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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 unicellular 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 glyco-engineering technology allows the generation of yeast strains 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 endoplasmic reticulum and Golgi 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.

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].

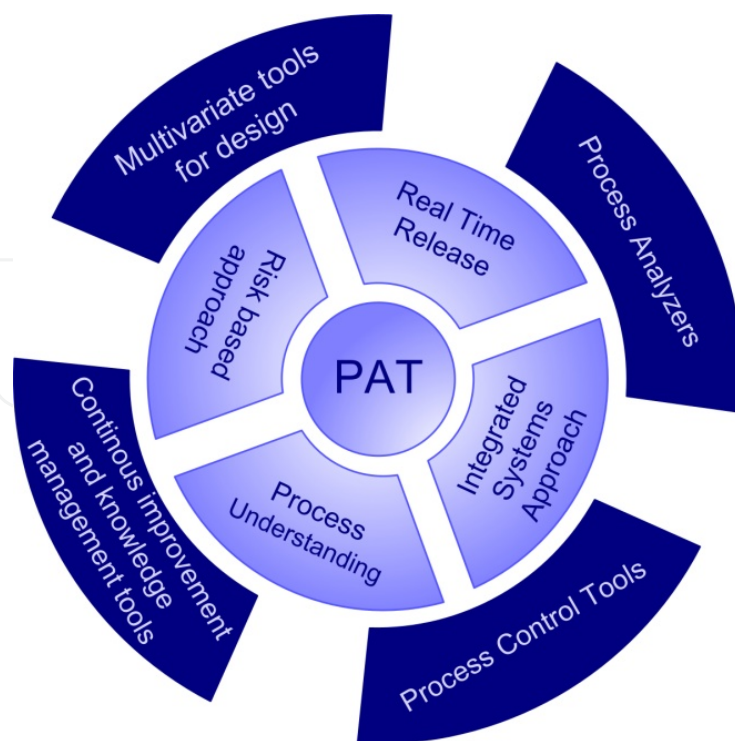
There are three types of *P. pastoris* host strains available which vary with regards to their ability to utilize methanol. The wild-type or methanol utilization plus phenotype (Mut^+), and the strains resulting from deletions in the *AOX1* gene, methanol utilization slow (Mut^s), or both *AOX* genes, 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 ($-727 \text{ kJ C-mol}^{-1}$) [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.



PAT - Process Analytical Technology

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.

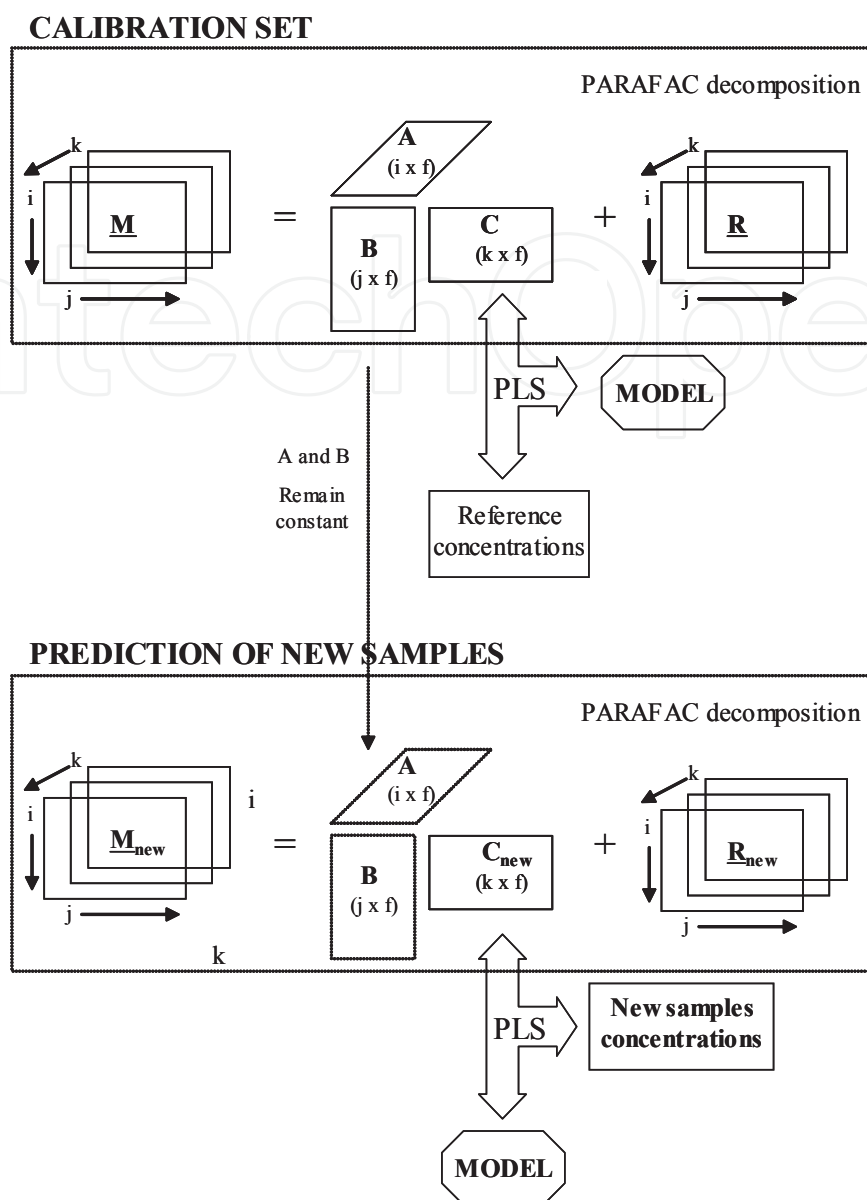


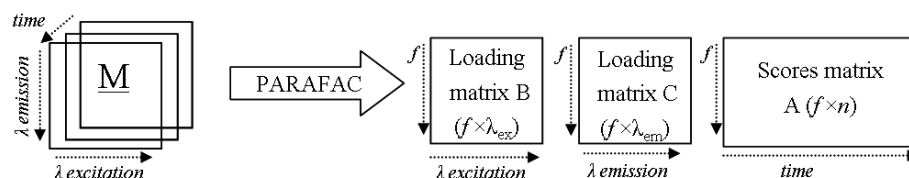
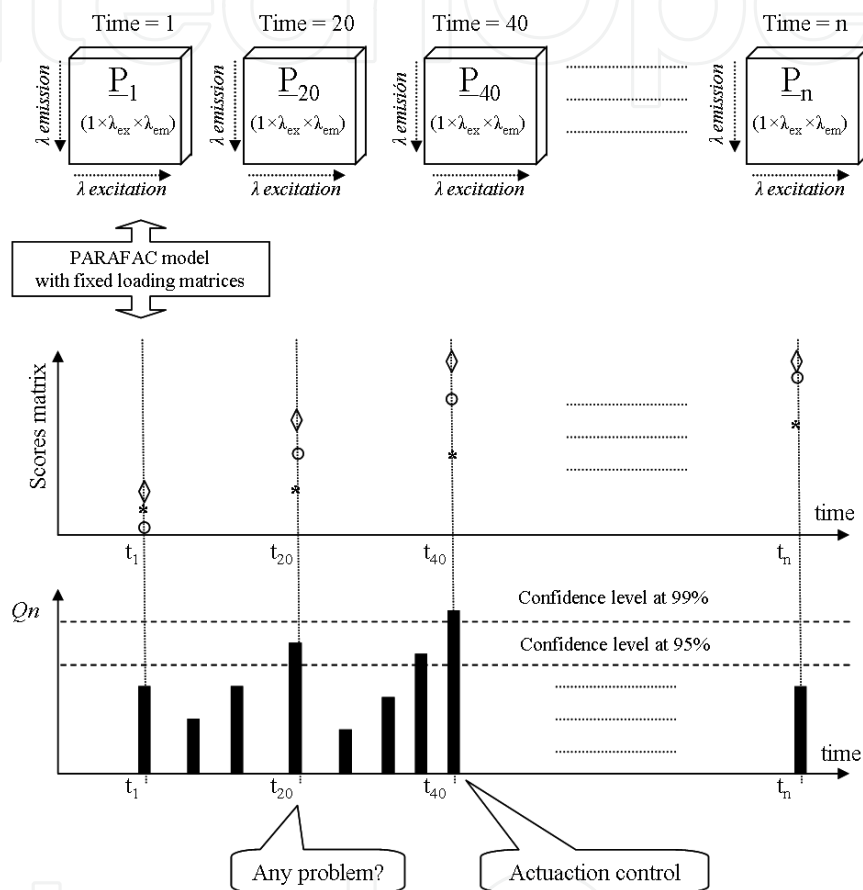
Figure 2. Scheme of the calibration and prediction processes for PARAFAC combined with PLS regression for state variables determination.

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

a) Creating the PARAFAC model with a NOC batch

b) Monitoring the process at n times**Figure 3.** Summary of the application of on-line PARAFAC approach (NOC = Normal Operating Conditions).

Raven Biotech, 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⁻). 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 CO₂ consumption rate (CER), TP is started. The objective of TP is increase biomass level to generate

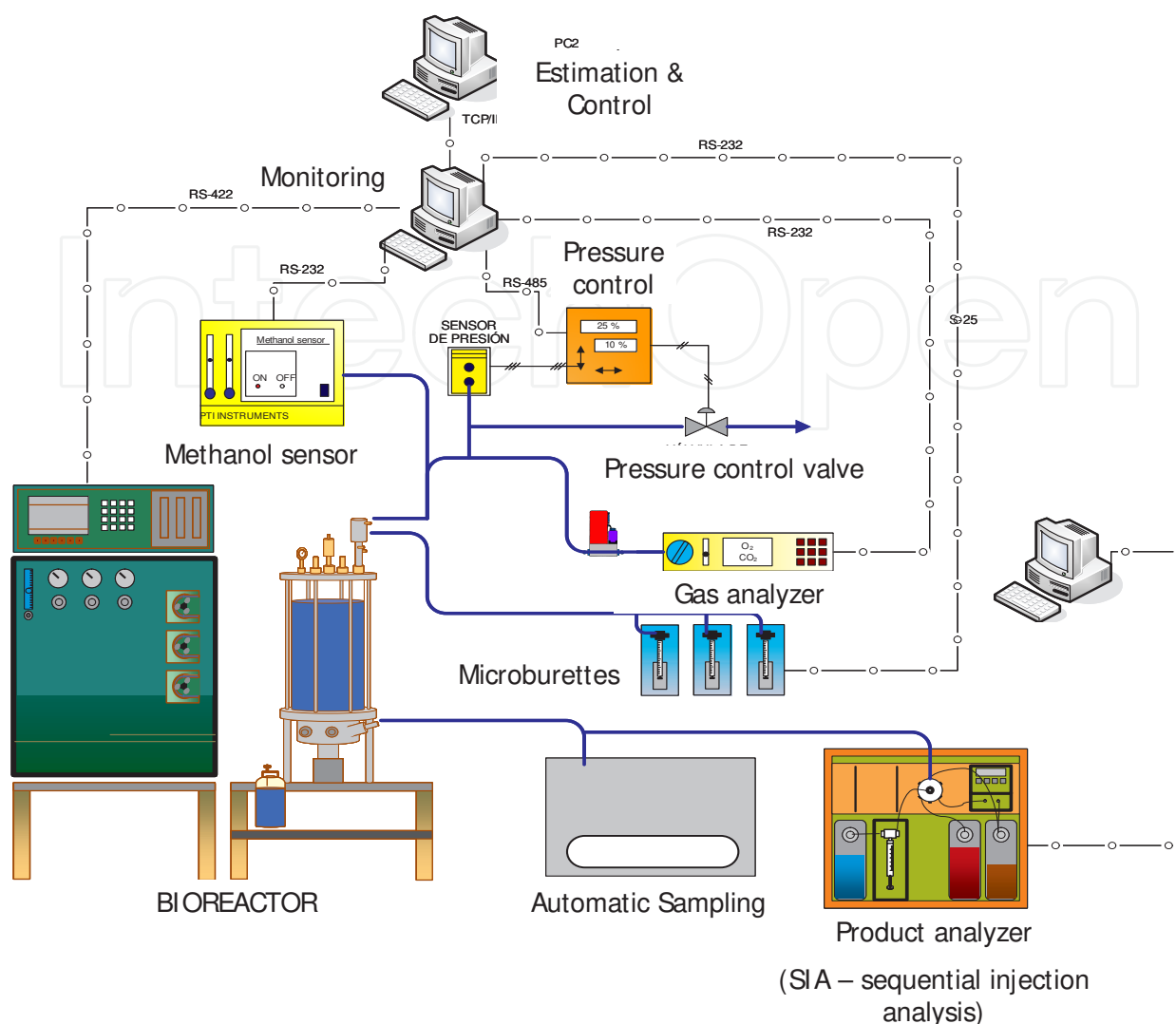
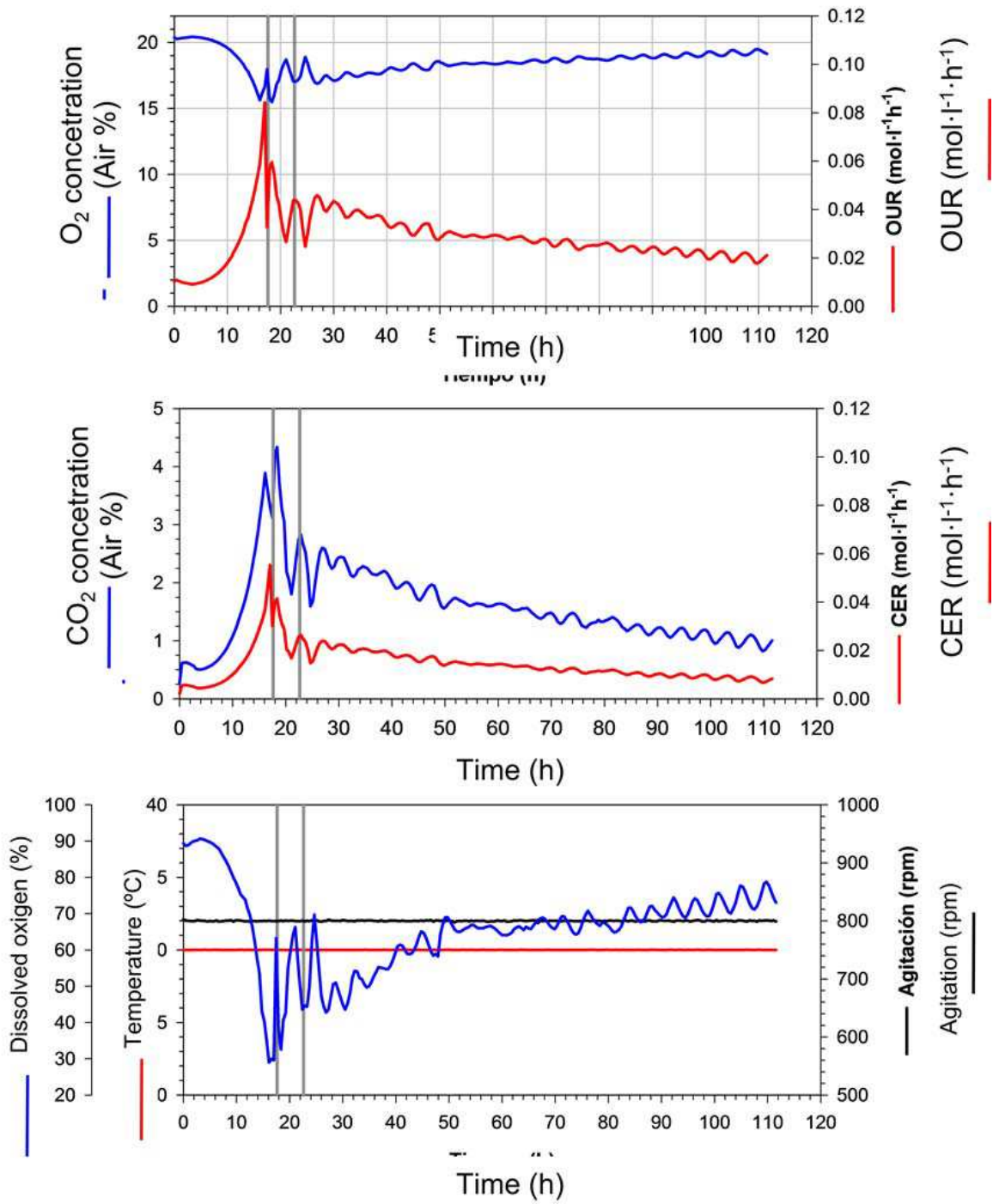


Figure 4. Bioprocess scheme for on-line monitoring and control of *Pichia pastoris* producing recombinant *Rhizopus oryzae* lipase.

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 this 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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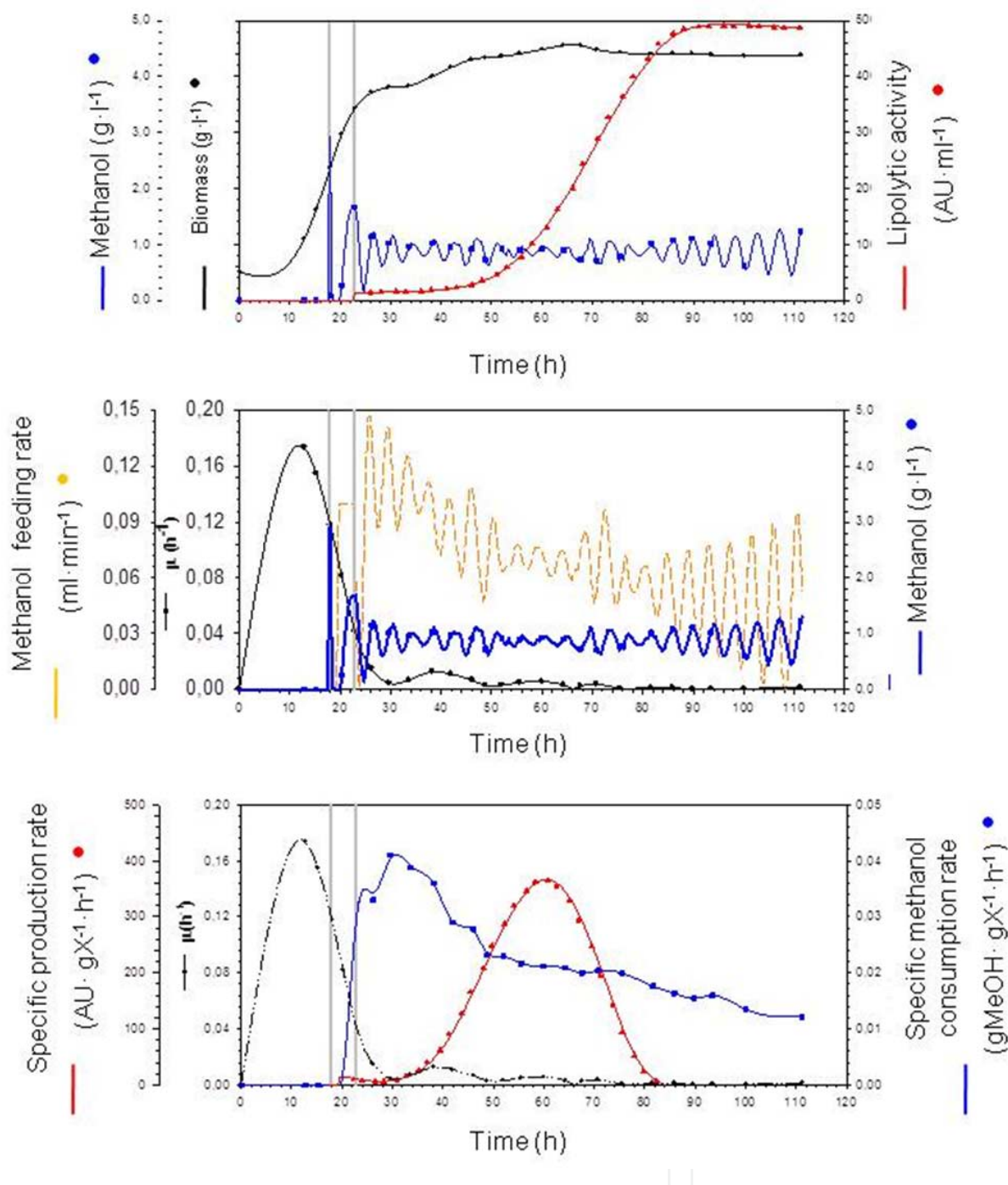


Figure 5. A.- Example of the on-line monitoring of *Pichia pastoris* producing recombinant *Rhizopus oryzae* lipase. Real time performance of standard fermentation parameters. B.- Example of the on-line monitoring of *Pichia pastoris* producing recombinant *Rhizopus oryzae* lipase. Real time evolution 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 rate 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-stat 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 (μ) 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 μ_{\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 μ_{\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.

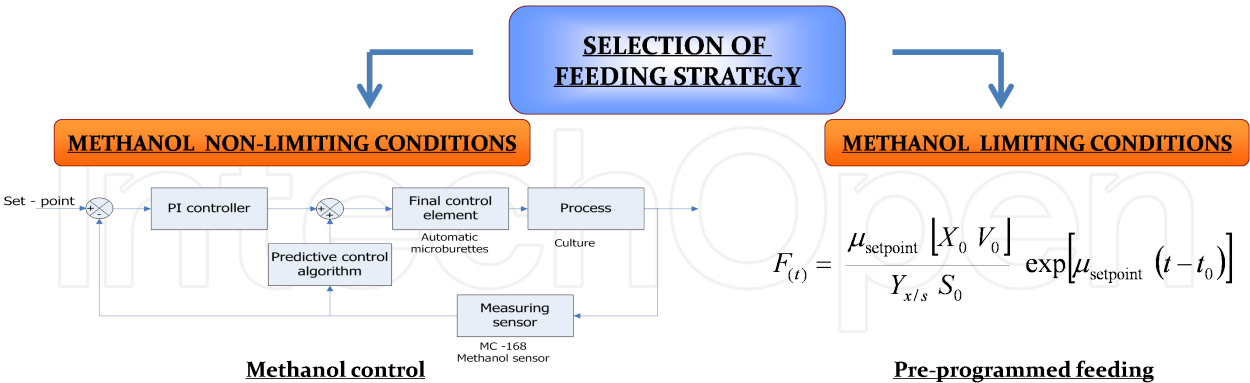


Figure 6. Scheme of methanol feeding strategies: open loop and closed loop control.

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

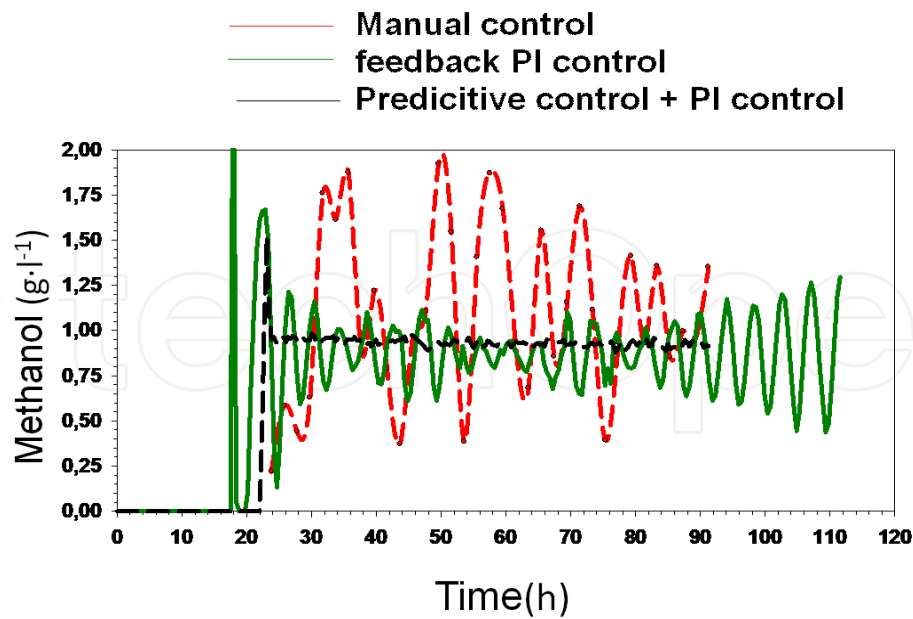


Figure 7. Comparison of the performance of the different methanol control algorithms in *Pichia pastoris* bioprocess producing recombinant lipase.

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 τ_I and the derivative time constant τ_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 kind 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.

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 kJ·C-mol⁻¹ [100] is lower than the enthalpy of combustion of methanol, -727 kJ·C-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 g·l⁻¹ [78]. The effect of different methanol-glycerol ratios at constant feeding rate has also been studied in the production of mouse α -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 g·l⁻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 productivity 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 g l⁻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 μ (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 μ_{\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 μ_{Gly} per μ_{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], dihydroxyacetone 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
<i>PAOX1</i>	22	<i>PGAP</i>	121
<i>PFLD1</i>	116	<i>PTEF1</i>	122
<i>PDHAS</i>	101	<i>PYPT1</i>	123
<i>PEX8</i>	118	<i>PPGK1</i>	124
<i>PICL1</i>	119	<i>PTH11</i>	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- α 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 *PTH11*.

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 heterologous 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 recombinant 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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References

- [1] Hitzeman RA, Hagie FF, Levine HL, Goeddel DW, Ammerer G, Hall BD. Expression of a human-gene for interferon in yeast, *Nature* 1981;293 717-722.
- [2] Higgins DR, Cregg JM. Introduction to *Pichia pastoris*. In: Higgins DR, Cregg, JM (ed) *Pichia* protocols Methods in Molecular Biology, Vol. 103. Totowa, NJ: Humana Press Inc; 1998.
- [3] Gellisen G, Hollenberg CP. Application of yeasts in gene expression studies: a comparison of *Saccharomyces cerevisiae*, *Hansenula polymorpha* and *Kluyveromyces lactis* – a review. *Gene* 1997; 90 87-97.
- [4] Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H, Oliver SG. Life with 6000 genes. *Science* 1996;274 546-567.
- [5] Böer E, Steinborg G, Kunze G, Gellisen G. Yeast expression platform. *Applied Microbiology and Biotechnology* 2007;77 513-523.
- [6] Giga-Hama Y, Thoda H, Takegawa K, Kumagai H. *Schizosaccharomyces pombe* minimum genome factory. *Biotechnol Appl Biochem* 2007;46 147-155.
- [7] Van Ooyen AJ, Dekker P, Huang M, Olsthoorn MM, Jacobs DI, Colussi PA, Taron CH. Heterologous protein production in the yeast *Kluyveromyces lactis*. *FEMS Yeast Research* 2006;6 381-392.
- [8] Cereghino JL, Cregg JM. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiology Reviews* 2000;24 45-66.

- [9] Sakai Y, Akiyama M, Kondoh H, Shibano Y, Kato N. High level secretion of fungal glucoamylase using the *Candida boindii* gene expression system. *Biochimica Biophysica Acta* 1996;1308 81-87.
- [10] J Raymond CK, Bukowski T, Holderman SD, Ching AF, Vanaja E, Stamm MR. Development of the methylotrophic yeast *Pichia methanolica* for the expression of the 65 kilodalton isoform of human glutamate decarboxylase. *Yeast* 1998;14 11-23.
- [11] Kang HA, Gellisen G. *Hansenula polymorpha*. In: Gellison G (ed) Production of recombinant proteins – novel microbial and eukaryotic expression systems. Weinheim: Wiley-VCH; 2005.
- [12] Madzac C, Nicaud JM, Gaillardin C (2005). *Yarrowia lipolytica*. In: Gellison G (ed) Production of recombinant proteins – novel microbial and eukaryotic expression systems. Weinheim: Wiley-VCH; 2005.
- [13] Böer E, Gellisen G, Kunze G (2005) *Arxula adeninivorans*. In: Gellison G (ed) Production of recombinant proteins – novel microbial and eukaryotic expression systems. Weinheim: Wiley-VCH; 2005.
- [14] Madzac C, Gaillardin C, Beckerich JM. Heterologous protein expression and secretion in the non-conventional yeast *Yarrowia lipolytica*: a review. *J Biotechnol* 2004;109 63-81.
- [15] Yin J, Li G, Ren X, Herrler G. Select what you need: A comparative evaluation of the advantages and limitations of frequently used expression systems for foreign proteins. *Journal of Biotechnology* 2007;127 335-347.
- [16] Böer E, Steinborn G, Kunze G, Gellisen G. Yeast expression platforms. *Applied Microbiology and Biotechnology* 2007;77 513-523.
- [17] Graf A, Dragosits M, Gasser B, Mattanovich D. Yeast systems biotechnology for the production of heterologous proteins. *FEMS Yeast Research* 2009;9 335-348.
- [18] Idiris A, Tohda H, Kumagai H, Takegawa A. Engineering of protein secretion in yeast: strategies and impact on protein production. *Applied Microbiology and Biotechnology* 2010;86 403-417.
- [19] Porro D, Gasser B, Fossati T, Maurer M, Branduardi P, Sauer M, Mattanovich D. Production of recombinant proteins and metabolites in yeasts. *Applied Microbiology and Biotechnology* 2011;89 939-948.
- [20] Çelik E, Çalik P. Production of recombinant proteins by yeast cells. *Biotechnology Advances* 2012;30 1108-1118.
- [21] Valero F., Heterologous expression system for lipases: A review. *Methods in Molecular Biology* 2012;861 161-178.
- [22] Cregg JM, Vedvick TS, Raschke WV. Recent advances in the expression of foreign genes in *Pichia pastoris*. *Bio/Technology* 1993;11 905-910.

- [23] Lin Cereghino GP, Cregg JM. Applications of yeast in biotechnology: protein production and genetic analysis. *Current Opinions in Biotechnology* 1999;10 422-427.
- [24] Cos O, Ramón R, Montesinos JL, Valero F (2006) Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: A review. *Microbial Cell Factories* 2006;5 1-20.
- [25] Heterologous protein expressed in *Pichia pastoris*. http://www.kgi.edu/documents/faculty/James_Cregg/heterologous_proteins_expressed_in_pichia_pastoris.pdf. (accessed 3 September 2012)
- [26] Yokohama S. Protein expression systems for structural genomics and proteomics. *Current Opinion in Chemical Biology* 2003;7 39-43.
- [27] De Pourcq K, De Schutter K, Callewaert N. Engineering of glycosylation in yeast and other fungi: current state and perspectives. *Applied Microbiology and Biotechnology* 2010;87 1617-1631.
- [28] Beck A, Cochet O, Wurch T. GlycoFi's technology to control the glycosylation of recombinant therapeutic proteins. *Expert Opinion Drug Discovery* 2010;5(1) 96-111.
- [29] Cregg JM, Lin Cereghino J, Shi J, Higgins DR. Recombinant protein expression in *Pichia pastoris*. *Molecular Biotechnology* 2000;16 23-52.
- [30] Daly R, Hearn MT., Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. *Journal of Molecular Recognition* 2005;18 119-138.
- [31] Hohenblum H, Gasser B, Maurer M, Borth N, Mattanovich D. Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris*. *Biotechnology and Bioengineering*. 2004;85 367-375.
- [32] Jahic M, Veide A, Charoenrat T, Teeri T, Enfors SO. Process technology for production of heterologous proteins with *Pichia pastoris*. *Biotechnology Progress* 2006;22 1465-1473.
- [33] Cereghino GPL, Cereghino JL, Ilgen C, Cregg JM. Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. *Current Opinion in Biotechnology* 2002;13 329-332.
- [34] Harder W, Veenhuis M. Metabolism of one carbon compounds. In: Rose AH, Harrison JS (ed.) *The Yeast*. London: Academic press; 1989. P 289-316.
- [35] Ellis SB. Isolation of alcohol oxidase and two other methanol regulatable genes from the yeast *Pichia pastoris*. *Molecular Cell Biology* 1985;5 1111-1121.
- [36] Cregg, JM, Shen S, Johnson M, Waterham HR. Classical genetic manipulation. In: Higgins DR, Cregg, JM (ed) *Pichia protocols Methods in Molecular Biology*, Vol. 103. Totowa, NJ: Humana Press Inc; 1998 p17-26.

- [37] Weast RC. Handbook of Chemistry and Physics. Boca Ratón (Florida): CRC Press Inc; 1980.
- [38] Jungo C, Marison I, von Stockar U. Mixed feed of glycerol and methanol can improve the performance of *Pichia pastoris* cultures: A quantitative study based on concentration gradients in transient continuous cultures. *Journal of Biotechnology* 2007;128 824-837.
- [39] Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM. Heterologous protein production using the *Pichia pastoris* expression system. *Yeast* 2005;22 249-270.
- [40] Junker BH, Wang HY 2006. Bioprocess monitoring and computer control: key roots of the current PAT initiative. *Biotechnology and Bioengineering* 2006;95(2) 325-336.
- [41] FDA. Guidance for industrial PAT-a Framework for innovative pharmaceutical manufacturing and quality assurance. Food and drug administration Rockville.
- [42] Wechselberger P, Seifert A, Herwig C. PAT method to gather bioprocess parameters in real-time using simple input variables and first principle relationships. *Chemical Engineering Science* 2010;65 5734-5746.
- [43] Teixeira AP, Duarte TM, Carrondo MJT, Alves PM. Synchronous fluorescence spectroscopy as a novel tool to enable PAT applications in Bioprocesses. *Biotechnology and Bioengineering* 2011;108(8) 1852-1861.
- [44] Surribas A, Geissler D, Gierse A, Scheper T, Hitzmann B, Montesinos JL, Valero F. State variables monitoring by in situ multi-wavelength fluorescence spectroscopy in heterologous protein production by *Pichia pastoris*. *Journal of Biotechnology* 2006;124 412-419.
- [45] Surribas A, Amigo JM, Coello J, Montesinos JL, Valero F, Maspoch S. Parallel factor analysis combined with PLS regression applied to the on-line monitoring of *Pichia pastoris* cultures. *Analytical Bioanalytical Chemistry* 2006;385 1281-1288.
- [46] Amigo JM, Surribas A, Coello J, Montesinos JL, Maspoch S, Valero F. On-line parallel factor analysis. A step forward in the monitoring of bioprocesses in real time. *Analytical Bioanalytical Chemometrics and Intelligent Laboratory Systems* 2008;92 44-52.
- [47] Barrigón JM, Ramón R, Rocha I, Valero F, Ferreira EC, Montesinos JL. State and specific growth estimation in heterologous protein production by *Pichia pastoris*. *Aiche Journal* 2012;58(10) 2966-2979.
- [48] Cunha AE, Clemente JJ, Gomes R, Pinto F, Thomaz M, Miranda S, Pinto R, Moosmayer D, Donner P, Carrondo MJT: Methanol induction optimization for scFv antibody fragment production in *Pichia pastoris*: *Biotechnology and Bioengineering* 2004;86 458-467.

- [49] Schenk J, Marison IW, von Stockar U. A simple method to monitor and control methanol feeding of *Pichia pastoris* fermentations using mid-IR spectroscopy. *Journal of Biotechnology* 2007; 128 344-353.
- [50] Cos O, Serrano A, Montesinos JL, Ferrer P, Cregg JM, Valero F. Combined effect of the methanol utilization (Mut) phenotype and gene dosage on recombinant protein production in *Pichia pastoris* fed-batch cultures. *Journal of Biotechnology* 2005; 116 321-335.
- [51] Potvin G, Ahmad A, Zhang Z. Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review. *Biochemical Engineering Journal* 2012;64 91-105.
- [52] Sreekrishna K. *Pichia*, Optimization of protein expression. In: Flickinger MC (ed.) *Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology*. John Wiley & Sons Inc 2010 p 1-16.
- [53] Ramón R, Feliu JX, Cos O, Monteinso JL, Berthet FX, Valero F. Improving the monitoring of methanol concentration during high cell density fermentation of *Pichia pastoris*. *Biotechnology Letters* 2004;26 144-1452.
- [54] Surribas A, Cos O, Montesinso JL, Valero F. On-line monitoring of the methanol concentration in *Pichia pastoris* cultures producing an heterologous lipase by sequential injection analysis. *Biotechnology Letters* 2003;25 1795.1800.
- [55] Gurramkonda C, Adnan A, Gäbel T, Lünsdorf H, Ross A, Nemani SK, Swaminathan S, Khanna N, Rinas U. Simple high-cell density fed-batch technique for high-level recombinant protein production with *Pichia pastoris*: application to intracellular production of hepatitis B surface antigen. *Microbial Cell Factories* 2009;8 13.
- [56] Zhang Y, Yang B. In vivo optimizing of intracellular production of heterologous protein in *Pichia pastoris* by fluorescent scanning. *Analytical Biochemistry* 2006;357 232-239.
- [57] Baker KN, Rendall MH, Patel A, Boyd P, Hoare M, Freedman RB, James DC. Rapid monitoring of recombinant protein products: a comparison of current technologies. *Trends in Biotechnology* 2002;20 149-156.
- [58] Chaa HJ, Shin HS, Lim HJ, Cho HS, Dalal NN, Pham MQ, Bentley WE. Comparative production of human interleukin-2-fused with green fluorescent protein in several recombinant expression system. *Biochemical Engineering Journal* 2005;24 225-233.
- [59] Cos O, Montesinos JL, Lafuente J, Sola C, Valero F. On-line monitoring of lipolytic activity by sequential injection analysis. *Biotechnology Letters* 2000;22 1783-1788.
- [60] Cornelissen G, Leptien H, Pump D, Scheffler U, Sowa E, Radeke HH, Luttmann R. Integrated bioprocess development for production of recombinant proteins in high cell density cultivations with *Pichia pastoris*. *CAB8 Computer Applications in Biotechnology* 2001.

- [61] Invitrogen corporation <http://www.invitrogen.com> (accessed 3 September 2012)
- [62] Brierley RA, Bussineau C, Kosson R, Melton A, Siegel RS. Fermentation development of recombinant *Pichia pastoris* expressing heterologous gene: bovine lysozyme. *Annal New York Academic of Science* 1990;589: 350-362.
- [63] Cos O, Resina D, Ferrer P, Montesinos JL, Valero F. Heterologous protein production of *Rhizopus oryzae* lipase in *Pichia pastoris* using the alcohol oxidase an formaldehyde dehydrogenase promoters in batch and fed-batch cultures. *Biochemical Engineering Journal* 2005;26 86-94.
- [64] Jahic M, Rotticci-Mulder JC, Martinelle M, Hult K, Enfors SO. Modeling of growth and energy metabolism of *Pichia pastoris* producing a fusion protein. *Bioprocess Biosystem Engineering* 2002;24 385-393.
- [65] Chiruvolu V, Eskridge K, Cregg J, Meagher M. Effects on glycerol concentration and pH on growth of recombinant *Pichia pastoris* yeast. *Applied Biochemistry and Biotechnology* 1998;75 163-173.
- [66] Zhang W, Inan M, Meagher MM. Fermentation strategies for recombinant protein expression in the methylotrophic yeast *Pichia pastoris*. *Biotechnology Bioprocess Engineering* 2000;5 275-287.
- [67] Maurer M, K hleitner M, Gasser B, Mattanovich D. Versatile modeling and optimization of fed-batch processes for the production of secreted heterologous proteins with *Pichia pastoris*. *Microbial Cell Factories* 2006;5(37) 1-10.
- [68] Sing S, Gras A, Vandal CF, Ruprecht J, Rana R, Martinez M, Strange PG, Wagner R, Byrne B. large-scale functional expression of WT and truncated human adenosine A2A in *Pichia pastoris* bioreactor cultures. *Microbial Cell Factories* 2008;7 28.
- [69] Khatri NK, Hoffmann F. Impact of methanol concentration on secreted protein production in oxygen-limited cultures or recombinant *Pichia pastoris*. *Biotechnology and Bioengineering* 2005;93 871-879.
- [70] Khatri NK, Hoffmann F. Oxygen-limited control of methanol uptake for improved production of a single-chain antibody fragment with recombinant *Pichia pastoris*. *Applied Microbiology and Biotechnology* 2006;72 492-498.
- [71] Charoenrat T, Ketudat-Cairns M, Sthendahl-Andersen H, Jahic M, Enfors SO. Oxygen-limited fed-batch process: an alternative control for *Pichia pastoris* recombinant protein processes. *Bioprocess and Biosystem Engineering* 2005;27 399-406.
- [72] Baumann K, Maurer M, Dragosits M, Cos O, Ferrer P, Mattanovich D. Hypoxic fed-batch cultivation of *Pichia pastoris* increases specific and volumetric productivity of recombinant proteins. *Biotechnology and Bioengineering* 2008;100 177-183.

- [73] Potgieter TI, Kersey SD, Mallem MR, Nylen AC, d'Anjou M. Antibody expression kinetics in glycoengineered *Pichia pastoris*. *Biotechnology and Bioengineering* 2010; 106(6) 918-927.
- [74] Berdichevsky M, d'Anjou M, Mallem MR, Shaikh SS, Potgieter TI. Improved production of monoclonal antibodies through oxygen-limited cultivation of glycoengineered yeast. *Journal of Biotechnology* 2011;155 217-224.
- [75] Ye J, Ly J, Watts K, Hsu A, Walker A, McLaughlin K, Berdichevsky M, Prinz B, Kersey DS, d'Anjou M, Pollard D, Potgieter T. Optimization of a glycoengineered *Pichia pastoris* cultivation process for commercial antibody production. *Biotechnology Progress* 2011;27(6) 1744-1750.
- [76] Chung, JD. Design of metabolic feed controllers: Application to high-density fermentations of *Pichia pastoris*. *Biotechnology and Bioengineering* 2000;68 298-307.
- [77] D'Anjou MC, Daugulis AJ. A rational approach to improving productivity in recombinant *Pichia pastoris* fermentation. *Biotechnology and Bioengineering* 2000;72 1-11.
- [78] Hu XQ, Chu J, Zhang Z, Zhang SL, Zhyang YP, Wang YH, Guo MJ, Chen HX, Yuan ZY. Effects of different glycerol feeding strategies on S-adenosyl-l-methionine biosynthesis by PGAP-driven *Pichia pastoris* overexpressing methionine adenosyltransferase. *Journal of Biotechnology* 2008;137 44-49.
- [79] Oliveira R, Clemente JJ, Cunha AE, Carrondo MJT. Adaptive dissolved oxygen control through the glycerol feeding in a recombinant *Pichia pastoris* cultivation in conditions of oxygen transfer limitation. *Journal of Biotechnology* 2005;116 35-50.
- [80] Zhang W, Bevins MA, Plantz BA, Smith LA, Meagher MM. Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of *Botulinum* neurotoxin serotype A. *Biotechnology and Bioengineering* 2000;70 1-8.
- [81] Ren HT, Yuan J, Bellgardt KH. Macrokinetic model for methylotrophic *Pichia pastoris* based on stoichiometric balance. *Journal of Biotechnology* 2003;106 53-68.
- [82] Sinha J, Plantz BA, Zhang W, Gouthro M, Schlegel VL, Liu CP, Meagher MM. Improved production of recombinant ovine interferon- τ by Mut⁺ strain of *Pichia pastoris* using an optimized methanol feed profile. *Biotechnology Progress* 2003;19 794-802.
- [83] Trinh LB, Phue JN, Shiloah J. Effect of methanol feeding strategies on production and yield of recombinant mouse endostatin from *Pichia pastoris*. *Biotechnology and Bioengineering* 2003;82 438-444.
- [84] Bawa Z, Darby RAJ. Optimising *Pichia pastoris* induction. *Methods in Molecular Biology* 2012;866 181-190.
- [85] Katakura Y, Zhang WH, Zhuang GQ, Omasa T, Kishimoto M, Goto W, Suga KI. Effect of methanol concentration on the production of human beta(2)-glycoprotein I do-

main V by a recombinant *Pichia pastoris*: A simple system for the control of methanol concentration using a semiconductor gas sensor. *Journal of Fermentation and Bioengineering* 1998;86 482-487.

- [86] Guarna MM, Lesnicki GJ, Tam BM, Robinson J, Radziminski CZ, Hasenwinkle D, Boraston A, Jervis E, Macgillivray RTA, Turner RFB, Kilburn DG. On line monitoring and control of methanol concentration in shake-flasks cultures of *Pichia pastoris*. *Biotechnology and Bioengineering* 1997; 56 279-286.
- [87] Zhang WH, Smith LA, Plantz BA, Siegel VI, Meagher MM. Design of methanol feed control in *Pichia pastoris* fermentations based upon a growth model. *Biotechnology Progress* 2002;18 1392-1399.
- [88] Cos O, Ramón R, Montesinos JL, Valero F. A simple model-based control for *Pichia pastoris* allows a more efficient heterologous protein production bioprocess. *Biotechnology and Bioengineering* 2006;95(1) 145-1154.
- [89] Curvers S, Brixius P, Klauser T, Thömmes J, Weuster-Botz D, Takors R, Wandrey C. Human chymotrypsinogen B production with *Pichia pastoris* by integrated development of fermentation and downstream processing. Part I. Fermentation. *Biotechnology Progress* 2001;17 495-502.
- [90] Surribas A, Stahn R, Montesinos JL, Enfors SO, Valero F, Jahic M. Production of a *Rhizopus oryzae* lipase from *Pichia pastoris* using alternative operational strategies. *Journal of Biotechnology* 2007; 130 291-299.
- [91] Jahic M, Wallberg F, Bollok M, García P, Enfors SO. Temperature limited fed-batch technique for control of proteolysis in *Pichia pastoris* bioreactor cultures. *Microbial Cell Factories* 2003;2 1-6.
- [92] Siren N, Weegar J, Dahlbacka J, Kalkkinen N, Fagervik K, Leisola M, von Weymarn N. Production of recombinant HIV-1 Nef (negative factor) protein using *Pichia pastoris* and a low-temperature fed-batch strategy. *Biotechnology and Applied Biochemistry* 2006; 44 151-158.
- [93] Yang M, Johnson SC, Murthy PN. Enhancement of alkaline phytase production in *Pichia pastoris*: Influence of gene dosage, sequence optimization and expression temperature. *Protein Expression and Purification* 2012;84(2) 247-254.
- [94] Dragosits M, Frascotti G, Bernard-Granger L, Vazquez F, Giuliani M, Baumann K, Rodriguez-Carmona E, Tokkanen J, Parrilli E, Wiebe MG, Kunert R, Maurer M, Gasser B, Sauer M, Branduardi P, Pakula T, Saloheimo M, Penttilä M, Ferrer P, Tutino ML, Villaverde A, Porro D, Mattanovich D. Influence of Growth Temperature on the Production of Antibody Fab Fragments in Different Microbes: A Host Comparative Analysis. *Biotechnology Progress* 2011;27(1) 38-46.

- [95] Dragosits M, Stadlmann J, Albiol J, Baumann K, Maurer M, Gasser B, Sauer M, Altmann F, Ferrer P, Mattanovich D. The Effect of Temperature on the Proteome of Recombinant *Pichia pastoris*. *Journal of proteome research* 2009;8(3) 1380-1392.
- [96] Trentmann O, Khatri NK, Hoffmann F: reduced oxygen supply increases process stability and product yield with recombinant *Pichia pastoris*. *Biotechnology Progress* 2004;20 1766-1775.
- [97] Narendar KK, Hoffmann F. Impact of methanol concentration on secreted protein production in oxygen-limited cultures of recombinant *Pichia pastoris*. *Biotechnology and Bioengineering* 2006;93 871-879.
- [98] Jungo C, Schenk J, Pasquier M, Marison IW, von Stockar U. cultures: A quantitative analysis of the benefits of mixed feeds of sorbitol and methanol for the production of recombinant avidin with *Pichia pastoris*. *Journal of Biotechnology* 2007;131 57-66.
- [99] Von Stockar U, Gustafsson L, Larsson C, Marison I, Tissot P, Gnaiger E. Thermodynamic considerations in constructing energy balances for cellular growth, *Biochemistry and Biophysics Acta* 1993;1183 221-240.
- [100] Tschopp JF, Brust PF, Cregg JM, Stillman CA, Gingeras TR. Expression of the lacZ gene from two methanol-regulated promoters in *Pichia pastoris*, *Nucleic Acids Res.* 15 (1987) 3859-3876.
- [101] Arnau C, Casas C, Valero F. The effect of lycerol mixed substrate on the heterologous production of *Rhizopus oryzae* lipase in *Pichia pastoris* system. *Biochemical Engineering Journal* 2011;57 30-37.
- [102] Gao M-J, Zheng Z-Y, Wu J-R, Dong S-J, Jin Z-L, Zhan X-B, Lin C-C. Improvement of specific growth rate of *Pichia pastoris* for effective porcine interferon- α production with an on-line model based glycerol feeding strategy. *Applied Microbiology and Biotechnology* 2012;93-1437-1445.
- [103] Zalaid D, Dietzsch C, Herwig C, Spadiut O. A dynamic fed batch strategy for a *Pichia pastoris* mixed feed system to increase process understanding. *Biotechnology Progress* 2012;28(3) 878-886.
- [104] Huang , Yang P, Luo H, Tang H, Shao N, Yuan T, Wang Y, Bai Y, Yao B. High-level expression of a truncated 1,3-1,4- β -D-glucanase from *Fibrobacter succinogenes* in *Pichia pastoris* by optimization of codons and fermentation. *Applied Microbiology and Biotechnology* 2008;103 78-95.
- [105] Boze H, Laborde C, Chemardin P, Richard F, Venturin C, Combarnous Y, Moulin G. High-level secretory production of recombinant porcine follicle-stimulating hormone by *Pichia pastoris*, *Process Biochemistry.* 2001;36 907-913.
- [106] Choi DB, Park EY. Enhanced production of mouse α -amilase by feeding combined nitrogen and carbon sources in fed-batch culture of recombinant *Pichia pastoris*. *Process Biochemistry* 2006;41 390-397.

- [107] Resina D, Cos O, Ferrer P, Valero F. Developing high cell density fed-batch cultivation strategies for heterologous protein production in *Pichia pastoris* using the nitrogen source-regulated *FLD1* promoter. *Biotechnology and Bioengineering* 2005;91:760–767.
- [108] Sreekrishna K, Brankamp RG, Kropp KE, Blankenship DT, Tsay JT, Smith PL, Wierschke JD, Subramaniam A, Birkenberger LA. Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. *Gene* 1997;190 55-62.
- [109] Inan M, Meagher MM. Non-repressing carbon sources for alcohol oxidase (AOX1) promoter of *Pichia pastoris*. *Journal of Bioscience and Bioengineering* 2001;92 585-589.
- [110] Thorpe ED, D'Anjou MC, Daugulis A. Sorbitol as a non-repressing carbon source for fed-batch fermentation of recombinant *Pichia pastoris*. *Biotechnology Letters* 1999;21 669-672.
- [111] Xie JL, Zhou QW, Pen D, Gan RB, Qin Y. Use of different carbon sources in cultivation of recombinant *Pichia pastoris* for angiotensin production. *Enzyme and Microbial Technology* 2005;36 210-216
- [112] Arnau C, Ramón R, Casas C, Valero F. Optimization of the heterologous production of *Rhizopus oryzae* lipase in *Pichia pastoris* system using mixed substrates on controlled fed-batch bioprocess. *Enzyme and Microbial Technology* 2010;46 494-500.
- [113] Çelik E, Çalik P, Oliver SG. Fed-batch methanol feeding strategy for recombinant protein production by *Pichia pastoris* in the presence of co-substrate sorbitol. *Yeast* 2009;26 473-484.
- [114] Hang H, Ye XY, Guo M, Chu J, Zhuang Y, Zhang M, Zhang S. A simple fermentation strategy for high-level production of recombinant phytase by *Pichia pastoris* using glucose as the growth substrate. *Enzyme and Microbial Technology* 2009;44 185-188.
- [115] Paulova L, Hyka P, Branska B, Melzoch K, Kovar K. Use of a mixture of glucose and methanol as substrates for the production of recombinant trypsinogen in continuous cultures with *Pichia pastoris* Mut⁺. *Journal of Biotechnology* 2012;157 180-188.
- [116] Shen S, Sulter G, Jeffries TW, Cregg JM. A strong nitrogen source-regulated promoter for controlled expression of foreign genes in the yeast *Pichia pastoris*. *Gene* 1998;216(1) 93-102.
- [117] Liu H, Tan X, Rissell KA, Veenhuis M, Cregg JM. *ER3*, a gene required for peroxisome biogenesis in *Pichia pastoris*, encodes a peroxisomal membrane protein involved in protein import. *Journal Biological Chemistry* 1995;270 10940-10951.
- [118] Menendez J, Valdes I, Cabrera N. The ICL1 gene of *Pichia pastoris*, transcriptional regulation and use of its promoter. *Yeast* 2003;20(13) 1097-1108.

- [119] Menendez J, Valdes I, Cabrera N. The ICL1 gene of *Pichia pastoris*, transcriptional regulation and use of its promoter. *Yeast* 2003;20(13) 1097-1108.
- [120] Stadlmayr G, Mecklenbräuer A, Rothmüller M, Maurer M, sauer M, Mattanovich D, Gasser B. Identification and characterization of novel *Pichia pastoris* promoters for heterologous protein production. *Journal of Biotechnology* 2010;150 519-529.
- [121] Waterham HR, Digan ME, Koutz PJ, Lair SV, Cregg JM. Isolation of the *Pichia pastoris* glyceraldehydes-3-phosphate dehydrogenase gene and regulation and use of its promoter. *Gene* 1997;186(1) 37-44.
- [122] Ahn J, Hong J, Lee H, Park M, Lee E, Kim C, Choi E, Jung J. Translation elongation factor 1-alpha gene from *Pichia pastoris*: molecular cloning, sequence, and use of its promoters. *Applied Microbiology and Biotechnology* 2007; 74(3) 601-608.
- [123] Sears I, O'Connor J, Rossanese O, Glick B. A versatile set of vectors for constitutive and regulated gene expression in *Pichia pastoris*. *Yeast* 1998;14(8) 783-790.
- [124] De Almeida JR, de Moraes LM, Torres FA. Molecular characterization of the 3-phosphoglycerate kinase gene (PGK1) from the methylotrophic yeast *Pichia pastoris*. *Yeast* 2005;22(9) 725-737.
- [125] Harder W, Veenhuis M. Metabolism of one carbon compounds. In: Rose AH, Harrison JS (ed.). *The Yeasts*. London: Academic Press; 1989. 289-326.
- [126] Thorpe ED, D'Anjou MC, Daugulis AJ. Sorbitol as a non repressing carbon-source for fed-batch fermentation of recombinant *Pichia pastoris*. *Biotechnology Letters* 1999;21: 669-672.
- [127] Resina D, Cos O, Ferrer P, Valero F. Developing high cell density fed-batch cultivation strategies for heterologous protein production in *Pichia pastoris* using the nitrogen source-regulated *FLD1* promoter. *Biotechnology and Bioengineering* 2005; 91(6) 760-767.
- [128] Resina D, Bollock M, Khatri NK, Valero F, Neubauer P, Ferrer P. Transcriptional response of *Pichia pastoris* in fed-batch cultivations to *Rhizopus oryzae* lipase production reveals UPR induction. *Microbial Cell Factories* 2007;6(21) 1-11.
- [129] Resina D, Maurer M, Cos O, Arnau C, Carnicer M, Marx H, Gasser B, Valero F, Mattanovich D, Ferrer P. Engineering of bottlenecks in *Rhizopus oryzae* lipase production in *Pichia pastoris* using the nitrogen source-regulated *FLD1* promoter. *New Biotechnology* 2009;25(6) 396-403.
- [130] Boer H, Teeri TT, Koivula A. Characterization of *Trichoderma reesei* cellobiohydrolase Cel7A secreted from *Pichia pastoris* using two different promoters. *Biotechnology and Bioengineering* 2000;69 486-494.
- [131] Goodrick JC, Xu M, Finnegan R, Schilling BM, Schiavi S, Hoppe H, Wan NC. High-level expression and stabilization of recombinant human chitinase produced in a con-

tinuous constitutive *Pichia pastoris* expression system. Biotechnology and Bioengineering 2004;31 330-334.

- [132] Wu JM, Lin JC, Chieng LL, Lee CK, Hsu TA. Combined used of GAP and AOX1 promoter to enhance the expression of human granulocyte-macrophage colony-stimulating factor in *Pichia pastoris*. Enzyme and Microbial Technology 2003;33 453-459.
- [133] Delroise JM, Dannau M, Gilsoul JJ, El Mejdoub T, Destain J, Portetelle D, Thonart P, Haubruge E, Vandebol M. Expression of a synthetic gene encoding a tribolium castaneum carboxylesterase in *Pichia pastoris*. Protein Expression and Purification 2005; 42:286-294.
- [134] Zhang A-L, Luo J-X, Zhang T-Y, Pan Y-W, Tan Y-H, Fu C-Y, Tu F-z. Recent advances on the GAP promoter derived expression system of *Pichia pastoris*. Molecular Biology Reports 2009;36 1611-1619.
- [135] Döring F, Klapper M, Theis S, Daniel H. Use of the glyceraldehydes-3-phosphate dehydrogenase promoter for production of functional mammalian membrane transport proteins in the yeast *Pichia pastoris*. Biochemistry Biophysics Research Communication 1998;250. 531-535.