Rational development of bioprocess engineering strategies for recombinant protein production in *Pichia pastoris* (*Komagataella phaffi*) using the methanol free *GAP* promoter. Where do we stand?

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List of abbreviations

- Antibody fragment (Fab)
- Compound annual growth rate (CAGR)
- Constitutive *GAP* promoter (glyceraldehyde-3-phosphate dehydrogenase, P_{GAP})
- Dissolved oxygen concentration (pO_2)
- Food and Drug Administration (FDA)
- Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)
- Heat yield ($Y_{Q/X}$)
- Methanol inducible *AOX1* promoter (alcohol oxidase 1, P_{AOX1}).
- Oxygen to biomass yield ($Y_{O2/X}$)
- Post-translational modifications (PTM)
- Product to biomass yield ($Y_{P/X}$)
- Product to substrate yield ($Y_{P/S}$)
- Recombinant protein production (RPP)
- Respiratory quotient (*RQ*)
- Single-cell protein (*SCP*)
- Space-time-yield (*STY*)
- Specific ethanol production rate ($q_{ethanol}$)
- Specific growth rate (μ)
- Specific product formation rate (q_P)
- Specific substrate uptake rate (q_S)
- Temperature (T)
- Unfolded protein responses (UPR)
- Volumetric oxygen mass transfer coefficient (k_La)
- Volumetric productivity (Q_P)

Abstract

The increasing demand for recombinant proteins for a wide range of applications, from biopharmaceutical protein complexes to industrial enzymes, is leading to important growth in this market. Among the different efficient host organism alternatives commonly used for protein production, the yeast *Pichia pastoris* (*Komagataella phaffi*) is currently considered to be one of the most effective and versatile expression platforms. The promising features of this cell factory are giving rise to interesting studies covering the different aspects that contribute to improving the bioprocess efficiency, from strain engineering to bioprocess engineering. The numerous drawbacks of using methanol in industrial processes are driving interest towards methanol-free alternatives, among which the *GAP* promoter-based systems stand out. The aim of this work is to present the most promising innovative developments in operational strategies based on rational approaches through bioprocess engineering tools. This rational design should be based on physiological characterization of the producing strains under bioprocess conditions and its interrelation with specific rates. This review focuses on understanding the key factors that can enhance recombinant protein production in *Pichia pastoris;* they are the basis for a further discussion on future industrial applications with the aim of developing scalable alternative strategies that maximize yields and productivity.

Introduction

Currently, proteins are used in a wide range of applications for industries, including pharmaceuticals, diagnostics, food, detergents, biofuels, textiles, polymers, and paper and pulp [1,2]. By using recombinant DNA technology, proteins of interest can be produced in different cell factories obtaining expression levels hundreds of times higher than those produced naturally [3]. The market for recombinant proteins, including from biopharmaceutical protein complexes to industrial enzymes, is growing steadily. The market for biopharmaceuticals is expected to reach $386.7 billion by the end of 2019 at a compound annual growth rate (CAGR) of 10.6% [4,5],

while for industrial enzymes, it is predicted to reach $6.3 billion in 2021 at a CAGR of 4.7% [6]. High demand and continuous market expansion provide incentives to improve the protein production platforms, both to enable the production of novel proteins and to reduce the manufacturing costs of the existing processes [7].

The choice of a suitable cell factory for the production of a recombinant protein is therefore very important and probably one of the most challenging and critical steps that should be addressed at the beginning of the bioprocess design [8,9]. In a perfect scenario, one universal expression system would enable the expression of all possible recombinant genes in a fast, cheap, and accurate manner with respect to yield, folding and biological activity [10]. However, the limitations of the current existing systems require empirical and specific determination of the most suitable cell factory among the potential candidates. The selection of the expression system must be based on production parameters, such as costs, yield, production timescale, scale up capacity and downstream processes, as well as on the properties and use of the product [11]. Different applications have different requirements for both quantity and quality. The characteristics of the target protein must also be taken into account in terms of structure and the requirement to incorporate post-translational modifications (PTM) [10]. Glycosylation represents the most complex and widespread PTM, being associated with 40% of all approved products [12]. Selecting a wrong expression host can result in the protein being misfolded or poorly expressed, lacking the necessary PTM's or containing unsuitable modifications.

In general, bacteria provide an excellent expression system for proteins that do not require synthesis in a glycosylated or extensively modified form, allowing fast and inexpensive production processes [8]. Among the different alternatives, *Escherichia coli* has been considered the workhorse for recombinant protein production (RPP), but *Bacillus* species, as well as other bacterial organisms, such as *Pseudomonas fluorescens, Staphylococcus carnosus* and *Streptomyces lividans,* are also efficiently used as cell factories for RPP [13,14]. Interestingly,

107 significant progress has been made over the past recent years towards the development of *E. coli*
108 strains able to perform eukaryotic-like PTM's such as glycosylation, as well as engineering
109 efficient protein secretion systems. In contrast to bacteria, mammalian cell lines should be able
110 to overcome most of the limitations of producing recombinant eukaryotic proteins. Consequently,
111 due to the capacity to perform human PTM's, as well as to correctly fold and assemble human
112 proteins, they have become the dominant recombinant production system for medical applications
113 [16]. They still present important drawbacks, such as low growth rates and product yield, high
114 costs derived from the use of complex media and sera or potential viral contaminations [17]. In
115 addition, in terms of bioprocessing, the operational mode, process control and scaling-up steps
116 are also challenging [18]. Consequently the specific yields obtained with these bioprocesses based
117 on mammalian cell lines are often low [17].
118
119 In this context, eukaryotic microorganisms such as yeast emerge as an ideal intermediate. They
120 combine the eukaryotic ability for protein processing, such as folding, assembling and introducing
121 PTMs, with important microbial advantages such as the capacity to grow quickly to high cell
122 densities in chemically defined media. In addition, yeasts can secrete recombinant proteins into
123 the extracellular media, greatly facilitating downstream processing [19]. Yeasts were first
124 implemented as a recombinant protein platform in 1981 using *Saccharomyces cerevisiae* [20].
125 Since then, other yeasts have been developed into alternative cell factories presenting some clear
126 advantages, with prime examples including *Pichia pastoris,* recently reclassified as *Komagataella*
127 *phaffi*, as well as other yeasts, such as *Hansenula polymorpha* [21] or *Kluyveromyces lactis* [22].
128 *P. pastoris* is currently considered to be one of the most effective and versatile systems for the
129 production of heterologous proteins [23]. This methylotrophic organism was developed as a host
130 system in 1985 [24]. Its primary advantages are summarized in **Table 1**. Currently, over 5,000
131 recombinant proteins have been expressed in the *P. pastoris* system reaching expression levels
132 approximately 80% of the total secreted protein or up to 30% of the total cell protein [23]
133 [www.pichia.com]. Due to its positive features, over 300 industrial processes have been licensed

and more than 70 commercial products made in *P. pastoris* are currently on the market. The range of application fields is very wide from industrial enzymes (nitrate reductase), to animal feed additives (phytase) and biopharmaceutical proteins. Interestingly, two biopharmaceuticals have been approved by the US Food and Drug Administration (FDA) and the European Commission (Kalbitor® and Jetrea®) [25] and over 20 therapeutic product candidates are still in the clinical pipeline [15,26]. Due to the high potential of *P. pastoris,* several reviews have been published recently [27–32]. However, none covers specifically novel aspects focused on bioprocess engineering to improve the current fermentation strategies, especially those focused on avoiding the use of methanol, since this compound involves numerous drawbacks for industrial application. Up-to-date fermentation technology has not reached yet the same maturity as traditional chemical processes, which is essential to increase the product accessibility in low and middle resource countries [33,34].

This review aims to update and discuss the most promising innovative developments in methanol-free *P. pastoris* cultivation strategies, mostly focused on constitutive *GAP* promoter (glyceraldehyde-3-phosphate dehydrogenase, P_{GAP}), and based on rational approaches through the efficient application of bioprocess engineering tools. Special attention has been given to the potential scalability and industrial implementation feasibility towards improvement of the overall bioprocess efficiency and profit.

Operational strategies for *P. pastoris* bioprocesses: from conventional approaches to rational innovative strategies

Even though the current interest in *P. pastoris* as a cell factory for different production processes is rapidly increasing [2], the fermentation technology developed for this yeast is still far from achieving the maturity of other common organisms used in the microbial fermentation industry,

such as *E. coli* or *S. cerevisiae* [35]. As with other production processes, a fermentation aims to produce the maximum amount of product in the minimum process time (space-time-yield, *STY*) and volumetric productivity, (Q_P [35]). For the design and implementation of innovative bioprocess strategies, it is important to select reference parameters independent of process-specific settings, such as specific growth rate (μ), specific substrate uptake rate (q_S), specific production rate (q_P) or yields. Instead, other parameters non-comparable between different systems, such as biomass concentration, reactor volume, process time or addition feed rates should be avoided [35]. In general, the efficiency of the fermentation processes is importantly influenced by different operational parameters including temperature (T), pH, osmolality, dissolved oxygen concentration (pO_2) and medium composition. However, excluding pO_2 [36–38], the selection of the optimal set-point as well as the control strategies for these parameters along cultivation are often considered straightforward for *P. pastoris* bioprocesses. The best ranges are widely known, extensively reviewed and used by most of the authors [39,40].

Promoter selection plays an essential role in the bioprocess development, since it critically determines the fermentation strategy. From the different promoters available for *P. pastoris* described in the literature [41,42], which are in continuous rapid evolution, the most commonly used in industrial processes are still constitutive P_{GAP} and the methanol inducible *AOX1* (alcohol oxidase 1, P_{AOX1}). The advantages of the former are primarily related to the non-utilization of methanol as the carbon source and/or inducer; thus, it can be substituted by alternative standard carbon sources, such as glucose or glycerol. Problems related to the storage, delivery and safety of large quantities of methanol would be avoided [40], as well as operational problems associated with methanol metabolism, which increases the complexity of the culture strategies that allow it to reach optimal production performances [43,44]. The P_{GAP} expression system, therefore, contributes greatly to the development of cost-effective bioprocesses for large-scale RPP [45]. In the following sections, the primary operational strategies related to P_{GAP} are summarized.

Carbon source selection. How do the alternatives affect the process?

The two most frequently used carbon sources for P_{GAP}-based systems are glucose and glycerol. However, fructose, sorbitol, mannitol, ethanol, methanol and trehalose also allow the expression of P_{GAP}-driven proteins. In general, in the literature similar production rates have been achieved using both substrates or slightly higher using glucose [46]. As an example, when comparing both alternatives systematically to produce a human antibody fragment (Fab) in batch process, similar production was obtained. However, growing in conditions of glucose excess, $Y_{X/S}$ was lower and by-products, such as ethanol, arabitol and acetate, were detected in the culture broth [47]. Thus, since glycerol does not generate by-products, it should be recommended for batch cultures. On the other hand, when comparing glucose and glycerol in carbon-limited fed-batch strategy at constant μ, no differences in yields and productivities were observed. However, due to both the lower heat yield ($Y_{Q/X}$) and $Y_{O2/X}$ for glucose, this substrate should be recommended for the fed-batch phase [48]. For industrial bioprocesses, economic factors such as substrate costs and the potential low-cost alternatives derived from industry by-products should also be taken into consideration. For yeast cell factories, molasses from the sugar industry, glycerol from the biodiesel industry, corn steep liquor from the starch industry, spent sulphite liquor from the forest products industry and cheese whey from the dairy industry are considered possible alternatives [49].

Chemostat and fed-batch as tools for physiological characterization

Currently, in bioprocess engineering, a physiological characterization of the producing strains that includes production rates and yields is considered essential for a proper bioprocess development [35,50,51]. In this regard, is essential to know how changes in environmental culture factors affect the cell factory, including the rearrangement of central carbon, amino acid metabolism and other basic cell functions. These effects can have a direct impact on cell growth, folding stress and vesicular transport, with possible implications in protein secretion, and other

aspects affecting the production rates and yields [52–55]. To determine these relationships, fed-batch or continuous cultivations are generally performed. In general, continuous mode is the strategy most frequently used to obtain physiological data, due to the robustness that allows working in the stationary state and thus very stable culture conditions, as well as providing an equivalent physiological state for all the cells in the culture. Due to these features, the chemostat is currently considered an excellent tool for systems biology to provide mechanistic explanations for the effect of different process variables in cell factory performance [56]. Although interesting studies have been recently published, fed-batch cultures are often considered less robust and more laborious and time consuming [57–59]. Interestingly, the use of automated micro-fermentation platforms using either microtitre plates as a bioreactor, or micro-bioreactors on a mL scale, is emerging as an interesting potential alternative to minimize the time consuming steps of bioprocess physiological characterization when comparing different strains or producer clones [60–64].

Fed-batch, the most frequently used operating mode for recombinant protein production processes

To date, the fed-batch mode has been widely used for recombinant production processes, since it allows extension of process time, as well as attaining higher biomass and product titre by gradual feeding of the selected substrate(s) [65]. Currently, simple nutrient feeding strategies for the fed-batch phase using pulse additions, constant rates, ramp and/or step-based profiles, which have been commonly used in the basic protocols, may be considered obsolete. The implementation of these simple feeding strategies during the fed-batch phase is not designed to cover the physiological requirements of the cells and thus gives rise to bioprocesses which are far from their optimal performance. A successful feeding strategy for fed-batch cultivation should aim to allow requirements for cell growth alongside the bioprocess. Therefore, physiological knowledge of the host cell is essential to increase its performance in order to reach optimal production rates and

yields. For instance, exponential feeding profiles provide a straightforward and effective strategy to achieve pseudo-stationary state conditions. In this case, culture reaches a constant and controlled specific growth rate, as well as fairly constant substrate(s) specific consumption and product production rates, i.e. providing a controlled environment to cover cell growth requirements and RPP [40,48].

Effect of specific growth rates on protein production

"Production kinetics", which correlate specific product formation (q_P) and specific biomass growth rate (μ), is usually considered a crucial factor for bioprocess efficiency, regardless of the selected criterion for bioprocess optimization [66]. Thus, since the involved rates are non-extensive variables, they are comparable at different fermentation systems. The product formation kinetics reflect the equilibrium between the various steps until the product is secreted and are subject to numerous physiological factors [39]. Each cell factory producing a specific recombinant protein in a given fermentation mode has a kinetic profile that should be taken into consideration to reach an optimum bioprocess production [67]. Thus, when developing a production process, the q_P *vs* μ relationship has to be empirically determined.

Compromise between yield and productivity is essential during bioprocess development in order to reach optimal performance. Different approaches are proposed to address this scenario in order to maximize the goal criteria. Usually, the feeding profile is optimized as a decision variable, while in other applications the goal of optimization is to find the best values for μ or q_S in which volumetric productivity or space-time yield (*STY*) is maximized. To apply more precise and robust control schemes, the use of a closed-loop control and software sensors, as well as predictive and adaptive control, generally requires multiple on-line measurements to follow the optimal time profiles of the specific growth rate, biomass or substrate concentration [68–72]. However, if an advanced control system is implemented in an industrial application, it must increase performance

while keeping the cost lower than the derived benefit and also do this without significantly increasing process complexity and operational and labour costs [73]. Specific case-studies of monitoring and direct feed-back control in *P. pastoris* are available in the literature [40,74,75]. As an example, a human Fab production applying the exponential feeding strategy showed that the implementation of a constant high μ resulted into the maximum values for yield and productivity [48]. Alternatively, an optimal decreasing μ trajectory was implemented in order to improve production of the same Fab [76]. When comparing the alternatives, as time proceeds during the cultivation process, optimal μ profiles allow maximizing volumetric productivity, while also providing suitable product yields. However, in terms of specific productivity, a high constant μ achieved two-fold higher rates due to the lower fermentation time.

An updated revision of the different operational fed-batch strategies under P_{GAP} presented in previous studies [23,40,67] is shown in **Table 2**. For processes based on the P_{AOX1}, different alternatives to optimize the production of some recombinant proteins can also be found in the literature [77–80]. For this system, the non-monotonic product formation kinetics may be coupled to the methanol metabolic burden or to secretory pathway saturation due to its outstanding expression levels [35,81,82]. Thus, using optimal profiles is advisable when the production kinetics are nonlinear [83].

Exploiting host physiology knowledge for rational development of bioprocess engineering strategies

Current trends in bioprocess engineering are directed towards a conceptual approach that aims to design processes based on the properties of both the physiology of the host cell system and the product, as well as the characteristics of the bioreactor equipment available [58,84]. In practice, improved cultivation strategies are usually rationally designed from the physiological characterization of producer strains [35,85]. As a simple but successful example of bioprocess

296 engineering development based on knowledge of host cell physiology is the activation of cell
297 stress responses [11]. The recent development of these strategies derives from several reported
298 cases in which increasing cell stress may couple with overexpression of the protein [26].
299 Therefore, when identifying a stress condition that enhances protein expression, a systematic
300 study on the cellular machinery of cell factories can lead to determination of optimal conditions
301 that allow protein overexpression.
302
303 Currently, detailed transcriptomic, proteomic and metabolomic studies of cellular reactions to
304 environmental stress factors have been performed and published with different microorganisms,
305 including *P. pastoris* and *S. cerevisiae* [53,86]. In *P. pastoris* cultivations, the effects of different
306 media osmolality, temperature, specific growth rate and oxygen supply were compared at the
307 transcriptome and proteome levels, revealing strong transcriptional and expression regulation of
308 core metabolic genes, and in turn, also of the recombinant protein of interest driven by P_{GAP}
309 [53,86–89]. From those, the effects of biomass growth and oxygen supply have been specifically
310 studied in order to develop innovative cultivation strategies in both chemostat and fed-batch
311 modes that exploit the stress conditions leading to recombinant protein overexpression.
312
313 ***Carbon-starving conditions to enhance recombinant protein production***
314
315 It was first reported in 2007 that a significant increase in P_{GAP} -driven protein expression occurred
316 on short-time glucose depletion periods in shake-flask cultures. This work did neither hypothesize
317 about the causes of overexpression nor aimed to exploit this phenomena in large-scale cultures
318 [90]. In a later study, different time combinations of carbon feeding and starvation periods were
319 compared in order to develop a strategy to increase the production in fed-batch cultures. All the
320 strategies compared achieved important increases in terms of product titre and yields ($Y_{P/S}$ and
321 $Y_{P/X}$) without affecting growth parameters. However, due to increased bioprocess times, in terms
322 of productivity values, the results were not always better than the conventional exponential
323 profile. Overall, best results were obtained when combining both the shortest feeding and carbon-

starving periods. The hypothesis proposed to describe the mechanisms leading to the protein overexpression was based on the described yeast response adaptation to changing nutritional states [91,92]. These rapid and complex mechanisms of transcription and translation regulation promote adaptation to new environments, which may include P_{GAP}-regulated genes [93,94]. A similar strategy was previously proposed for the yeast *Hansenula polymorpha,* which also enhanced RPP compared to conventional feeding profiles [95]. Based on the favourable results, as well as the simplicity of the new operational strategy, the development of feeding profiles that include carbon-starving periods in order to promote target gene overexpression, should be considered highly promising for industrial yeast-based RPP.

Implementation of oxygen-limiting conditions to increase RPP

Oxygen availability has been widely studied in *P. pastoris* recombinant production processes, because its limited transfer capacity can become a bioprocess-limiting factor. Different studies have assessed the dissolved oxygen effect on protein production observing slight differences [36,96]. However, for P_{GAP}-based bioprocesses, it has been reported that oxygen-limited cultures can lead to up to 3-fold increases in terms of q_P, [51,53,87]. Interestingly, oxygen-limiting conditions led to similar improvements in a P_{AOX1} system and *S. cerevisiae* [36,97–99]. A primary test assessing oxygen-limiting effect in both chemostat and fed-batch cultivation was carried out for a P_{GAP}-driven Fab production [87]. This work described a decrease in biomass production, the generation of ethanol and a significant increase in both q_P and volumetric productivity, Q_P. This environmental stress led to a shift from a respiratory to respiro-fermentative metabolism resulting in the physiological effect mentioned. The metabolic impact increased progressively as oxygen availability decreased. Inter-disciplinary systems biology studies, including transcriptomic, proteomic and metabolomic analyses, were subsequently carried out to understand the cell responses to hypoxic conditions [53,55,100]. Recently, a publication further discussed how to

implement practically the desired oxygen-limiting conditions for different bioreactors regardless of oxygen transfer capacity [51].

Maximum levels of protein production were always achieved for the most severely oxygen-limiting conditions prior to respiratory pathway collapse. The target gene overexpression may be due to the overall increased transcriptional levels observed for glycolytic pathway genes. In addition, overexpression may also be coupled to further hypoxic effects such as increased transcription of the unfolded protein responses (UPR) genes and changes in membrane fluidity. Overall, the metabolic shift to respiro-fermentative pathways leads to a decrease in biomass yields, generation of secreted by-products (primarily ethanol), and an increase in q_S, q_{CO2} and respiratory quotient (*RQ*). Specifically for hypoxic *Pichia* cultures, the trends of specific ethanol production rate ($q_{ethanol}$) and *RQ* respect to oxygen availability could lead to feasible indirect reporting parameters of oxygen availability for the cells [51,101]. Importantly, it would allow implementation of oxygen-limiting conditions to different fermenters regardless of their oxygen transfer capacity. At the practical level, since the control of $q_{ethanol}$ would require a more complex estimation, *a priori* the *RQ* on-line determination should be considered more feasible.

The effect of lack of essential nutrients on RPP

Both alternatives previously discussed demonstrate a common factor, namely the induction of an environmental stress to the cells caused by the lack of essential nutrients for growth. P_{GAP} natively regulates glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), a key NAD-binding enzyme in the glycolysis and gluconeogenesis pathways, and thus the central carbon metabolism. Adaptation responses to stress are expected to regulate yeast central carbon metabolism, the metabolic fluxes through the glycolysis pathway, and, in turn, transcription of the P_{GAP}-regulated genes. Thus, it is not surprising that environmental stress conditions have a direct impact on the levels of RPP. However, an excessive impact of both environmental stress conditions studied can even lead to a detrimental effect on product formation. Therefore, deep and systematic studies to characterize

377 the impact on host cell physiology and its productivity are necessary to exploit overexpression of
378 the desired product (**Figure 1**). In turn, bioprocess engineering will aim to design, develop and
379 implement new operational cultivation strategies that allow maximizing the recombinant
380 production yields and product.
381

382 **Towards industrial implementation of *P. pastoris* bioprocesses**
383
384 Scale up of complex bioprocesses is always challenging, since this step in a bioprocess
385 development is not straightforward. Usually, important variation in bioprocess performance and
386 efficiencies are observed, as described in several studies [33,38,75,102–104]. One of the most
387 important factors that can lead to significant loss of bioprocesses efficiency is that, while lab-
388 scale bioreactors are considered perfectly mixed, lack of homogeneity is an issue in large-scale
389 cultivations. The difference in mixing often leads to significant differences in mass and heat
390 transfer in the processes [105]. As a result, spatial gradients of important parameters, such as
391 dissolved gases, pH, temperature or concentration of substrates are all likely to emerge, leading
392 to potential conditions of oxygen limitation or other essential nutrient starvation [106]. Spatial
393 heterogeneity, in turn, leads to important difficulties in monitoring and controlling large-scale
394 cultivation, because the process sensors can only display an average of the whole system [33].
395 These environmental conditions may impose stress conditions on the cells, which can often
396 importantly affect biomass growth and product generation of industrial-scale bioreactors and
397 consequently bioprocess efficiency being positive or negative. Specifically hypoxia, transient
398 anoxia and nutrient starvation are of great importance in process optimization [38,50,51].
399 Therefore, the most commonly selected parameters to keep constant between scales for these
400 highly aerobic and high cell density systems are impeller tip speed, volumetric power input,
401 oxygen transfer rates, volumetric oxygen mass transfer coefficient (k_La) or minimum dissolved
402 oxygen concentration. Although bioprocess engineering development of P_{GAP} systems is still in

its infancy compared with P_{AOX1} [40], scale up under P_{GAP} should be easier due to the use of glucose/glycerol as main substrate instead of methanol.

Why to avoid the use of methanol on the way to large-scale production

From an industrial point of view, *a priori* primary advantages of using alternative systems to methanol-inducible bioprocesses can be anticipated. Primarily, problems related to storage, delivery and safety of large quantities of methanol would be avoided [40]. In addition, using methanol as a main substrate involves operational problems at high-cell density cultures derived from the methanol catabolism, in particular, high values for oxygen requirements and heat production [44,48,107]. Avoiding the use of methanol also reduces the accumulation of the by-products of methanol metabolism, as well as cell lysis and subsequent release of native protein and potential proteolysis of secreted products of interest [108,109]. Furthermore, accounting for downstream issues, additional steps in order to achieve the total elimination of methanol in the final product, would be rendered necessary [45].

Thus, alternative methanol-free systems are expected to contribute greatly to the development of cost-effective solutions for industrial RPP [40]. Recent strain engineering approaches that exploit the exceptional capacity features of the P_{AOX1}, but in simpler methanol-free de-repressed bioprocesses, could become promising alternatives. Up-to-date conventional methanol inducible strains have been retrofitted into methanol-free systems regulated by glucose/glycerol. For instance, it has been achieved by the derepressed overexpression of key identified P_{AOX1}-related transcription factors (Mxr1, Mit1 and Prm1), which by themselves are suitable to activate the P_{AOX1}-based RPP machinery strongly [110–112]. However, although some reports are starting to address basic parameters such as substrate comparison and production kinetics determination, the bioprocesses developed for these strains are currently still not widely described in the literature [59,113]. Finally, although methanol involves operational drawbacks from an industrial point of

view, the productivity of the bioprocess, product quality and its corresponding analysis of economic viability will determine the decision about which the expression system to be used.

Design criteria in bioprocess optimization

The current trend in bioprocess optimization is to move away from standard protocols towards concepts that enable an operator to adapt particular recommendations to any specific clones/strain or bioreactor [67]. Strain and process engineering strategies should be combined in order to achieve optimal results, avoiding complex media to reduce costs and facilitate downstream processes [23,114], with the aim of increasing economic viability of the bioprocess. However, bottlenecks are generally product-specific and need to be identified in each particular case taking into account economic considerations [29].

When optimizing a bioprocess efficiency, usually $Y_{P/S}$, Q_P and the final titre are selected as design criteria. Significant reduction in capital and operating costs can be achieved when reaching high values of these indexes. These criteria are also importantly affected by the field of application of the product. Usually, $Y_{P/S}$ is selected to obtain low added value products, whereas Q_P and titre are often prioritized to produce high added value products [36,115]. In fermentation, in order to boost bioprocess efficiency it is essential to produce the maximum product amount within the minimum time (Q_P or space time yield, *STY*). This goal will be achievable by reaching the maximum biomass amount jointly with high q_P within the shortest process time required to recover a product that meets quality specifications demanded for its application [35]. In addition, for high value-added products, the requirement for a high final titre are usually also crucial, since it allows the reduction of costs for downstream processing. Product quality takes into account target attributes, such as biological activity, half-life, immunogenicity and safety, protein glycosylation, and aggregation [67,116]. These may be compromised by release of intracellular proteases and/or host proteins which occurs when the culture suffers cell lysis or death [39,117]. Correct folding and

functionality of the product are primarily defined by the cell physiological characteristics but can be modified by cultivation parameters [88,118,119].

Since in most industrial fermentation processes for RPP product formation is growth-associated, biomass is often considered an unavoidable but crucial waste by-product. In fact, the optimal q_P is usually reached when working at high μ [35]. Furthermore, due to the different biological and different physical restrictions (primarily heat and mass transfer), it must also be taken into consideration that the maximum amount of biomass reachable in the bioprocesses must be limited as much as possible. Consequently, higher product amounts may be generated when performing the bioprocesses at lower growth rates, which therefore lead to longer fermentation times before the process is stopped due to system limitations. In this context, for careful optimization of bioprocess performance indicators such as *STY* or product yield, the trade-off between q_P and μ turns to be crucial [50]. In addition, the use of biomass as single-cell protein (*SCP*), in order to obtain value-added products for food and agriculture industries, can become a viable and interesting circular-economy practice [120,121]

The industrial trend: From fed-batch to continuous production processes?

Currently, most protein production processes using yeast as cell factories are still carried out with fed-batch fermentations, which allows higher biomass and product concentration, yields and productivity by preventing substrate inhibition and catabolite repression [122]. *P. pastoris* cultivations in industrial processes are often carried out in large stirred tank bioreactors (up to 10 m^3), achieving high amounts of biomass and product [123]. In general, the primary costs of yeast fermentations are usually related to substrate and energy. When reaching very high cell densities, the aeration requirements are coupled with the need for efficient cooling systems. However, considering the overall bioprocess, purification represents a very high proportion of the total

bioproduct cost, especially for those with high added-value [123]. Finally, the biomass separation from high cell density cultures is also a challenging task in downstream processing [124].

Although until now the protein production processes using yeasts are primarily conducted in batch or fed-batch mode, the current trend, as in other industries, is leading to the transition from batch to continuous manufacturing. This trend aims to exploit the benefits of continuous production such as flexible operation, higher productivity and quality, decreased cost, smaller facilities and the integration and the simplification of the processes. However, the use of this cultivation mode still presents important drawbacks, including losses of productivity due to genetic instability of the cells [125], higher risk of contamination and poor short-term flexibility to handle multiple products due to long run times [126–128]. Continuous cultivations are currently being used for industrial recombinant human insulin production, carried out using *S. cerevisiae* as a cell factory [129]. For *P. pastoris*, several recombinant proteins have been successfully produced in continuous cultures at laboratory bench-scale for both constitutive (P_{GAP}) and methanol-inducible (P_{AOX1}) expression [38,88,130–133]. A summary of several continuous processes under P_{GAP} is presented in **Table 3** [76,134–136]. The change of operational mode from fed-batch to continuous should be considered an effective strategy for improving the bioprocess efficiency. Indeed, the FDA has encouraged the development of continuous processing for biopharmaceuticals manufacturing [126,137,138].

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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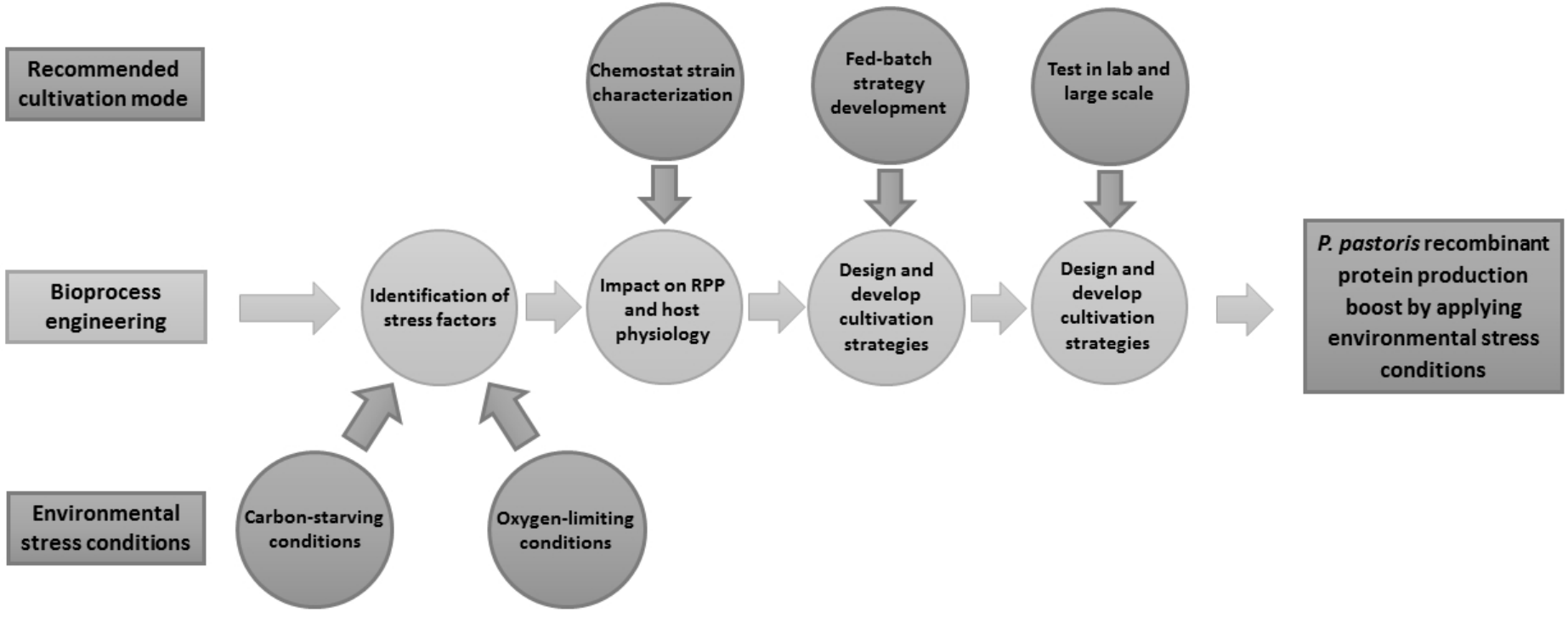
Figure captions

Figure 1: Scheme to exploit the adaptive cell responses to environmental stress conditions to boost the recombinant protein production in *P. pastoris.*


Recommended cultivation mode
Bioprocess engineering
Environmental stress conditions
Chemostat strain characterization
Fed-batch strategy development
Test in lab and large scale
Identification of stress factors
Impact on RPP and host physiology
Design and develop cultivation strategies
Design and develop cultivation strategies
P. pastoris recombinant protein production boost by applying environmental stress conditions
Carbon-starving conditions
Oxygen-limiting conditions

Tables

Table 1. Summary of the main advantages of *P. pastoris* as a recombinant protein expression system. (GRAS = Generally Recognised as Safe. DCW = Dry Cell Weight).

Genetic Engineering	Protein processing	Bioprocess engineering
Simple and stable genetic manipulation	Eukaryotic capacity for folding, assembling and performing PTM's	GRAS organism lacks detectable endotoxins
Numerous tools available for genetic manipulation including CRISPR/Cas9 system.	Protein processing and secreting pathway similar to high eukaryotes	Non-fermentative growth on glucose
Different strong and efficient promoters	Ability to secrete efficiently target proteins combined with low levels of secretion for native proteins	Growth in chemically defined medium up to 120 g DCW L^{-1}
Reported high yield and stable producing strains	Reduced hyperglycosilation and reported human-like glycoengineered strains	Well-established large-scale production and downstream processing

Table 2. Summary of recombinant protein production processes in fed-batch cultures under *GAP* promoter in *P. pastoris*.

Protein expressed	Promoter P_{GAP} vs P_{AOX1} comparison	Strain	Operational strategy	Volume (L)	μ (h^{-1})	Production (mg L^{-1})	Q_p (mg L^{-1} h^{-1})	q_p (mg g_x^{-1} h^{-1})	$Y_{X/S}$ (g g^{-1})	$Y_{P/X}$ (mg g^{-1})	Final DCW (g/L)	Ref.
Fungal endoglucanase	Rather similar	X33	Pseudo Constant feeding rate	15	n.d.	Between 5000-7000	86	0.5	1.03* n.d.	37.5	160	[139]
β-Mannanase	AOX1 > GAP	GS115	Constant feeding rate	1	n.d.	960 Equiv. 2980 UL^{-1}	10 Equiv 31 U L^{-1} h^{-1}	n.d.	n.d.	n.d.	n.d	[140]
GGH / GLP-1	GAP > AOX1	X33	Constant glucose concentration 5 g L^{-1}	5	n.d.	246	3.8	0.1	--	5.9	41.2	[141]
rhIK-2-HSA	AOX1 > GAP	X33	DO depending	5	n.d.	250	2.5	--	--	--	75	[142]
Angiostatin	GAP > AOX	GS115	Continuous feeding	20	n.d.	176	4.9	--	--	--	--	[143]
Fab fragment (anti-HIV antibody 2F5)	---	X33	Constant feeding rate	1.75	Variable	41.2	--	0.02	--	--	--	[144]
Fab fragment (anti-HIV antibody 2F5)	---	X33	Fed-batch optimal profile	2	Variable	45	0.67	0.04	---	0.47	94	[76]
Fab fragment (anti-HIV antibody 2F5)	---	X33	Exponential feeding rate	5	Controlled 0.05-0.15	25	0.73	0.09	0.45	0.25	100	[48]
Glucose isomerase	---	X33	DO control 15%	3	--	4500 U L^{-1}	--	--	0.22	126 U g^{-1}	44	[37]
hGM-CSF	---	GS115	Exponential feeding rate	2	0.2	250	7.35	0.07	1.05	2.4	98	[145]
hSA	---	X33 SMD1168H	Fed-batch optimal profile	3.5	Variable	44	2.2	0.06-0.02	--	--	98	[146]
Streptavidin	---	X33	Constant feeding rate	0.5	--	221	3.06	--	--	--	30	[147]
***Rhizopus oryzae* lipase**	---	GS115	Constant feeding rate	7	--	2600 U mL^{-1}	30.95 U mL^{-1} h^{-1}	--	--	--	140	[148]

*Some of the presented values were estimated from the published available data.

Table 3. Summary of recombinant protein production processes in continuous mode under *GAP* promoter in *P. pastoris*.

Protein expressed	Strain	Volume (L)	μ (h^{-1})	Production (mg L^{-1})	Q_p (mg L^{-1} h^{-1})	q_p (mg g_x^{-1} h^{-1})	$Y_{X/S}$ (g g^{-1})	$Y_{P/X}$ (mg g^{-1})	Final DCW (g/L)	Ref.
Serum albumin (human)	SMD1168H	1	0.015-0.15	30.04 (μ = 0.15)	---	0.15	0.52	1.16	---	[88]
Fab fragment (anti-HIV antibody 2F5)	X33	2	0.0086-0.2	---	---	0.046	0.56	---	---	[76]
Fab fragment (anti-HIV antibody 2F5)	X33	1	0.025-0.15	---	---	0.057	0.43	---	---	[50]
hGM-CSF (human)	X33	1	0.025-0.2	82	---	0.5	0.8	2.5	35	[136]
rh-chitinase	X33 SMD1168	1.5	0.05	360	18	0.15	0.33	3.6	100	[149]
rh-chitinase	X33	15	0.042	250	6	0.055	0.37	2.3	110	[150]
phytase	X33	2	0.05-0.035	---	---	1,4 kU/g·h	--	--	--	[134]
α-amylase	GS115	20		125	---	---	---	---	---	[135]

*Some of the presented values were estimated from the published available data.