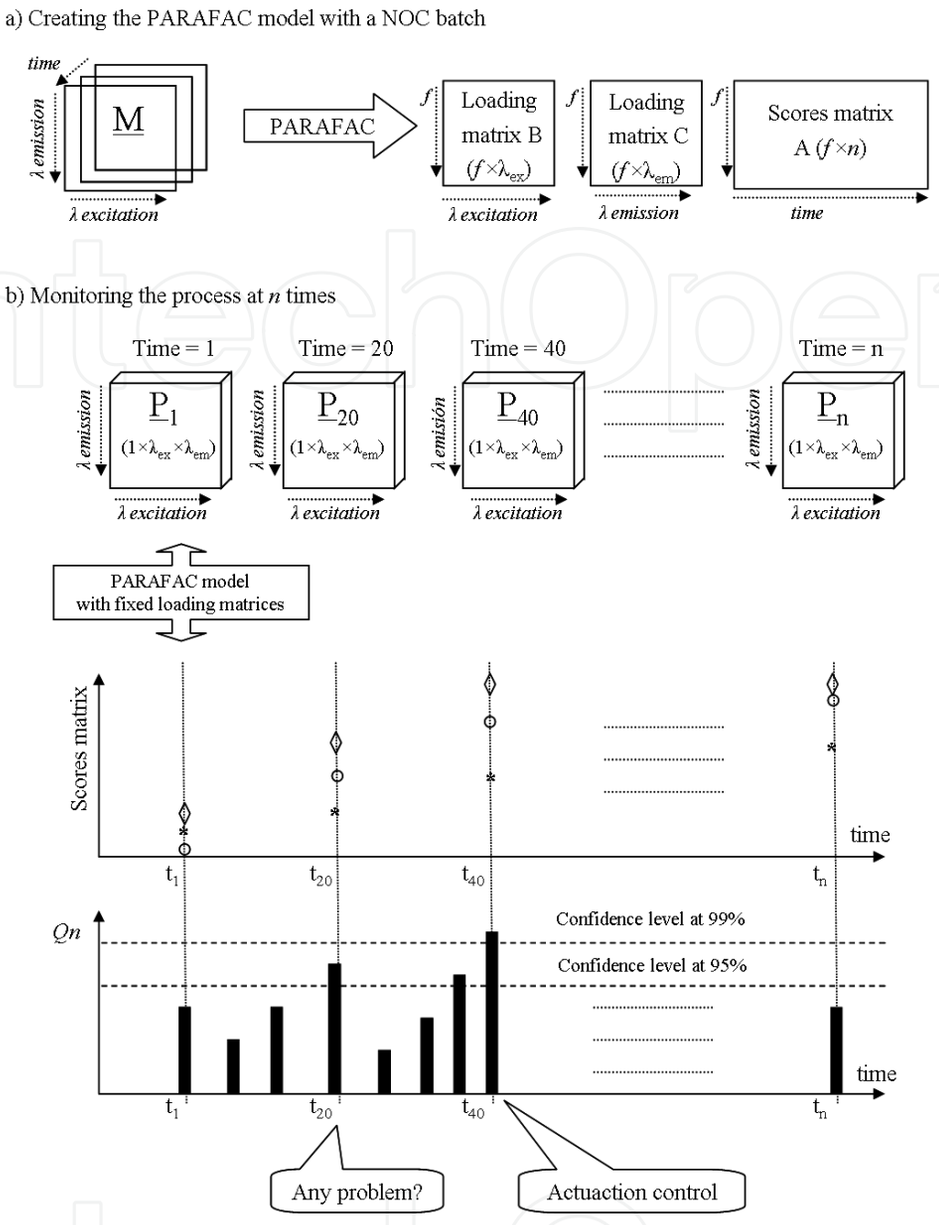


**Figure 3.** Summary of the application of on-line PARAFAC approach (NOC = Normal Operating Conditions).

Raven Biotec, Figaro Biotech, PTI Instruments [52]. These equipments are quite robust and with minimum maintenance although some precautions should be taking into account to obtain a precise measurement [53].

Other alternatives are sequential injection analysis [54] Fourier transform mid-infrared spectroscopy [49] and flame ionization [55].

Process optimization only can conclude with effective measurement of heterologous protein production. Classical methods as ELISA, SDS-PAGE and Western blots or bioactivity assay are time-consuming, labour-intensive, and not applicable for the determination of the product in real time [51]. Methods including perfusion chromatography, specific biosensors and

| Methods | Advantages | Disadvantages |
|---|---|---|
| NLOBE | Easy tuning, 1 tuning parameter. | Strong dependence of initial values and kinetic yields. |
| AO-SODE | Rapid and stable response. Easy tuning, 2 tuning parameters. | Accurate knowledge of reaction scheme and stoichiometric coefficients are necessary. |
| RLS-VFF | Minimal knowledge of the system. | Sensible to rapid changes of μ. |

**Table 1.** Comparison of three different observers for the estimation of biomass, substrate and specific growth rate.

immunonephelometric assays are limited to proteins secreted in the extracellular culture broth, but not intracellular protein production [56,57]. To circumvent this problem fusing a GFP signal marker to the recombinant protein could be detected by fluorescence [58]. However the co-expression of this protein fusion could provoke a lost in the production of the recombinant product. When the recombinant protein has an associated colorimetric reaction, for instance enzymes, analytical approaches using flow injection analysis (FIA) or sequential injection analysis (SIA) are widely used [59].One of the most fully automated *Pichia* bioprocess has been developed by the group of professor Luttmann [60]. An example of on-line monitoring and control of *Pichia* bioprocess producing *Rhizopus oryzae* lipase is presented in Figure 4. The real time evolution of the main parameters, variables and specific rates of this bioprocess are presented in Figure 5a and 5b.

## 4. Operational strategies using *PAOX1* Mut$^+$ phenotype

Some of the operational strategies using the phenotype Mut$^+$ are focused in order to circumvent operational problems previously commented. Invitrogen Co., only provides an operational manual for the fed-batch growth on *Pichia* (Manual Invitrogen) [61] mainly derived from the protocols of Brierley and coworkers [62]. Fed-batch fermentation protocols include three different phases. A glycerol batch phase (GBP), a transient phase (TP) and finally, a methanol induction phase (MIP). Normally GBP and TP are similar for both phenotypes (Mut$^+$ and Mut$^s$). The objective of the GBP is the fast generation of biomass previous to the induction of methanol. The specific growth rate and yield of *Pichia* growing on glycerol are from 0.18 $h^{-1}$ and 0.5 g DCW per gram of glycerol [63] to 0.26 $h^{-1}$ and 0.7 g DCW per gram of glycerol [67]. Brierley and coworkers recommended a maximum glycerol concentration of 6% [62]. Higher concentration inhibits growth [68]. The specific growth rate and yield is higher than growing on methanol (0.12 $h^{-1}$) and 0.27 g DCW per gram of methanol) [62]. When higher initial biomass concentration is required a second step with an exponential feeding rate of glycerol is implemented. It is important that in GBP dissolved oxygen (DO) reaches values higher than 20-30% to avoid the production of ethanol.

Once the GBP is finished, indicated by a spike in measured DO or a decreased in $CO_2$ consumption rate (CER), TP is started. The objective of TP is increase biomass level to generate

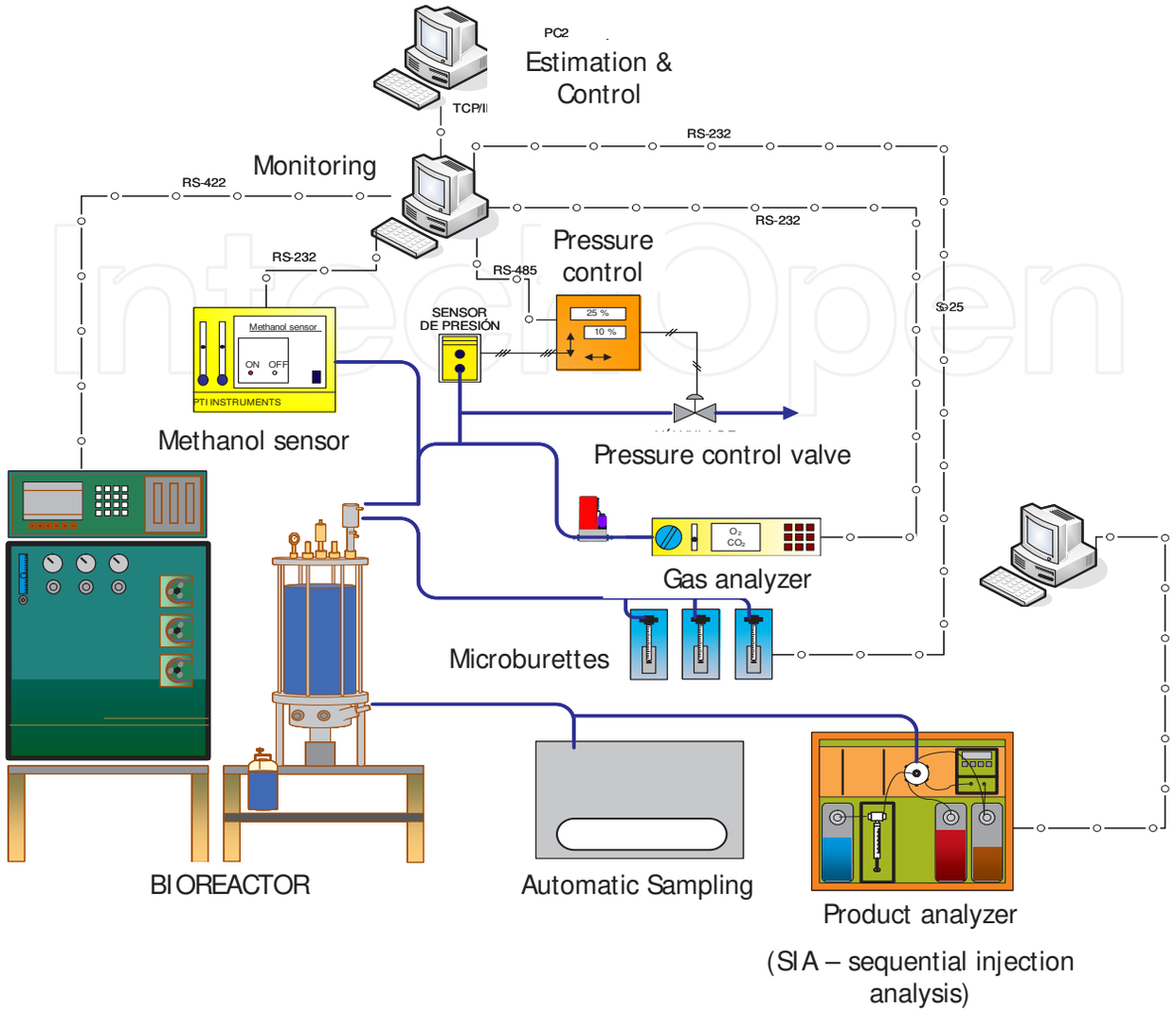


**Figure 4.** Bioprocess scheme for on-line monitoring and control of *Pichia pastoris* producing recombinant *Rhizopus oryzae* lipase.

high cell density cultures jointly with the derepression of AOX1 promoter due to the absence of an excess of glycerol prior to MIP. Different strategies are collected in a set of reviews [32, 34, 51, 52].

The selected operational strategy used in the MIP is one of the most important factors to maximizing heterologous protein production [67]. These strategies using a $Mut^+$ phenotype have to circumvent the associated problems to the maximum methanol consumption capacity previously pointed out.

At his point, the monitoring and control of the inducer substrate, methanol, are the most important key parameter. High levels of this inducer substrate can generate inhibitory effect on cell growth [67], and low levels of methanol may not be enough to initiate the AOX transcription [8]. The inhibition profile on methanol follows an uncompetitive inhibition growth model, with a reported critic methanol concentration between 3 and 5.5 g/L depending

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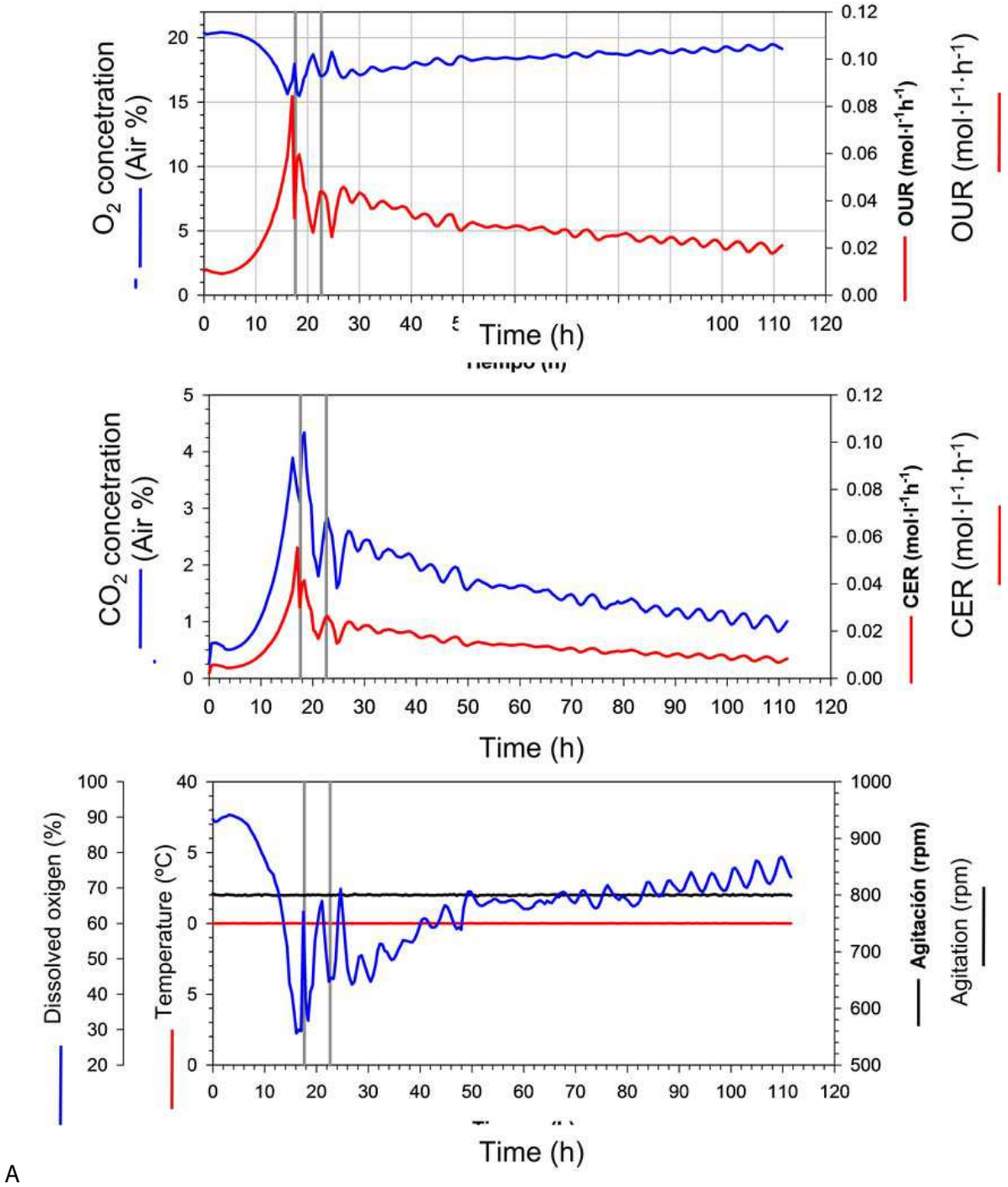
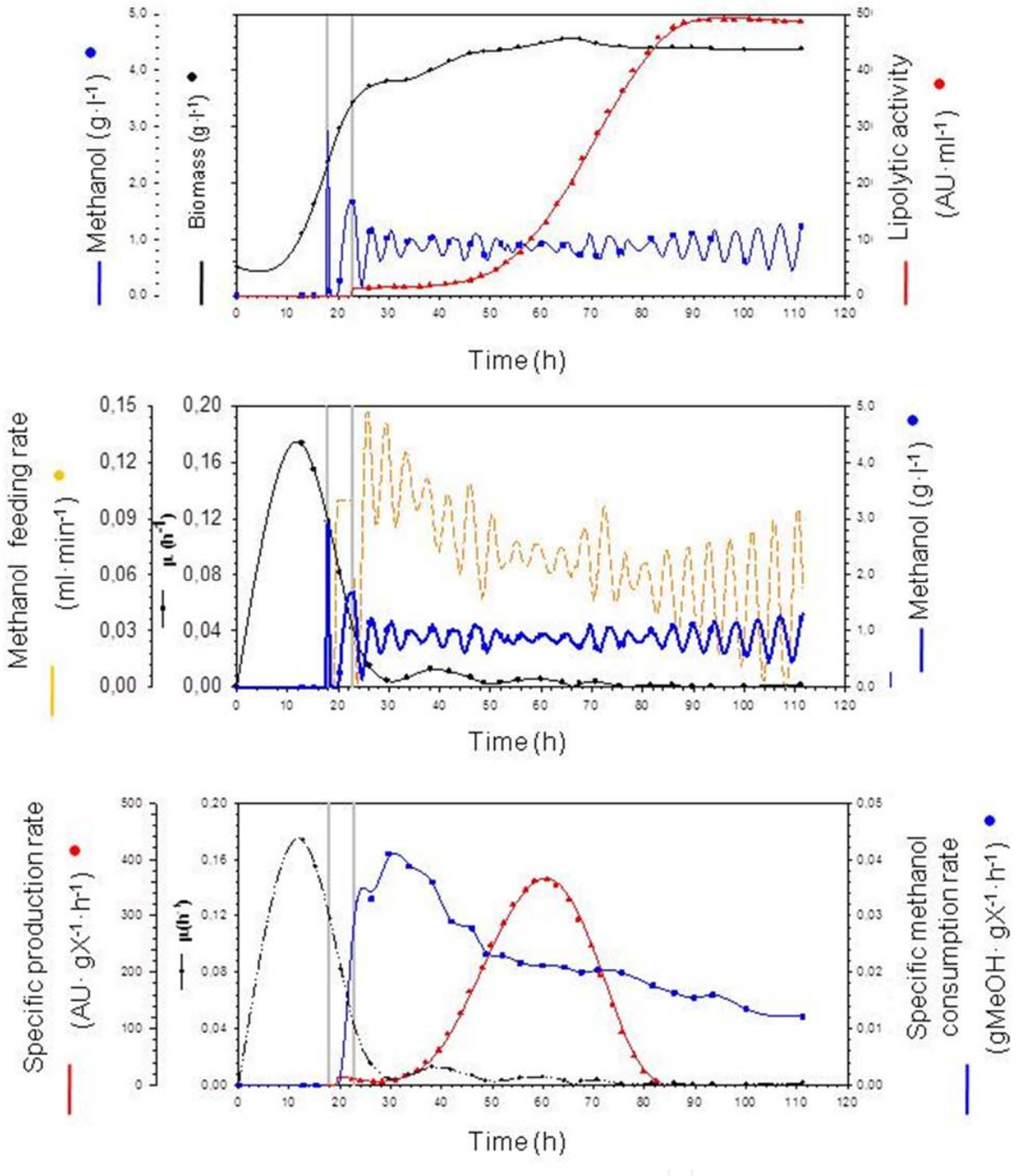



A




**B**

**Figure 5.** A.- Example of the on-line monitoring of *Pichia pastoris* producing recombinant *Rhizopus oryzae* lipase. Real time performance of standard fermentation parameters. B.- Example of the on-line monitoring of *Pichia pastoris* producing recombinant *Rhizopus oryzae* lipase. Real time evolution of biomass, substrate and product and their corresponding specific growth rate, methanol consumption rate and lipase production rate.

on the target protein [34]. Thus, a set-point methanol concentration around 2 g/L seems an optimal value to maximize protein production. Although keeping a constant methanol concentration during the induction phase has positive effects on the production of foreign

protein [65], some authors pointed out that the design of an optimal methanol or specific growth rat profile along the MIP maximize the productivity of the process [68].

It is quite difficult to compare the performance of different fed-batch strategies with different heterologous protein. On the other hand, the selection of the fed-batch strategy depends on the facilities to monitor methanol or other key variables as biomass or recombinant product.

Simple strategies, like the addition of a pulse of methanol at different time intervals, must be limited in basic studies to obtain a quantity of recombinant protein for preliminary characterization or structural studies, but is not realistic from an industrial point of view.

Several strategies have been proposed to optimize the methanol feeding rate with the final objective of maximizing protein production and to get a reproducible bioprocess:

## 5. DO-stat control

*Pichia* cells utilize methanol through the oxidative pathway only when oxygen is non-limiting [34]. Thus, DO must be controlled above a minimal level around 20% [69]. However, oxygen limitation was successfully used to control the methanol uptake during single-chain antibody fragment production [70,71] and other groups have proposed using oxygen as the growth-limiting nutrient, instead of methanol to circumvent the problem of high oxygen demand and observed 16-55% improvements in product concentrations [72,73]. Recently, an oxygen-limited process has been developed and optimized for the production of monoclonal antibodies in glycoengineered *P. pastoris* strain using oxygen uptake rate as a scale-up parameter from 3L laboratory scale to 1200 L pilot plant scale. Scalability and productivity were improved reducing oxygen consumption and cell growth [74-76]. On the other hand, excessive high DO levels are cytotoxic reducing cell viability [77].

Although different DO-start control has been developed [77-80]. This strategy cannot distinguish the possible accumulation of methanol. In this situation DO signal increases due to the inhibitory effect of methanol on growth, and the response of the DO-controller should be to increase the feeding rate of methanol aggravating the problem. This is particularly problematic in the beginning of the induction phase where *AOX1* is not yet strongly induced and the AOX activity in the cells is growth-rate limiting but constantly increasing as a result of the induction [32].

## 5. Methanol open-loop control

In this simple strategy, the methanol feeding rate profile (exponential) is obtained from mass balance equations with the objective to maintain a constant specific growth rate ($\mu$) under methanol limiting conditions (no accumulation of methanol should be observed). To implement preprogrammed exponential feeding rate strategy, biomass concentration and volume at the beginning of the MIP have to be known and to assume that a constant biomass/substrate

yield is maintained along the induction phase. This strategy has problems in terms of robustness and process stability, because, although open-loop system could be easy to implement they do not respond to perturbations of the bioprocess. To avoid this problem the set point of μ is fixed far from the $\mu_{max}$ diminishing the productivity of the process. Nevertheless this simple strategy has been applied successfully in different bioprocesses [81-84]. On the other hand, when the recombinant protein affects the growth of the host reaching $\mu_{max}$ lower than the wild strain, like in the production of *Rhizopus oryzae* lipase under methanol limiting conditions, the production is stopped few hours later of the beginning of MIP (personal communication of the author).

## 6. Methanol closed-loop control

In previous strategies methanol concentration is neither measured on-line not directly controlled [51]. Thus, an accurate monitoring and control of methanol concentration is required. As previously has been commented, different analytical approaches has been implemented in order to on-line monitoring of methanol concentration in *Pichia's* fermentation. Analytical devices based on liquid-gas equilibrium by analyzing exhaust gas from the fermented are the most used. There are as set of methanol sensors available in the market from Raven Biotech, Figaro Electronics, PTI Instruments, and Frings America [52, 85]. The first attempts have been based to maintain the methanol concentration along the induction phase at a constant and optimal concentration to maximize protein production or productivity bioprocess. However, in the last years, some approaches are implementing in order to define an optimal variable methanol set-point function of the different stages of the induction phase. A scheme of both methanol feeding strategies, open and closed loop, is presented in Figure 6.

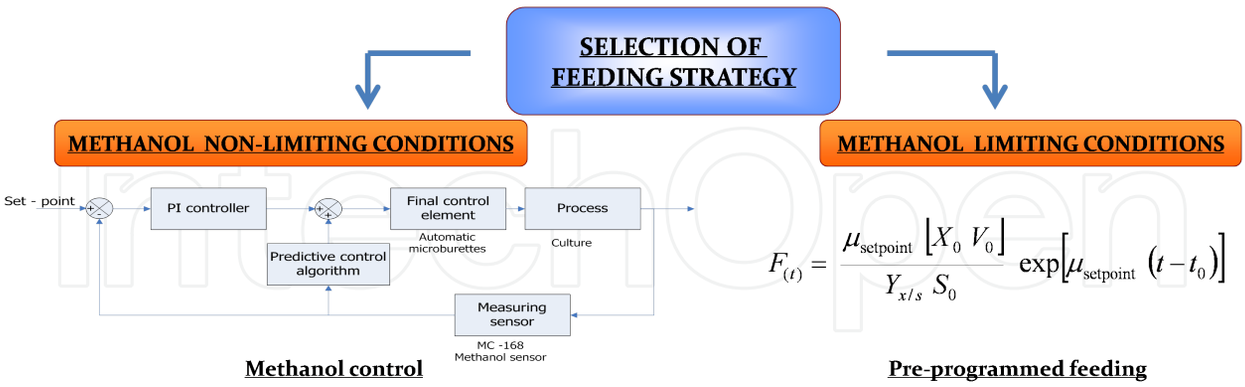



**Figure 6.** Scheme of methanol feeding strategies: open loop and closed loop control.

Different methanol control concentration algorithms and strategies have been proposed. Although the on-off control is the simplest feed-back control strategy and it has been used by different authors [81, 85-88] *Pichia* fermentation, as bioprocesses in general, is characterized by a complex and highly non-linear process dynamics. For this reason this control strategy is inadequate for precise control of methanol concentration in the bioreactor because it can result

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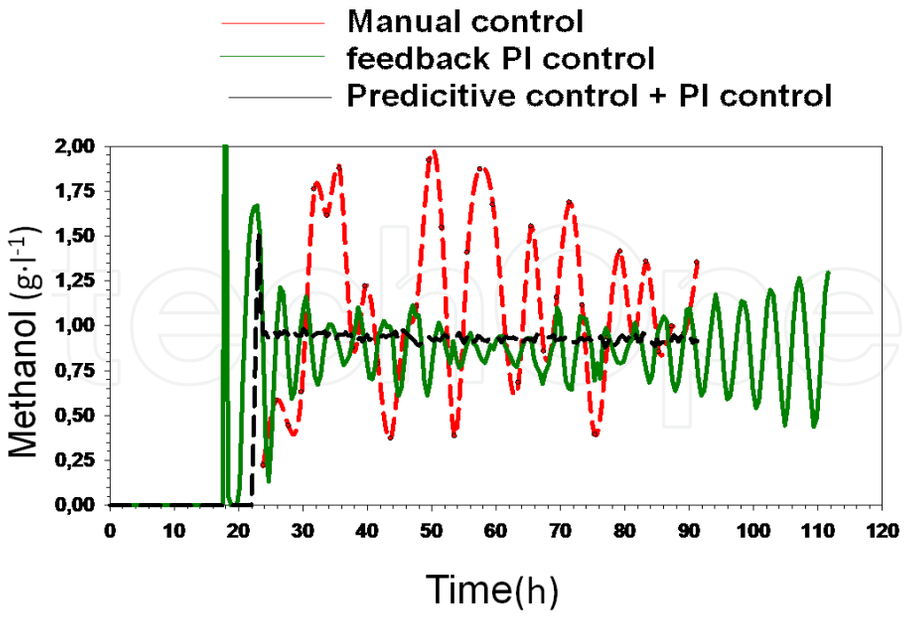


**Figure 7.** Comparison of the performance of the different methanol control algorithms in *Pichia pastoris* bioprocess producing recombinant lipase.

in a fluctuating methanol concentration around the set-point [34]. In $Mut^s$ phenotype, where the methanol consumption rate is lower than in $Mut^+$ phenotype, this control algorithm has better performance.

A proportional-integral (PI) or proportional-integral derivative (PID) control algorithms are more effective approach. Nevertheless, the optimal settings of the PID controller (gain $K_C$, the integral time constant $\tau_I$ and the derivative time constant $\tau_D$) are hardly ascertained by trial and error tuning or other empirical methods. Some authors have developed a PID control Bode stabilization criterion to achieve the parameters associated to this king of control, obtaining good results on methanol regulation in short time fermentations [77,88]. Because of the dynamics of the system, the optimal control parameters may vary significantly during the fermentation. Moreover, the existence of an important response time for both, the on-line methanol determination and the biological system has promoted the development of other control alternatives [34].

A predictive control algorithm coupled with a PI feedback controller has been implemented successfully in heterologous *Rhizopus oryzae* lipase production. It is based on the methanol uptake on-line calculation from the substrate mass balance in fed-batch cultivations, requiring the first-time derivative of methanol concentration for each time interval. This predictive part is coupled to a feed-back term (PI) to regulate the addition aiming a stabilizing the signal around the set-point [89]. Although this strategy was implemented in $Mut^s$ phenotype, it has been implemented in $Mut^+$ phenotype successfully. A comparison of the performance of the different control algorithms is presented in Figure 7.

Model based on-line parameters estimation and on-line optimization algorithms have been developed to determine optimal inducer feeding rates. Continuous fermentation using

methanol was performed via on-line methanol measurement and control using a minimal-variance-controller and a semi-continuous Kalman-Filter [90].

## 7. Strategies to minimize oxygen demand

The standard fed-batch fermentation without oxygen limitation is namely methanol non-limited fed-batch (MNLFB). Independently of the strategy selected, high cell density cultures with Mut$^+$ *P. pastoris* phenotype in laboratory bioreactors presents the problems of oxygen supply, since the bioreactor oxygen transfer capacity is unable to sustain the oxygen metabolic demand [91]. When the biomass reaches values higher than 60 gDCW/L oxygen limitations appears, even using mixtures of air and oxygen or pure oxygen. Different approaches have been published to overcome this disadvantage:

Temperature-limited fed-batch (TLFB). In this strategy the common methanol limitation is replaced by temperature limitation in order to avoid oxygen limitation at high cell density limitation [92]. Temperature controller was programmed to maintain a DO set-point around 25%,. When DO is lower than the set-point the culture temperature was decreased [32]. Using this approach cell death values decrease drastically and also protein proteolysis where reduced, although specific growth rate diminishes and, sometimes, it affect negatively to the productivity of the bioprocess [92]. This strategy has been applied successfully in different heterologous protein production [92-96].

Methanol limited fed-batch strategy (MLFB). The strategy is applied once the DO value under non limited conditions achieves values lower than the set-point (around 25%). At this point methanol feeding rate is controlled in order to assure the DO set-point. At this point methanol concentration starts to diminish from the methanol set-point to limiting conditions, although specific productivity can diminish the production of the heterologous product is not stopped [84, 91, 97-98].

## 8. Operational Strategies using *PAOX1* Mut$^s$ Phenotype

Probably Mut$^+$ phenotype under *PAOX1* is the most common *P. pastoris* strain used. However, as it has been commented along the chapter, it presents important operational problems related to oxygen and heat demand and methanol security requires. From the biological point of view, Mut$^s$ phenotype can be used, since they require less oxygen supply and heat elimination. However, the specific growth rate using methanol as sole carbon source is too low compared with Mut$^+$, and low levels of biomass are produced [34,50]. Although from the bioprocess engineering point of view the slow operational conditions facilitates the control and reproducibility of the bioprocess, the fermentation time increase and sometimes the productivity of the process decreases drastically.

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## 9. Mixed substrates

All the strategies previously described for $Mut^+$ phenotype can be applied to $Mut^s$ phenotype, but to increase cell density and process productivity, as well as to reduce the induction time, a typical approach is the use of a multicarbon substrate in addition to methanol. It is a simple strategy to increase the energy supply to recombinant cells and the concentration of the carbon sources in the culture broth [81, 86, 88].

One of the most selected substrates is glycerol. Several authors have reported that the use of mixed feeds of glycerol and methanol during the induction phase increase productivity and feeding rates [99]. The advantages to use glycerol as co-substrate is that enthalpy of combustion of glycerol -549,5 kJC-$mol^{-1}$ [100] is lower than the enthalpy of combustion of methanol, -727 kJC-$mol^{-1}$ [37]. Thus, less heat will be released using mixing substrates compared with methanol alone. On the other hand, oxygen consumption is also reduced since less oxygen is necessary for the oxidation of glycerol [38]. Therefore, any method which reduces the heat and oxygen consumption rate without affecting productivity would clearly advantageous.

However, glycerol is reported to repress the expression of alcohol oxidase and subsequently the expression of the target protein [101]. Thus, the rational design of operational strategies for the addition of both substrates in fed-batch fermentation, while avoiding glycerol repression, is the key point of the bioprocess. Different strategies have been developed in $Mut^+$ phenotype [24, 32, 52, 102-105]. One of the most applied is a pre-programmed exponential feeding rate with an optimum methanol-glycerol ratio [38, 106], or similar strategy maintaining a residual methanol concentration between 1- 2 $gl^{-1}$ [78]. The effect of different methanol-glycerol ratios at constant feeding rate has also been studied in the production of mouse $\alpha$-amylase [107].

One important feature showed in these works is that, although the maximum specific growth rate of *P. pastoris* is around 0.2 $h^{-1}$, the optimum specific growth rate in $Mut^+$ phenotype is around 0.06 $h^{-1}$, too low compared with the maximum value. It seems that although glycerol is under limiting conditions high specific growth rate diminish the productivity of the bioprocess.

For this reason the use of different carbon sources other than glycerol may improve operational strategies on fed-batch cultures [99]. In contrast with glycerol, sorbitol accumulation during the induction phase does not affect the expression level of recombinant protein [108].

In shake flasks, inhibitory effect of sorbitol on cell growth appears at concentrations around 50 $gl^{-1}$ [99]. Hence, control of residual sorbitol concentration during the induction phase is less critical than mixed feeds of glycerol and methanol. On the other hand less oxygen will be consumed during mixed substrate growth on sorbitol and methanol than using the combination glycerol and methanol or on methanol as sole carbon source [99]. However sorbitol has the disadvantage that the maximum specific growth rate is too low around 0.02 $h^{-1}$ similar value that obtained in $Mut^s$ phenotype growing on methanol as sole carbon source. Thus, time fermentation is long and sometimes the increase in the production not is reflected in the producitivity of the bioprocess.

Some different operational strategies have been implemented using sorbitol as co-substrate [99, 102, 106,109-114].

Arnau et al., [102,113] designed an operational strategy using a Mut$^s$ phenotype comparing both co-substrates sorbitol and glycerol in the production of *Rhizopus oryzae* lipase [102,113]. The induction phase started with a preprogrammed exponential feeding rate of sorbitol or glycerol with the objective to maintain a constant specific growth rate under limiting substrate conditions. Methanol set-point was maintained using a predictive control algorithm coupled with a PI feedback control [89]. A set of different specific growth rates and methanol set-points were tested. When sorbitol was used as co-substrate the different specific growth rates tested did not have significance influence on specific production rate of the bioprocess, probably because the use of co-substrate improved the energetic state of the cells overcoming partially the unfolding protein response (UPR) and secretion problems observed in the production of this recombinant fungi lipase. The key parameter in terms of protein production was the methanol set-point selected. Optimal methanol concentration was 2 $gl^{-1}$, lower and higher concentrations diminished specific production rates. The product/biomass yield and the volumetric and specific productivity were 1.25-1.35 fold higher than using methanol as sole carbon source [113].

Irrespective of any economical reasons to use sorbitol or glycerol as co-substrate, one of the key advantages of using glycerol instead of sorbitol is its higher $\mu$ (0.2 $h^{-1}$ versus 0.02 $h^{-1}$) and the subsequent potential increase in the productivity of the bioprocess. However, for Mut$^s$ phenotype this potential advantage is ineffective, because when glycerol exceeds the $\mu_{max}$ of *P. pastoris* growing on methanol as a sole carbon source (around 0.014$h^{-1}$) a repression of *AOX* promoter is clearly observed, represented by a drastic decrease in methanol consumption rates. Additionally, when the relation $\mu_{Gly}$ per $\mu_{MeOH}$ was larger than 4, an important decrease of all productivity ROL parameters was observed. On the other hand, the presence of proteolytic activity detected when glycerol was used as co-substrate is another important drawback [102]. In conclusion sorbitol presented better results than glycerol as co-substrate in the heterologous production of *Rhizopus oryzae* lipase).

*PAOX1* is strongly repressed by glucose at the transcription level. This is the cause that few authors present positive results using this substrate. Nevertheless, a real-time parameter-based controlled glucose feeding strategy has been developed successfully in the recombinant production of phytases [115], Mixtures of glucose and methanol has also been used in continuous cultures producing recombinant trypsinogen [116].

## 10. Alternative promoters

An important set of inducer promoters derived from genes which code for enzymes involved in the methanol metabolism are used as alternative promoters to the classical. *PAOX1*. A summary of the main alternative promoters is presented in table 2. Formaldehyde dehydrogenase promoter *PFLD1* inducible by methanol or methylamine [116], dihidroxyacetone synthase promoter *PDHAS* [101], and peroxisomal matrix protein gene promoter *PEX8*

inducible by methanol or oleate [118] are some examples. Other inducer promoter is the isocitrate lyase 1 *PICL1*. This promoter is inducible with ethanol and repressed by glucose in the exponential phase, but not in the stationary phase [119].

| Inducible promoters | Reference | Constitutive promoters | Reference |
|---|---|---|---|
| *PAOX1* | 22 | *PGAP* | 121 |
| *PFLD1* | 116 | *PTEF1* | 122 |
| *PDHAS* | 101 | *PYPT1* | 123 |
| *PEX8* | 118 | *PPGK1* | 124 |
| *PICL1* | 119 | *PTHI1* | 120 |

**Table 2.** Summary of the main inducible and constitutive alternative promoters to *PAOX1*.

However, these alternative promoters have similar operational problems than *PAOX1*, especially when methanol is not substituted as inducer due to safety problems. This is the cause of a strong demand for alternative regulated promoters [120]. Between them, the constitutive glyceraldehydes-3-phosphate dehydrogenase promoter *PGAP is* the most common used [121]. Other constitutive promoters are the translation elongation factor 1-$\alpha$ promoter *PTEF1* [122], the promoter of YPT1, a GTPase involved in secretion [123] and the promoter of the 3-phosphoglycerate kinase *PPGK1*, from a glycolytic enzyme [124].

Stadlmayr *et al.*, [120] have identified 24 novel potential regulatory sequences from microarray data and tested their applicability to drive the expression of both, intracellular and secretory recombinant proteins with a broad range of expression levels. Although the production of model proteins not exceed the values obtained with the constitutive promoter *PGAP*, higher transcription levels at certain growth phases were detected with the translation elongation factor EF-1 promoter *PTEF1* and the promoter of a protein involved in the synthesis of the thiamine precursor *PTHI1.*

Between them only the inducer *PFLD1* and the constitutive *PGAP* have been applied for the routine production process, specially the last one.

The *FLD1* gene codes for an enzyme that plays an important role in the methanol catabolism as carbon source, as well as in the methylated amines metabolism as nitrogen source [125]. *PFLD1* is a strongly an independently induced either by methanol as carbon source or methylamine as nitrogen source [117]. Preliminary experiments to get an alternative carbon source to methanol showed that sorbitol, a carbon source that no repress the synthesis of methanol metabolism enzymes, also allows the induction of *PFLD1* by methylamine [126]. It suggests that the use of sorbitol as carbon source combined with methylamine as nitrogen source could be the basis for the development of methanol-free fed-batch fermentation. In fact, a methanol-free high cell density fed-batch strategy has been developed for the recombinant production of *Rhizopus oryzae* lipase. These fed-batch strategy has the same phases that a

standard *PAOX1* promoter. GBP is similar but glycerol and ammonia as carbon and nitrogen sources are presented in a stoichiometric relation to achieve the exhaustion of both substrates at the end of the GBP. The TP consist in a sorbitol methylamine batch (SMBP) as a transition phase. The objective of the SMBP is the adaptation of the cells to the carbon and nitrogen sources used in the induction phase. Finally, the methylamine induction phase (MAIP) where a pre-programmed feeding rate strategy ensured a constant specific rate under sorbitol limiting conditions or maintaining a set-point of methanol at high specific growth rate have been implemented [127]. The result showed that the recombinant protein production is favored with the second strategy. When the performance of the bioprocess were compared to classical *PAOX1* promoter, the results were quite similar in terms of process productivity [63]. The production of this recombinant lipase under *PFLD1* triggers the unfolding protein response (UPR) detected at transcriptional levels [128].To overcome this problem two cell engineering strategies have been developed and applied successfully: the constitutive expression of the induced form of the *Saccharomyces cerevisiae* unfolded protein response transcriptional factor Hac1 and the deletion of the *GAS1*gene encoding a β-1,3 glucanosyltransglycosylase, GPI-anchored to the outlet leaflet of the plasma membrane, playing a key role in yeast cell wall assembly [129]. This is an example that how the co-expression of proteins or the deletion of genes affect to bioprocess engineering.

The great advantage of the constitutive GAP promoter is that the cloned heteroloogus protein will be expressed along with cell growth if the protein is not toxic for the cells [130]. The use of this promoter is more suitable for large-scale production because the hazard and cost associated with the storage and delivery of large volumes of methanol are eliminated [131], and also for the implementation of continuous cultures, continuous cultures practically not described using *PAOX1* [134]. Thus, the features of the GAP expression system may contribute significantly to the development of cost-effective methods for large-scale production of heterologous recombinants proteins [132-133]. The efficiency of *PGAP* compared with *PAOX1*depends generally of the protein expressed, although some times the better optimization of operational strategy can mask the results.

In general, the substrates used with this promoter are glucose or glycerol. The standard operational strategy is a batch phase using glycerol and a fed-batch phase in an open-loop control using glucose. The selection of the optimal sequence of both substrates is under studies. For instance, the production of rPEPT2 growing on glucose was approximately 2 and 8 times higher than in cells grown on glycerol and methanol [135].

When using this expression system, specific production rate increases asymptotically to a maximum value with increasing μ [68]. Maurer *et al.,* have developed a model to describe growth and product formation, optimizing the feeding profile of glucose limited fed batch cultures to increase volumetric productivity under aerobic conditions [68]. Under hypoxic conditions, where growth is controlled by carbon source limitation, while oxygen limitation was applied to modulate metabolism and heterologous protein productivity, an increase in the specific productivity has been observed. This strategy has additional benefits including lower aeration and lower final biomass concentration [73].

In conclusion PGAP is the most promise alternative to the classical PAOX1 promoter.

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