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REVIEW

Pichia pastoris: A Recombinant Microfactory for Antibodies and Human Membrane Proteins

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During the last few decades, it has become evident that the compatibility of the yeast biochemical environment with the ability to process and translate the RNA transcript, along with its capacity to modify a translated protein, are relevant requirements for selecting this host cell for protein expression in several pharmaceutical and clinical applications. In particular, *Pichia pastoris* is used as an industrial host for recombinant protein and metabolite production, showing a powerful capacity to meet required biomolecular target production levels in high-throughput assays for functional genomics and drug screening. In addition, there is a great advantage to using *P. pastoris* for protein secretion, even at high molecular weights, since the recovery and purification steps are simplified owing to relatively low levels of endogenous proteins in the extracellular medium. Clearly, no single microexpression system can provide all of the desired properties for human protein production. Moreover, chemical and physical bioprocess parameters, including culture medium formulation, temperature, pH, agitation, aeration rates, induction, and feeding strategies, can highly influence product yield and quality. In order to benefit from the currently available wide range of biosynthesis strategies using *P. pastoris*, this mini review focuses on the developments and technological fermentation achievements, providing both a comparative and an overall integration analysis. The main aim is to highlight the relevance and versatility of the *P. pastoris* biosystem to the design of more cost-effective microfactories to meet the increasing demands for recombinant membrane proteins and clinical antibodies for several therapeutic applications.

Key words: *Pichia pastoris*, antibodies, human membrane proteins, bioprocess design

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Using recombinant DNA technology to insert genes into a variety of organisms, a door to the production of functional proteins has been opened. Initially, commercial production of heterologous proteins was achieved using *Escherichia coli* as a host, owing to the availability of complete information about its genetic and biochemical systems [31]. However, the need to express more complex recombinant proteins led to the use of more complex transformation systems, ranging from prokaryotes to eukaryotes and even transgenic animals and plants [31]. Among them appears the *Pichia pastoris* expression system. With its proven ability to express more than 300 proteins, from human endostatin to spider dragline silk protein, *Pichia* has become a consistent choice for heterologous protein production [13, 16]. It is known that the cell machinery of the host organism and the production of a functional protein are inseparable and closely related concepts. *P. pastoris* as a eukaryotic organism is capable of producing soluble, correctly folded recombinant proteins that have undergone all of the posttranslational modifications required for functionality [25]. *P. pastoris* has the ability to produce proteins of therapeutic and commercial interest in concentrations ranging from milligrams to grams per liter [57].

This review will illustrate the importance of the host cell machinery and cell culture conditions in the production of functional proteins. Moreover, since *P. pastoris* is the center point of our discussion, we will illustrate the different challenges faced by this host in reaching the standards to be considered in the production of proteins such as antibodies and membrane proteins.

Product purity, yield, and cost also have to be taken into account in the design of the process. Compared with mammalian cells, *Pichia* does not need a complex growth medium or culture conditions [59]. Fermentations can be readily scaled up to meet greater demands, and parameters influencing protein productivity and activity, such as pH, aeration, and carbon source feed rate, can be controlled. Additionally, as *Pichia* does not secrete high levels of

native proteins, the purification of secreted formulations is much more simple than in other systems [73]. Moreover, the simplicity of the techniques needed for molecular genetic manipulation and their similarity to those used with *Saccharomyces cerevisiae* have made *Pichia* one of the most well-characterized experimental systems in modern biology [18].

Pichia pastoris as a Recombinant Microfactory

As a eukaryotic fungal organism, *Pichia* is easy to manipulate and cultivate. Initially used by Philips Petroleum Company for the production of single cellular proteins, this organism is nowadays a nonconventional yeast in which proteins have been successfully expressed in laboratory- and large-scale fermentation procedures to produce recombinant proteins [15, 81]. Its unique features, which we will discuss below, combined with the possibility of designing suitable flowsheets for each specific protein, make *Pichia* a microfactory to be reckoned with.

Morphological and biomolecular characteristics. Being an organism that is easy to manipulate, *P. pastoris* can reach high levels of expression and produce complex biomolecules that need to undergo posttranslational modification. Since *Pichia* has no native plasmids, the expression of foreign genes is achieved by chromosomal integration, using integrative plasmids or autonomous vectors. Like *S. cerevisiae*, *P. pastoris* exhibits a propensity for homologous recombination between genomic and artificially introduced DNAs. The integration of a foreign gene expression cassette can occur *via* gene insertion or gene replacement (Fig. 1). For optimal genetic stability, cleavage of a *P. pastoris* vector within a sequence shared by the host genome stimulates homologous recombination events that efficiently target integration of the vector into a specific genomic locus, AOX1 [19] (Fig. 1A). This results in the site-specific eviction of the AOX1 structural gene, affording a strain that grows slowly in methanol. Alternatively, the vector can be linearized and targeted to integrate into the genome by non-disruptive insertion into HIS4, generating strains that grow normally in methanol (Mut^+) (Fig. 1B) [22, 31].

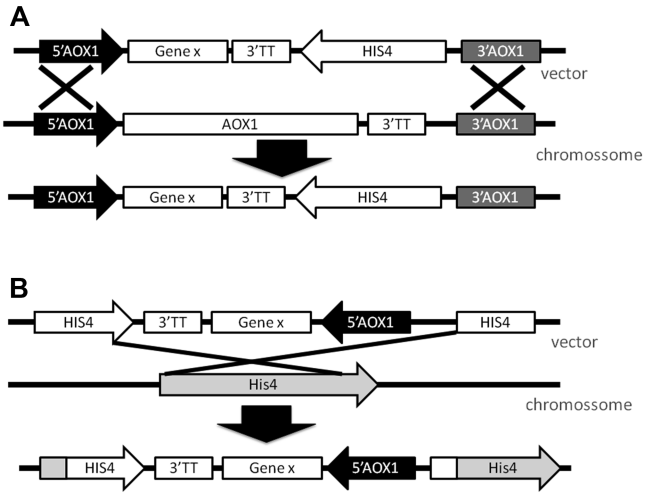

Fig. 1. Integration into *P. pastoris* genome by (**A**) gene replacement, and (**B**) gene insertion at his4.

All *P. pastoris* expression strains are derivatives of NRRL-Y 11430 (Northern Regional Research Laboratories, Peoria, IL, USA). Most of them have a mutation in the HIS4 gene for the selection of expression vectors containing HIS4 upon transformation. Table 1 shows the available strains applied in several biotechnological domains. The choice of an ideal strain is mostly determined by the target application [38].

An understanding of the phenotype is important in deciding upon the culture conditions to be used. *Pichia* is a yeast capable of metabolizing methanol. A unique set of enzymes, alcohol oxidase (AOX), catalase (CAT), and dihydroxyacetone synthase (DHAS) locked in peroxisomes, enable the yeast to convert methanol as a source of carbon and energy (Fig. 2) [34]. The AOX enzyme is very important since it is responsible for the initial step, the oxidation of methanol to formaldehyde. There are two

Table 1. *Pichia pastoris* strains.

Strain	Genotype	Phenotype	Reference
Y-11430	Wild type	-----	NRRL[a]
X-33	Wild type	-----	[55]
Auxotrophic strain			
GS115	his4	Mut^+, His^-	[21]
Protease-deficient strain			
KM71	Δaox1::SARG4 his4 arg4	Mut^S, His^-	[79]
SMD1168	His4, pep4	Mut^+, His^-, $pep4^-$	[82]
SMD1165	His4, prb1	Mut^+, His^-, $prb1^-$	[1]
MC100-3	arg4his4aox1Δ::SARGA4aox2Δ::Phis4	Mut^-,His^-	[36]

[a]Northern Regional Research Laboratories, Peoria, IL, USA.

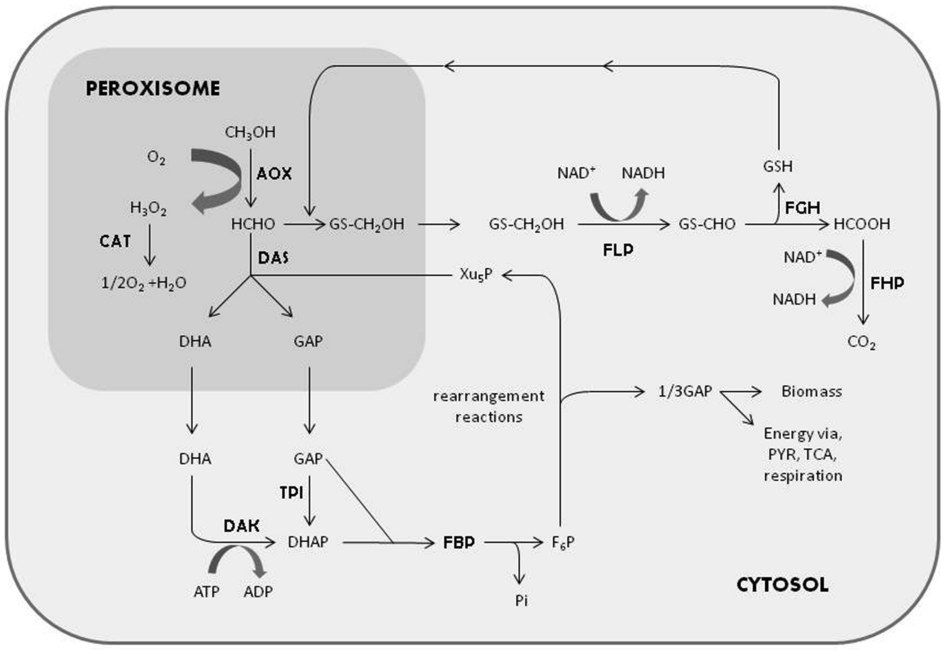

Fig. 2. Methanol metabolic pathway can be split into three steps.
1. Methanol is oxidized to formaldehyde by alcohol oxidase, alcohol dehydrogenase, or the catalase peroxidative pathway. 2. Formaldehyde is rapidly oxidized to formic acid mainly by formaldehyde dehydrogenase. Catalase and aldehyde dehydrogenase can also mediate this step. 3. Formic acid, which is the principal toxic agent in methanol poisoning, is metabolized by formate dehydrogenase. The catalase peroxidative pathway can also oxidize formic acid to CO_2.

genes encoding AOX, *aox1* and *aox2*, but the former is responsible for the vast majority of alcohol oxidase activity in the cell. Its regulation is similar to that of *gal1* in *S. cerevisiae*, involving two mechanisms: repression/derepression and induction. However, unlike *gal1* regulation, the absence of a repressing carbon source such as glucose in the medium does not result in substantial transcription of *aox1*. However, methanol is essential to the induction of high levels of transcription. The tight regulation and high level of *aox1* expression make AOX a strong promoter for the expression of heterologous proteins, being the most widely reported and utilized of all available promoters (Table 2) [14, 34]. Fundamentally, five promoters are used for recombinant membrane protein (MP) production in *P. pastoris*. Of these, three are inducible by methanol (AOX1, FLD1, and PEX8), and the other two are constitutive (GAP and YPT1) [88].

Most strains grow on methanol at the wild-type rate (Mut^+); however, cells with the Mut^S and Mut^- phenotypes, because of the deletion in one or both AOX genes, requires

Table 2. *Pichia pastoris* alternative promoters to AOX1.

Promoter	Features
GAP	Does not require methanol for induction, nor is it necessary to shift cultures from one carbon source to another, making strain growth more straightforward Is expressed constitutively, so is not a good choice for expression of proteins that may be toxic to the cell
FLD1	Can be induced by methanol as the sole carbon source (with ammonium sulfate as a nitrogen source), or Methylamine as the sole nitrogen source (with glucose as a carbon source) Is repressed with glucose and ammonium sulfate Offers the flexibility of inducing high levels of expression using either methanol or methylamine, an inexpensive nontoxic nitrogen source
PEX8	Moderate expression levels are desirable Expressed at low but significant level on glucose Induced modestly by methanol
YPT7	Moderate expression levels are desirable Provides a low but constitutive level of expression in media containing glucose, methanol, or mannitol as carbon sources

Table 3. Comparison of five different expression systems.

Features	*P. pastoris*	*S. cerevisiae*	*E. coli*	*B. bacillus*	Mammalian cells
Protein productivity	++	+	+	+	-
Secretion	++	+	-	++	+/++
Glycosylation	+	++	--		+
Cost	--	-	--	-	++

++ high; +/++ medium to high; + medium; - low; -- very low/absent

less methanol to induce expression, which is an advantage in large-scale fermentations, where large quantities of methanol are considered a significant hazard [57].

Advantages and troubleshooting. The type and size of the target protein will determine which system is more suitable for its expression. Large proteins are usually expressed in a eukaryotic system, and smaller proteins are preferably produced in prokaryotic systems. *Pichia*, as a eukaryotic organism, with its own protein synthesis pathway, reveals itself as a system capable of producing soluble, correctly folded recombinant proteins. Being faster, easier, and less expensive to use, this system presents several advantages over conventional systems (Table 3) [27].

In its capacity to secrete soluble proteins with complete posttranslational maturation, *Pichia* offers an advantage over the traditional *E. coli* host, which produces proteins as intracellular or periplasmic inclusion bodies. In this case, the proteins organized in the inclusion bodies must be further refolded and purified, especially if the proteins contain multiple disulfide bonds or require glycosylation, phosphorylation, and the absence of an amino-terminal methionine or oligomer formation for the correct assembly of the mature protein [53, 58].

As a methylotrophic yeast, *P. pastoris* possesses the strongest, most regulated promoter (AOX1), enabling the level of product expressed to be regulated by a simple manipulation of the culture medium. The expression of recombinant proteins is highly repressed while cultures are grown to high density in glucose or glycerol, which prevents selection of non-expressing mutant cells. Shifting the cultures to a methanol medium induces rapid, high-level expression [15]. However, the high levels of expression provided not only by AOX1, but also by the GAP promoter, can be toxic in some cases, and may overwhelm the protein-handling machinery of the cell, leading a significant portion of the protein to be misfolded or unprocessed [9]. The lack of moderately expressed promoters along with the existence of only a few selectable markers for *P. pastoris* transformation constitute a limitation of this system [15].

In terms of glycosylation, mammalian cell systems are the best option, being to date the only ones capable of yielding human-like glycoproteins. However, there is evidence of some differences between the human form and the glycoproteins produced through the use of mammalian cells. Among these differences is the addition of the sialic acid *N*-glycolylneuraminic acid, which is absent in humans and could lead to an immune response [41].

On the other hand, yeast can perform glycosylation by the use of an evolutionarily conserved *N*-linked oligosaccharide biosynthetic pathway. However, despite sharing the initial stage of glycosylation with mammalian cells (Fig. 3A), the final glycoprotein produced in Golgi present some differences [22, 40, 41]. In mammalian systems, mannose residues are removed, and fucose, galactose, *N*-acetylneuraminic acid, *N*-acetylgalactosamine, *N*-acetylglucosamine, and sialic acid are incorporated. Nevertheless, in yeast, mannoses are added and the oligomannose units can be linked at α-1,6 or α-1,3 mannose in the Manα-1,3-Manβ-1,4-GlcNAc2 inner core, which leads to a diverse structural heterogeneity of the proteins produced [22, 40]. These crucial differences enable the yeast not to form the expected complex glycans, but to produce hybrid glycans that could be immunogenic, or, in the case of therapeutic antibodies, may lack the desired effector function [40, 41].

However, *P. pastoris* is distinguished from the other yeasts, such as *S. cerevisiae*, by its capacity to perform a less extensive glycosylation, in which the oligosaccharide chains attached to the proteins are shorter and more authentic [52]. Glycoproteins produced in wild-type yeast contain potentially immunogenic high-mannose-type glycans; to overcome this problem, *P. pastoris* strains have been engineered with more human-type glycosylation properties, through the use of GlycoSwitch and GlycoFi technologies [40, 41, 67]. The implementation of these technologies allows the disruption of the genes responsible for yeast high-mannose glycans, such as OCH1, and the introduction of a series of glycosidases and glycotransferases (Fig. 3b). In a first step to humanizing the *N*-linked glycan synthesis, α-1,2 mannosidase trims Man8 to Man5, which will be glycosylated to the hybrid structure GlcNAcMan5 by the Golgi-residing fusion protein *N*-acetylglucosaminyltransferase I. Then, mannosidase II and *N*-acetylglucosaminyltransferase II are introduced in order to obtain GlcNAc2Man3. Finally, the last step involves the addition of Gal sugars to the nonreducing end of the terminal GlcNac, achieved by galactosyltransferase (GalT) [40, 41]. These techniques enable a reduced production time while improving glycan uniformity [22, 41, 49, 67]. However, the genetic engineering

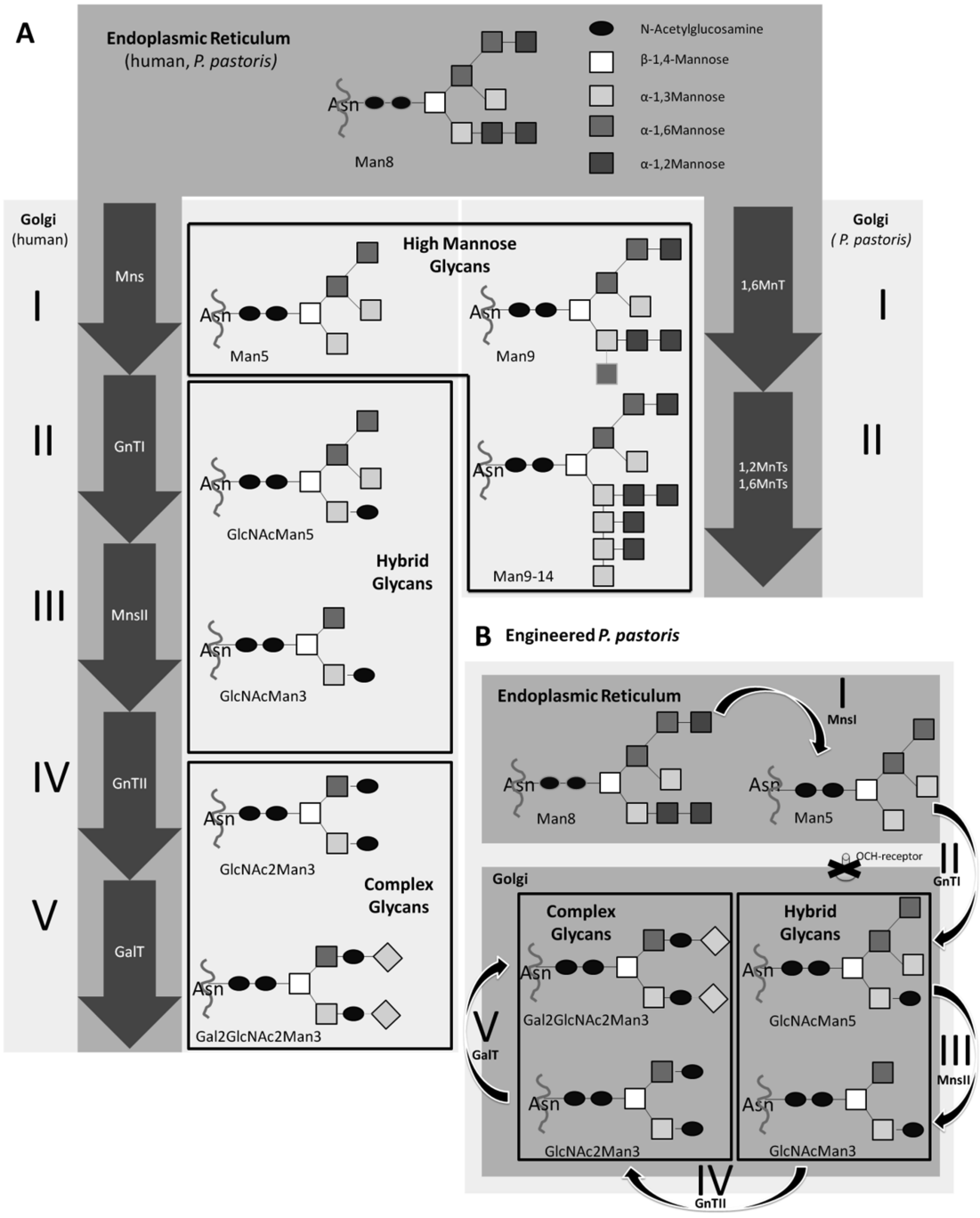

Fig. 3. Outline scheme of *N*-linked glycosylation pathways.
(**A**) In human and yeast *P. pastoris*. (**B**) In humanized *P. pastoris*. Mns: α-1,2-mannosidase; GnTI: β-1,2-*N*-acetylglucosaminyltransferase I; MnsII: mannosidase II; GnTII: β-1,2-*N*-acetylglucosaminyltransferase II; GalT: β-1,4-galactosyltransferase; MnT: mannosyltransferase.

of *Pichia* raises questions about its genetic stability. To date, several studies have been performed that revealed promising results in the maintenance of uniform *N*-linked glycans in consecutive generations, with commercially viable productivities and titers [49, 67].

Biosynthesis Strategies for Protein Production in *Pichia pastoris*

The *P. pastoris* expression system is able to produce many kinds of recombinant proteins and, in particular, seems to be well suited to the heterologous production of membrane

proteins (MP) and antibodies (Ab). In fact, the facility for genetic manipulation combined with its rapid growth on an inexpensive medium to high cell densities and the capacity to perform complex posttranslational modifications makes *P. pastoris* one of the most attractive hosts for recombinant protein production. Indeed, it has been reported that the production of full-length antibodies (mAbs) carrying the uniform N-glycan (critical for biological effector function) could be achieved using *P. pastoris* systems, whereas prokaryote-derived mAbs do not possess any glycosylation [65]. Likewise, *P. pastoris* stands out as a successful expression host for the production of a number of functional, properly folded, and inserted MPs that have not been produced successfully in other systems [70].

Bioprocess design: Medium formulation and operation conditions. Expected yields of the *P. pastoris* expression system in the production of heterologous proteins have met new levels of demands, and so, bioprocess design including all the parameters inherent to the bioprocess has a great influence on the final result and quality of the target protein. Hence, in this section, we present an overview of the current strategies used for designing and optimizing a bioprocess using *P. pastoris* for MP and Ab heterologous overexpression.

The culture medium composition exerts an effect on heterologous protein production in yeast through changes in cell growth and viability [52]. In general, all *P. pastoris* strains grow on defined medium supplemented with specific components for each strain. For instance, whereas the wild-type X-33 *P. pastoris* strain grows in minimal medium, strains GS115 and KM71H only grow on minimal medium supplemented with histidine and arginine, respectively [52]. Nevertheless, complex media such as YPDS or BMGY and BMMY may be employed either for selecting *P. pastoris* Zeo transformants or for protein expression, respectively. Moreover, as will be discussed in detail below, the culture medium may also be supplemented with specific components such as casamino acids or peptone in order to reduce or prevent proteolytic degradation of the target protein.

For high cell density growth and induction of *P. pastoris* strains, the culture medium composition is similar to those described by Philips Petroleum Company in the 1970s [16]. Essentially, the medium consists of biotin, ammonium hydroxide as the nitrogen source (also used to control the pH), glycerol or methanol as, respectively, the carbon and energy source, basal salts medium (BSM), and trace elements (zinc chloride, ferrous sulfate heptahydrate, and biotin being the most abundant constituents) [11, 16]. Alternative formulations to the standard BSM were proposed by Stratton *et al*. (the so-called FM22) [77] as well as d'Anjou and Daugulis [23]. However, common problems associated with these formulations were the precipitation of one or more salts of the BSM during media preparation, changing the effective concentration of the dissolved minerals in the medium as well as turbidity, thus compromising cell density assessments [16]. Nevertheless, a solution to this problem was presented by Oehler *et al*. [60] and Brady *et al*. [8]. Oehler and collaborators presented an alternative medium in which sodium hexametaphosphate, a non-precipitate-forming compound, replaced phosphoric acid as a source of phosphate [60], and Brady proposed the reduction of the basal salts concentrations to one quarter of that recommended [8] (see Table 4 for the detailed composition of BSM preparations). This alteration has proven to be effective since no adverse effects on cell growth rate, biomass yield, or protein expression levels have been detected. Moreover, in 2008, Ghosalkar *et al*. [37] optimized a chemically defined medium for maximal biomass production of recombinant *P. pastoris* in fermentor cultures using glycerol as the main carbon source, where 11.25 g DCW/l of biomass was produced [37].

Recently, Plantz *et al*. [65] evaluated the influence of metals on both cell growth (GS115 strain) and product yield (β-galactosidase) in *P. pastoris* expression systems. Interestingly, they found that the levels of metals reported by Stratton *et al*. [77] are in excess for the production of β-galactosidase by *P. pastoris*, some of which were by over two orders of magnitude [65]. Although it is possible that the medium requirements will depend on the type of protein expressed, those studies were important in showing that excess trace metals could have a potentially negative effect on both cell growth and product yield of *P. pastoris* expression systems [65].

Table 4. Fermentation basal salts medium (BSM) proposed by Invitrogen Corporation (*Pichia* fermentation process guidelines).

BSM component	Invitrogen corporation	Brady *et al.* [8]
Phosphoric acid 85%	2.67% (v/v)	----------
Calcium sulfate	0.93 g/l	0.23 g/l
Potassium sulfate	18.2 g/l	4.55 g/l
Magnesium sulfate heptahydrate,	14.9 g/l	3.73 g/l
Potassium hydroxide	4.13 g/l	1.03 g/l
Glycerol	40.0 g/l	40 g/l
Sodium hexametaphosphate	----------	6.5 g/l

Despite the sometimes significant achievements that can be reached with unusual culture conditions, success largely depends on the target protein; therefore, general guidelines concerning culture conditions for *P. pastoris* heterologous protein production are provided by Invitrogen Corporation, which are a good starting point. Thus, for bioreactors and shake flasks, the temperature should be between 28°C and 30°C (growth above 32°C is detrimental to protein production), the pH should not be higher than 6 (can be adjusted when the protein is secreted since it would possibly reduce proteolysis), and an antifoam agent should be added to the culture medium for secretory expression. Normally, each factor may have to be optimized since, for example, the ideal temperature for human muscarinic acetylcholine receptor M2 subtype production in strain SMD1163 was found to be 20°C [4]. In fact, enhancement of protein secretion at lower temperatures can be attributed to the general decrease in folding stress, as well as increased viability and decreased release of cellular proteases [32]. Whenever the target protein is produced in bioreactors, the agitation speed should range between 500 and 1,500 rpm, whereas for shake flasks it should be established in the range of 250–300 rpm. Aeration is also an important factor that affects high celldensity cultures of *P. pastoris* and should be kept in the range of 0.1–1.0 vvm (volume of oxygen per volume of culture per minute) in order to maximize the oxygen concentration in the medium. On the other hand, the observation that hypoxic conditions lead to an increased specific productivity of secreted protein by *P. pastoris* motivated Baumann *et al*. [6] to develop a hypoxic fed-batch protocol using the GAP promoter, in which the ethanol concentration in the culture was kept constant at approximately 1.0% (v/v), resulting in an increase of 2.3-fold in the volumetric productivity of an antibody Fab fragment.

Regarding the production of heterologous proteins in *P. pastoris* in bioreactors, it is desirable to achieve high cell density cultures and, consequently, to attain high levels of recombinant protein. In fact, several strategies in fed-batch mode are available for protein production in *P. pastoris* strains in bioreactors under the control of AOX1 [11]. All of these strategies comprise three or four stages. First, the strains are batch cultured in a defined medium containing glycerol (or another repressing carbon source) to generate biomass but repress heterologous gene expression [11, 13, 20]. Usually, in this stage, the feed rate is set to a growth-limited level to avoid glycerol accumulation where the feed time will depend on the desired cell density. The second stage is a fed-batch transition phase in which more glycerol is added to the culture but at a growth-limiting rate in order to increase the biomass even more and to derepress the cells for induction [13, 20]. The last stage is the addition of methanol to the culture at a slow rate to induce protein expression [13, 20]. In a four-stage process, an additional stage of batch-methanol addition is employed between the second and third stages (called transition phase), to prepare the cells prior to fed-batch operation [11]. As discussed in detail above, there are two phenotypically distinct strains, Mut^s and Mut^+, that differ in their capacity to use methanol as a carbon source. For Mut^+ strains, when we switch the medium composition to methanol, one must observe a dissolved oxygen (DO) spike to ensure that all of the glycerol of the fed-batch phase is consumed before initiating the transition phase [82]. On the other hand, in the induction phase for Mut^s strains, an excess of methanol not exceeding 0.3% is recommended [87]. Typically, for Mut^+ *P. pastoris* strains, the methanol concentration in the medium used for induction ranges between 0.5% and 1% (v/v) but it is an important factor that requires optimization for each target protein since it affects cell growth as well as protein levels [22]. Moreover, alternatives to the common co-feeding strategy with glycerol/methanol have also been reported. Çelik *et al*. [12] reported that the addition of sorbitol as a co-substrate at the induction phase of methanol fed-batch fermentation by a *P. pastoris* Mut^+ strain increased the levels of recombinant human erythropoietin with no adverse effects on alcohol oxidase activity. Specifically, for Mut^S phenotype strains, it may be necessary to use alternative nonrepressing carbon sources such as sorbitol, mannitol, alanine, or trehalose even after induction. Three strategies are commonly employed to keep the methanol concentration at optimal limits. In the first one, the methanol feeding rate is controlled according to the concentration in the culture medium, which is determined by gas chromatography [52]. Another strategy consists of controlling the dissolved oxygen content, since the methanol feeding rate increases with increasing dissolved oxygen [52]. The third strategy is related to the specific growth rate during the induction phase, but the kinetic growth model needs to be set up well [52]. In any event, the strategy has to be adapted when protein production is under the control of another promoter such as GAP in which the genes are constitutively expressed. In these cases, the strains are cultured in a medium with an appropriate carbon source (glycerol, methanol, oleic acid, glucose, among others) by a fed-batch process [89].

The design of a fed-batch bioprocess based on different *P. pastoris* strains should include the optimization of several parameters in order to set up the most accurate feeding profiles. Dietzsch *et al*. [29, 30] evaluated the adaptation time of the culture to methanol ($\Delta time_{adapt}$), the specific substrate uptake rate during the adaptation phase ($q_{s\ adapt}$), and the maximum specific substrate uptake rate ($q_{s\ max}$) for *P. pastoris* strains that were either Mut^+ or Mut^S. Their strategy consisted of a batch experiment with a 0.5% (v/v) methanol adaptation pulse (to determine $\Delta time_{adapt}$ and $q_{s\ adapt}$), at least four consecutive 1.0% (v/v) methanol pulses (to assess $q_{s\ max}$), and a dynamic fed-batch feeding

strategy based on q_s, where, after the adaptation of the culture to methanol (monitored by a maximum in off-gas activity), q_s set points can be increased to $q_{s\ max}$ without observable methanol accumulation [29, 30]. The authors showed that this strategy is less time-consuming than continuous cultures or consecutive fed batch cultivations, and therefore allows a faster process development [29, 30].

Besides presenting clear advantages, heterologous protein secretion at high *P. pastoris* cell densities leads to a major problem. The secreted target protein can be proteolytically degraded by host-specific proteases, which are induced readily by environmental stresses, especially during high-density bioprocesses [45]. In addition, the contribution of cell lysis to the presence of proteases in the culture medium has been reported [76]. Therefore, it becomes necessary to develop strategies to circumvent the proteolytic degradation of the secreted target protein. Basically, three strategies can be implemented to solve this problem. The addition of amino acid-rich supplements (peptone or casamino acids) into the culture medium and the reduction of the culture medium pH to 3.0 [10, 35] seem to reduce proteolytic degradation of the target protein, respectively, by acting as substrates for the proteases and changing the protease optimal pH [20]. The third strategy consists of the application of protease-deficient *P. pastoris* strains such as SMD 163, SMD 165, and SMD 168 [20, 38].

Concerning the production of isotopically labeled proteins in *P. pastoris* for NMR analysis, several reports have been published [35, 83]. Wood and Kamives [83] reported in 1999 a fermentation method that allows for both ^{15}N-labeling from $(^{15}NH_4)_2SO_4$ and ^{13}C-labeling from ^{13}C-glucose or ^{13}C-glycerol, a method that is less prohibitive than others. More recently, Fan *et al*. [35] reported a method for isotope labeling of a eukaryotic seven transmembrane helical protein produced in *P. pastoris*, suitable for solid-state NMR analysis.

An interesting study carried out by Routledge *et al*. [72] characterized the effect of five commonly used antifoaming agents on the total amount of GFP secreted from shake-flask cultures. They found that the addition of antifoaming agents to shake-flask cultures increases the total yield of the recombinant protein. Apparently, some antifoams (Antifoam A and Antifoam C; Sigma; J673A, Struktol) improve the specific yield of GFP by increasing the total amount of protein produced and secreted per cell, whereas other antifoams (P2000, Fluka; SB2121, Struktol) increase the total yield by increasing the density of the culture [72].

Monitoring the methanol concentration is of critical importance in a *P. pastoris* process since high levels of methanol can be toxic to the cells and low levels may not be sufficient to initiate transcription [57] Chromatographic methods such as gas chromatography and high performance liquid chromatography are the most common off-line methanol monitoring methods used, despite being expensive and time consuming. On the other hand, the on-line methods are generally based on the liquid–gas equilibrium and monitor methanol in the broth by analyzing the fermenter exhaust gas. Moreover, biomass is an important parameter that has to be controlled during the bioprocess. Hohenblum *et al*. [43] revealed for the first time the usefulness of flow cytometry as a tool for the analysis and optimization of recombinant protein production processes in *P. pastoris*. They applied *P. pastoris* strain GS115 to human trypsinogen biosynthesis in a fed-batch mode and evaluated the viability as well as the product that remained associated with the cell wall with propidium iodide and immunofluorescent staining, respectively. Interestingly, the authors claim that the viability of the culture dropped in an early phase of the fed batch and finally fell to 65% at the end of the fermentation. Concerning product localization, the authors described that after starting the methanol feed, first the cells accumulate product, and then, nearly 20 h later, they begin to release product into the supernatant at a higher rate. In addition, the authors concluded that the dead cells retain the product they contain as long as they are not lysed; however, they will not contribute to newly synthesized product [43].

Typical fermentation flowsheets.

Antibodies. Antibodies or immunoglobulins are glycoproteins with a special affinity for foreign molecules. Their ability to recognize and bind with high affinity to any type of antigen makes them a powerful weapon for physicians to fight cancer, inflammation, and infectious diseases [48]. With the rise of recombinant DNA technology and increasing knowledge of the structure and function of antibodies, the engineering of new antibody formats with improved features has become a reality. With most applications requiring only the antigen-binding site of the native antibody, we observe the creation of antibody fragments that are increasingly smaller and fused with effector molecules, resulting in so-called “magic bullets” [48]. The *P. pastoris* expression system, with its special features, such as the ability to grow at high cell densities in a well-established fed-batch procedure without the endotoxins that are present in bacteria and the potential viral contamination associated with mammalian cell cultures, is a powerful tool in antibody production [5]. During the 1990s, many reports were published regarding single-chain IgG fragments (scFv), fusion scFv and antibody fragments produced in *P. pastoris*. However, it was not until 1999 that Ogunijimi *et al*. [62] reported the first fully functional IgG produced in *P. pastoris*. Knowing that glycans attached to IgG play an important role in maintaining the structure, solubility, conformation, and optimal binding of the Fc domain to its many receptors, efforts were made to engineer *P. pastoris* strains in order to produce glycoproteins

with uniform human *N*-glycans [5, 53]. GlycoSwitch and GlycoFi technologies have been used with promising results, with titers of full-length antibody of up to 1 g/l and the recent value of 1.6 g/l being reported [5, 67, 84].

Taking the production process into concern, the conventional approach involves the expression of the gene of interest under the control of the AOX1 promoter (Table 5). The genes of interest are integrated into the *P. pastoris* genome *via* homologous recombination, which results in clones that either retain their methanol utilization ability (Mut^+) or exhibit slow growth on methanol (Mut^S) [25]. With this system, the typical flowsheet design observed consists of a two-stage procedure, beginning with a batch phase with a repressing carbon source, usually glycerol, followed by a fed-batch phase where cells are induced by the addition of methanol [56, 78]. Between phases, it is possible to visualize an adaptation or transition phase where methanol is slowly introduced in combination with the repressing carbon source, whose concentration is ramped down linearly, to allow a gradual adaptation of the culture from optimal cell growth to optimal protein production [86]. Zhang *et al*. [86] have developed a fed batch under glycerol-limited conditions before the transition phase, which was revealed to be of benefit to the induction of the AOX1 promoter, since metabolites such as acetate and ethanol, produced during the batch phase, were consumed. On the other hand, Damasceno *et al*. [25] found that by omitting the transition phase by increasing the initial glycerol concentration, biomass can accumulate at a faster rate, starting the induction phase sooner. With regard to the fed-batch stage, the addition of methanol could be done according to three different feeding strategies [44]. First, the methanol feeding rate is controlled according to the methanol concentration in the culture medium, which is assessed by on-line methanol sensors [24]. Second, feeding can be adjusted based on the demand of dissolved oxygen level in the culture medium [58]. Third, the methanol feeding rate is controlled according to the specific growth rate in the induction phase [24]. The use of limited exponential feed rates to manipulate specific growth rates has been successfully implemented by several investigators. However, it is not only the feeding strategy that limits the yield of antibody production; factors such as pH, temperature, and medium formulation are also relevant to the design of experiments. Nowadays, one can observe the increased use of design of experiments (DoE) to determine the correct values for these different inputs in the production of the desired formulation, and so improve process yields and reduce costs.

Typically, the expression and activity of a single-chain variable fragment (scFv) is highest at 72 h [68] after induction, but we can also see studies where the production peak occurs 96 h after induction [74]. The decrease in scFv levels and activity is most likely due to the presence of proteases in the medium. One way to conteract this problem is by manipulating the pH [25], which is usually adjusted to inhibit the activity of proteases in the culture medium during the production phase. Therefore, it is necessary to adjust the pH during cell growth to the bioproduction phase. Lowering the culture pH from the optimal range (pH 6–7) to a value of 3.0 has been reported to reduce proteolysis and thus increase the yield of the functional protein [74]. However, Panjideh *et al*. [64] observed that lowering the pH provided no benefits in their case, which reminds us that fermentation yields are the result of a combination of factors. For example, very high cell densities are usually employed since the yield is roughly proportional to this parameter [20]. However, high cell density increases the concentration of extracellular proteases and may have detrimental effects on cell physiology, which in turn limits the amount of the desired product [53, 74]. Eldin *et al*. [33] have reached the conclusion that the level of expression depends on the copy number and the site of integration, as well as culture conditions, and that success could be achieved by either growing cells to a high density prior to induction or by screening larger numbers of transformants for the highest level of expression.

In terms of growth temperature, the optimum for *P. pastoris* is 28–30°C [64]. However, at this range, proteases may destroy the recombinant protein, so lower temperatures may be advisable, a suggestion with which Shi *et al*. [74] agree. They found that *P. pastoris* could be cultivated effectively in BMGY medium at 30°C to accumulate biomass,

Table 5. Expression levels for antibody and antibody fragment conformations under AOX1 control in *Pichia pastoris* using different approaches.

P. pastoris strain	Form	Vector	Titer (mg/l)	Cultivation	Reference
SMD1168	mAb	pPICZαB	36	Shake flask	[62]
Glycoengineered	mAb	pGLY2988	~1,000	Fermentation	[67]
GS115	scFv	pPIC9K	45	Fermentation	[42]
GS115	scFv	pPIC9K	4-5	Fermentation	[25]
X-33	scFv-Fc	pPICZα	~373	Fermentation	[56]
X-33	scFv	pPICZαC	25	Fermentation	[54]
KM71	$[sc(Fv)_2]_2$	pPICZαA	2-3,5	Shake flask	[39]

followed by induction at temperatures as low as 15°C, leading to reduced protease levels and greatly enhanced periods of scFv production [74]. In accordance with these results, Jafari *et al*. [47] describe temperature as one important factor to take into account; one could deviate considerably from the generally recommended conditions as far as lowering the temperature to 11°C, with very high yields of recombinant protein production [47]. Lin *et al*. [56], in the production of a Fc fusion protein, found a strong positive correlation between temperature and growth rate. Furthermore, they found that increasing the glycerol concentration in the batch phase and supplementing the medium with salts gave a higher final optical density (OD). The same results were not achieved with increased temperature and salt supplementation. However, despite the faster growth that had been observed at increased temperatures (25°C, 185 mg/l), the highest protein yield was observed without salt supplementation and at a lower temperature than used in this study (20°C, 311 mg/l), which leads to the same conclusion that lower temperatures allow more efficient protein folding and secretion [56].

Several reports have indicated that medium composition influences heterologous protein expression in yeast by affecting cell growth and viability or the secretion of extracellular proteases [74]. Inclusion of L-arginine, EDTA, and casamino acids in the BMMY induction medium led to increased production of scFv, the best result being obtained with the addition of casamino acids [74]. With L-arginine, cell growth decreased, but scFv accumulation increased [17, 74]. Reducing the concentration of basal salts in the fermenter medium allows for a decrease in salt precipitation during the preparation of cleared supernatant for downstream purification, and also the reduction or elimination from the medium of a lipid-like substance produced during the course of fermentation, which can interfere with downstream processing [8, 13, 25]. To maximize the amount of recovered protein and reduce the complexity of the procedure, purification is very important. Since purification steps are expensive, we observe a growing concern in this regard in experimental design domains. Expression systems and medium composition are some of the factors that have to be taken into account in order to reduce the complexity of the downstream procedure. Thus, affinity chromatography techniques are frequently explored in order to apply only one purification step. Such a procedure is accomplished by the fusion of poly-His tags to non-antibody regions, with a special affinity to metal ions. Indeed, centrifugation, dialysis, and metal affinity chromatography with immobilized Ni^{2+} ions as a chelating resin have been employed, with successful results at under 90% purity [33, 39, 59]. When tags should not be used, affinity ligands such as Protein G and Protein A (natural immunoglobulins ligands) are used alone or coupled with other chromatographic techniques to remove bioprocess impurities [49, 71].

Human membrane proteins. The methylotrophic yeast *P. pastoris* has been used to produce crystallization-grade proteins for several MPs, from which high-resolution 3D structures have been determined [7, 29, 70]. In fact, *P. pastoris* is able to produce proteins with all kinds of membrane-spanning topologies, including enzymes, aquaporins, and ion channels [7]. As highlighted previously by Ramón and Marin [70], the production of functional, properly folded and inserted MPs is often achieved by using alternative approaches that are almost always specific for each target MP. The use of the mammalian Kozak sequence, optimization of codon usage, coexpression of MPs with the Hac1p, N-terminal fusion of signal sequences to the MP, lower temperature cultivation, and the addition of chemical chaperones such as DMSO are some of the successful strategies that have been employed to improve MP expression in *P. pastoris* [70]. Moreover, a set of guidelines and instructions with several tips to overexpress MP in the *P. pastoris* system using GPCR as a model was recently published by Bornert *et al*. [7].

Essentially, the first choice that has to be made when designing a bioprocess based on *P. pastoris* is the expression vector, which may have important implications in the following steps. There are many commercially available vectors for the expression of foreign genes in *P. pastoris* (for a good review of the different options, please refer to [20]); some may potentially produce the protein with fusion partners such as His_6 and c-myc, and others are indicated for intracellular or secreted expression, and there are vectors in which the expression is driven by different promoters (AOX1 or GAPp).

P. pastoris is able to produce proteins both intracellularly and secreted into the culture medium. When the latter option is preferred, the presence of a signal sequence is required to target the protein to the secretory pathway [20]. A variety of secretion signal sequences including the *S. cerevisiae* α-factor prepropeptide [20, 26], acid phosphatase signal sequence [20], SUC2 gene signal sequence [63], 128 kDa pGKL killer protein [50], and hydrophobins HFBI and HFBII from *Trichoderma reesei* [51] have been applied to protein secretion in *P. pastoris*, but the *S. cerevisiae* α-factor prepropeptide has been the most common and successful strategy [20]. Indeed, the secretion of proteins can be advantageous as *P. pastoris* secretes low levels of endogenous proteins, making the target protein the majority of the total protein in the medium [20]. However, most of the fused signal sequences do not allow the secretion of the target MP into the culture medium as happens for soluble proteins, but, in some cases, it may exert a positive effect on MP yield, as previously shown for the μ-opioid receptor [70, 73].

Fundamentally, five promoters (listed in Table 2) and six strains (listed in Table 1) are used for recombinant MP production in *P. pastoris*. The choice of the promoter as well as the strain will also influence the further steps, particularly the culture conditions and choice of medium.

Regarding the successful optimization of culture conditions in order to improve target MP yield, the work developed by Yurugi-Kobayashi *et al*. [85] provides one of the most interesting examples. When expressing CHMR2 in *P. pastoris*, they obtained an almost 10-fold increase in the yield of the target MP by lowering the culture temperature to 20°C and increasing the pH to 7 or 8, when compared with the standard values of 30°C and pH 6 [70, 85]. In addition, for the HRH2R human receptor, Andre *et al*. [2] achieved a 7-fold improvement by adding specific ligands to the culture medium that act as molecular chaperones during protein folding and membrane insertion. Moreover, the use of DMSO as a chemical chaperone led to a 6-fold increase in the production of several receptors when compared with standard conditions [2, 70].

The most common methods used for *P. pastoris* cell lysis employ glass beads, microfluidizers, and a French press (Invitrogen 2005) [3, 4], which are the more suitable choices for large amount of cells. Alternatively, Shepard *et al*. [75] reported in 2002 a method for *P. pastoris* intracellular protein recovery based on cell permeabilization using *N*,*N*-dimethyltetradecylamine and Triton X-100.

Usually, MPs are produced intracellularly, in which case the membranes need to be isolated. This is accomplished by performing an ultracentrifugation step, typically at 200,000 ×*g* for 60 min at 4°C or 30 min at 4°C after cell lysis [3, 4, 7].

On the other hand, when MPs are secreted into the culture medium, an appropriate strategy has to be designed in order to recover/concentrate the target MP. Ammonium sulfate precipitation, dialysis, or pressurized cell concentrators, as have been employed in human carboxypeptidase M recovery from *Pichia* culture broth [3, 11], are some of the techniques suggested by the Invitrogen Corporation. However, these techniques share common problems; they can unfold the target protein, which cannot easily be refolded, and can also give rise to losses of MP total yield. To address this issue, strategies based on microfiltration techniques through membranes might be a good option. Also, expanded bed adsorption has been used to recover human bile salt-stimulated lipase from fermentation broth [60].

Secretion of the target MP can have a great impact on the following purification steps. The high-cost and low-yield cell disruption or refolding process could be eliminated by direct capture of the properly folded product from the culture medium [26].

Although there are many vectors that are suitable for the secretory expression of heterologous proteins in *P. pastoris*, it cannot always be achieved for membrane proteins owing to their higher hydrophobicity compared with soluble counterparts. However, an interesting strategy has been proposed by Issaly *et al*. [46] concerning wheat puroindoline-a production in *P. pastoris* using the pYAM7SP8 vector containing the acid phosphatase signal sequence [46]. In this work, they added 0.01% (v/v) Triton X-114 directly to the culture medium and found that the ratio between secreted and membrane-bound protein was inverted, since most of the puroindoline-a was present in the culture supernatant instead of being associated with the membrane, thus improving the overall productivity of the process [46]. Another advantage of adding Triton X-114 to the feeding medium can be a possible reduction in proteolysis as seen with puroindoline-a [46] and urokinase-type plasminogen activator [80].

Construction of the target MP with an affinity tag allows purification by affinity chromatography [3, 4, 28]. In fact, the application of affinity chromatography as the main tool for MP capture from total crude extract or total membrane fraction remains one of the most common and successfully employed techniques for the first step of MP purification. However, molecular exclusion and ion-exchange chromatographies have also been successfully employed [18].

The main aim of developing an expression system based on *P. pastoris* strains for overexpression of an MP is to obtain recombinant MP of high quantity and purity that allows for structural and functional studies. To date and according to the Membrane Protein Data Bank, 49 structures of membrane proteins have been solved using *P. pastoris* as the host for MP production [69], making this microfactory a good option for performing heterologous protein expression for structural studies of MP [7].

Conclusion and Future Perspectives

In this review, we present an overview of *P. pastoris* as an expression system for heterologous protein production as well as strategies specifically adapted to Ab and MP overexpression. In fact, the growing number of human MP structures that have been solved using *P. pastoris* makes this microsystem one of the most efficient hosts for producing large amounts of MP for structural proteomics.

Although general strategies have already been described for Ab and MP production in *P. pastoris*, each protein may require the optimization of different parameters and, therefore, this process is sometimes based on trial-and-error experiments. However, is it possible to obtain extremely hydrophobic MP in the extracellular medium and avoid the usual first step of purification as happens for soluble proteins? Promising answers to this issue may consist in MP secretion as membrane vesicles or fused with solubility tags. Concerning Ab and Ab fragment production, relevant achievements have been made using improved *P. pastoris* strains and optimized bioprocesses. However, full-length

Ab folding and secretion still present an obstacle between successful Ab overexpression and Ab misfolding, so further studies are required. Moreover, correct antibody glycosylation has been revealed to be of extreme importance to antibody function, so it requires much further research. Can a full antibody be correctly glycosylated and flooded without the time-consuming process of developing new strains? This is a question that still has no answer and needs further investigation.

Moreover, an important bottleneck affecting protein production in *Pichia* that has still room for improvement remains the different glycosylation patterns provided by the organism. In addition, despite the AOX1 promoter being one of the best characterized and most efficient promoters driving protein expression in *Pichia*, the availability of additional protocols using other promoters to circumvent the disadvantages of using methanol as an inducer would be desirable. In terms of recombinant multi-subunit protein complexes, production in *P. pastoris* can also be accomplished. Through the coexpression of multiple protein chains or subunits in a single recombinant genetic construct that includes at least two expression cassettes, a biologically active target protein containing multiple chains or subunits can be synthesized in *P. pastoris*.

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