**MINI-REVIEW**

**Pichia pastoris: A highly successful expression system for optimal synthesis of heterologous proteins**

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**Abstract**
One of the most important branches of genetic engineering is the expression of recombinant proteins using biological expression systems. Nowadays, different expression systems are used for the production of recombinant proteins including bacteria, yeasts, molds, mammals, plants, and insects. Yeast expression systems such as *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Pichia pastoris* (*P. pastoris*) are more popular. *P. pastoris* expression system is one of the most popular and standard tools for the production of recombinant protein in molecular biology. Overall, the benefits of protein production by *P. pastoris* system include appropriate folding (in the endoplasmic reticulum) and secretion (by Kex2 as signal peptidase) of recombinant proteins to the external environment of the cell. Moreover, in the *P. pastoris* expression system due to its limited production of endogenous secretory proteins, the purification of recombinant protein is easy. It is also considered a unique host for the expression of subunit vaccines which could significantly affect the growing market of medical biotechnology. Although *P. pastoris* expression systems are impressive and easy to use with well-defined process protocols, some degree of process optimization is required to achieve maximum production of the target proteins. Methanol and sorbitol concentration, Mut forms, temperature and incubation time have to be adjusted to obtain optimal conditions, which might vary among different strains and externally expressed protein. Eventually, optimal conditions for the production of a recombinant protein in *P. pastoris* expression system differ according to the target protein.

**KEYWORDS**
expression system, optimization, *Pichia pastoris*, recombinant proteins, subunit vaccines

**1 | INTRODUCTION**

Nowadays, biological expression systems are used for the production of heterologous proteins in industrial and medical fields. These proteins can consist of recombinant vaccines, drugs, and agricultural products (Gomes, Byregowda, Veeregowda, & Balamurugan, 2016). One of the obstacles in this area is the production of a large number of recombinant proteins in both the medical field and research. Therefore, researchers apply both prokaryotic and eukaryotic cells to overcome the difficulties associated with the production of recombinants proteins (Balamurugan, Sen, Saravanan, & Singh, 2006). Already used expression systems include: bacteria, yeasts, molds, mammals, plants, and insects. Prokaryotic cells such as Gram-negative bacteria are among the first cells used in engineering genetic technology. One of the most important cells is *Escherichia coli*, that has been widely used for cloning recombinant DNA and subsequently, for the production of heterologous proteins (Baneyx, 1999). Bacterial expression system has several advantages including rapid multiplication, simple and inexpensive nutritional requirements, high-level expression, and fast and easy transformation process. However, this...
TABLE 1 Basic characteristics of different host systems for the expression of recombinant proteins

| Characteristics                        | Escherichia coli | Pichia pastoris | CHO cell |
|----------------------------------------|------------------|----------------|---------|
| Doubling time                          | 30 min           | 60–120 min     | 24 hr   |
| Cost of growth medium                  | Low              | Low            | High    |
| Complexity of growth medium            | Minimum          | Minimum        | Complex |
| Expression level                       | High             | Low to high    | Low to moderate |
| Extracellular expression               | Secretion to periplasm | Secretion to medium | Secretion to moderate |
| Protein folding                        | Refolding usually required | Refolding may be required | Proper folding |
| N-linked glycosylation                 | None             | High mannose   | Complex |
| O-linked glycosylation                 | No               | Yes            | Yes     |
| Phosphorylation & acetylation          | No               | Yes            | Yes     |
| Drawback                               | Accumulation of LPS | Codon bias     | Contamination with animal viruses |

Abbreviations: CHO, Chinese hamster ovary; LPS, lipopolysaccharide.

cell factory has some limitations such as intracellular aggregation and misfolding of heterologous proteins, production of lipopolysaccharide, lack of posttranslational modification, and protein degradation due to proteases (Rosano & Ceccarelli, 2014). Another part of expression systems is the eukaryotic cells which include mammalian and yeast cells. The most common mammalian cell lines are Chinese hamster ovary (CHO) cells. Currently, CHO cells are used to produce biopharmaceutical compounds, monoclonal antibodies, and Fc-fusion proteins. Apart from this, baby hamster kidney, human embryonic kidney 293 and NS0, SP2/0 (mouse-derived myeloma) cell lines have also received legal permissions (Picanço-Castro, Cristina Correa de Freitas, Bomfim, & Maria de Sousa Russo, 2014). Significant advantages of this system include proper protein folding, posttranslational modifications, and glycosylation of recombinant proteins in the correct sites which is important for protein stability (Khan, 2013).

Besides, mammalian expression systems grow slowly and the relevant nutrient requirement is costly. On the other hand, potential contamination of culture medium with some viruses has limited its use in large-scale production (Yin, Li, Ren, & Herrler, 2007). Yeasts are other eukaryotic cells that are widely used for the expression of several proteins in vaccine and pharmaceutical production. The mechanism of protein expression in these microorganisms is close to the ones in mammalian cells. Compared with bacteria, yeast cells have significant advantages including growth speed, posttranslational modification, secretory expression, and easy genetic manipulation. Furthermore, linearized foreign DNA can be inserted in a chromosome in high efficiency via cross recombination phenomena to generate stable cell lines (Daly & Hearn, 2005). Among yeast cells, Saccharomyces cerevisiae is used in the manufacture of hepatitis B and human papillomavirus vaccines, both of which produce a protective immune response against wild-type viruses (Bill, 2015). The expression proteins in S. cerevisiae are often N and O-glycosylated, which may affect protein immunogenicity (Rasala & Mayfield, 2015).

In recent years, to solve the problem of protein expression, methylotrophic yeasts such as Hansenula polymorpha and Pichia pastoris (P. pastoris; syn. Komagataella phaffii) have been developed. Among these, P. pastoris has become the most popular for its cost and expression host system. This microorganism can produce high yields of recombinant proteins with the high similarity of glycosylation to the mammalian cells (Balamurugan, Reddy, & Suryanarayana, 2007). Overall, the benefits of protein production by P. pastoris system include appropriate folding (in the endoplasmic reticulum [ER]) and secretion (by Kex2 as signal peptidase) of recombinant proteins to the external environment of the cell (S. Yang et al., 2013). Given the fact that some proteins produced by their original host are secreted out of the cell; P. pastoris is suitable for the production of recombinant proteins since it is equipped with a secretion system (Ahmad, Hirz, Pichler & Schwab, 2014). The basic characteristics of different host systems for the expression of recombinant proteins are summarized in Table 1.

2 | BACKGROUND OF P. PASTORIS AS A EUKARYOTIC EXPRESSION SYSTEM

Historically, P. pastoris yeast was first isolated from the exudates of a chestnut tree in France and was named Zygosaccharomyces pastoris by Guillermond (Zahr, Peña, Mattanovich, & Gasser, 2017). Yamada et al. then categorized the organism to a novel genus, Komagataella or Pichia (Naumov, Naumova, & Boundy-Mills, 2018). It has been proven that P. pastoris, as an engineered methylotrophic microorganism, can use methanol as sole carbon and energy source (Cereghino, Cereghino, Ilgen & Cregg, 2002). Unlike, Y-11430 strain (wild-type), which is not used for protein expression, GS115 is one of the most popular strains used as an important expression system particularly in industry and medicine fields (Julien, 2006). P. pastoris GS115 strain has two encoding genes (AOX1 and AOX2) of alcohol oxidase (AOX) enzyme. In the presence of methanol, the transcription of these genes is induced and finally produces a high amount of AOX enzyme (Vanz et al., 2012). Although both genes are used for the production of an AOX enzyme, AOX1 produces more enzyme. Therefore, by knocking out the AOX1 gene, the growth on methanol is slowed down drastically. This phenotype is called methanol utilization slow (Mut−). A knockout of the AOX2 gene will not decelerate growth on methanol.
and the growth rates are comparable to methanol utilizing plus (Mut+) phenotype (wild-type). However, by knocking out both genes, the strains are unable to grow on methanol (methanol utilizing minus [Mut-]; Cámara et al., 2017). In the KM71 strain, as a derivative of GS115, aox1 gene has been deleted; therefore, this strain is known as Mut strain (Charoenrat et al., 2013). Older strains such as KM7121, MC100-3, and MC101-1, cannot use methanol as a food source (Mut-), because no AOX genes were detected in these strains and therefore they were unable to grow in the presence of methanol (Cregg, Madden, Barringer, Thill, & Stillman, 1989).

3 THE CHARACTERISTIC FEATURES OF P. pastoris EXPRESSION SYSTEM

3.1 Advantages and drawbacks of the P. pastoris expression system

Pichia expression system has advantages for the expression of different recombinant proteins. As noted, P. pastoris is a methylo-trophic yeast which is known as a recombinant expression host system. One of the advantages of the Pichia system is its high similarity with advanced eukaryotic expression systems such as CHO cell lines. This yeast system is inexpensive, it also has relatively rapid expression times, cotranslational and posttranslational processing. By the use of industrial bioreactors, proteins of interest can be produced on a large scale from small culture volumes. Recently, studies have shown that the Pichia expression system is unique in the production of membrane proteins including calcium and potassium channels, nitrate and phosphate transporter, and histamine H1 receptor (Byrne, 2015). Furthermore, P. pastoris is a suitable microorganism in the secretory production of recombinant proteins directly into the supernatant of the culture medium. In the P. pastoris expression system due to its limited production of endogenous secretory proteins, the purification of recombinant protein is easy (Tachioka et al., 2016).

Another advantage of P. pastoris as a protein production host is its ability to perform posttranslational modifications such as O- and N-linked glycosylation and disulfide bond formation. Many therapeutic proteins are glycoproteins and require the attachment of carbohydrate structures to the protein backbone (glycosylation) to allow for correct folding, solubility, stability, and proper biological activity (Cereghino et al., 2002). There are two main types of glycosylation in yeast cells (N-linked and O-linked glycosylation) that takes place in the ER or Golgi apparatus. In yeast, the structure of N-linked glycans is typical of the hypermannose type, whereas, in humans, complex and hybrid structures are of the predominant type. For providing N-linked glycosylation, oligosaccharides are attached to the amide nitrogen of asparagine (Asn) residue through an N-glycosyl linkage within the consensus sequence Asn-X-Ser/Thr (where X is any amino acid except proline). For yielding the O-linked type of glycosylation, oligosaccharides are attached to serine or threonine residues through a glycosidic linkage. The O-linked saccharides are typically much smaller than N-linked saccharides (<5 residues). N-glycosylation in S. cerevisiae is characterized by hypermannosylation with α-1,2-, α-1,6-, and α-1,3-mannosyltransferases (Figure 1b). In comparison to S. cerevisiae, P. pastoris may have an advantage in the glycosylation of secreted proteins because it may not hyperglycosylated. In P. pastoris N-glycans (Man8-14GlcNAc2) are frequently shorter than the long oligosaccharide chains (Man >5GlcNAc2) found in the S. cerevisiae. Besides, S. cerevisiae core oligosaccharides have terminal α-1,3 glycan linkages whereas P. pastoris does not. Unlike S. cerevisiae, P. pastoris does not hyperglycosylated therapeutic proteins and does not contain potentially immunogenic terminal α-1,3-linked mannosides (Figure 1c). It is believed that the α-1,3 glycan linkages in glycosylated proteins produced from S. cerevisiae are primarily responsible for the hyper-antigenic nature of these proteins making them particularly unsuitable for therapeutic use. Moreover, very little O-linked glycosylation has been observed in P. pastoris (Bretthauer & Castellino, 1999). Due to its attractive characteristics for heterologous protein production (low incidence of hyperglycosylation), P. pastoris is an interesting organism for the production of therapeutic glycoproteins. The N-glycosylation plays an important role in achieving complete biological activities of therapeutic proteins such as interferon, erythropoietin, and monoclonal antibodies. The N-glycosylation pathway in mammalian cells consists of the addition of one or more N-acetylgalactosamine (GlcNAc) residues followed by the sequential addition of galactose (Gal) and sialic acid which creates a complex type of N-glycans (Figure 1a). While, hypermannosylation of recombinant protein in the P. pastoris expression system can lead to immunologic reaction and decreased serum half-life (Laukens, De Wachter, & Callewaert, 2015). Recently, new strategies have been designed to engineer the P. pastoris N-glycosylation pathway. With Pichia GlycoSwitch, the yeast’s own hyperglycosyl N-glycans are switched to the more human biantennary complex-type N-glycans. In glycoengineering strategy by disruption of an endogenous glycosyltransferase gene (OCH1) and introducing heterologous enzyme activities, Pichia has been engineered to produce human-like glycoproteins (Jacobs, Gey sens, Vervecken, Contreras, & Callewaert, 2009). Indeed, the first step of humanizing Picha glycosylation or GlycoSwitch® strategy is the knockout of the gene coding for α-1,6-mannosyltransferase (OCH1) which is responsible for the initiation of hypermannosylation. The next step in this process is the co-overexpression of several glycosyltransferases or glycosidase to produce human-like glycoproteins. Commonly employed Picha GlycoSwitch® strains (BioGrammatics, Carlsbad, CA) are SuperMan5, SuperMan5HIS, SuperMan5Sep4+, SuperMan5(aox1+, Muts), SuperMan5( pep4+, prb1+) and SuperMan5( pep4+, sub2+). These strains, OCH1 inactivated strains that express an ER-targeted α-1,2-mannosidase, express target protein with a mannos-5 structure at N-linked site (Ahmad et al., 2014; Figure 1d). The SuperMan5 strain is commonly used to express of vaccine antigens. Moreover, by introducing heterologous enzyme activities and adding of N-acetyl glucose amine (GN) or Gal, the SuperMan5 strain has been engineered to produce human-like glycoproteins (M5GN and M5GNGal strains). M5GN and M5GNGal strains (BioGrammatics) are commonly used to express vaccine antigens, cytokines, and antibodies.
However, like other expression systems, this eukaryotic system has some disadvantages. In the transformation stage, unlike the bacterial system, competent cells require large (μg-level) amounts of the plasmid. The number of *E. coli* transformants (10^8–10^11) is higher than *P. pastoris* transformants (10^3–10^5) per μg of DNA (S. Wu & Letchworth, 2004). The production of recombinant protein in this system is regulated through two promoters: Promoter of glyceraldehyde-3-phosphate dehydrogenase (P_GAP) and promoter of AOX (P_AOX1). Despite several
advantages, both promoters have no tenability (Rajamanickam, Metzger, Schmid, & Spadut, 2017). Protein production in yeast systems is dependent on the consumption of methanol. Based on EasySelect™ Pichia Expression Kit (Catalog no. K1750-01, Invitrogen, Carlsbad, CA), at least 0.5% concentration of methanol is required for the expression of recombinant proteins. The production could reach to the maximum level of 2–2.5% (wt/vol) of methanol (Z. Wang et al., 2010). Normally, the concentration of methanol up to 5% is tolerable for the organisms, but high levels of methanol concentrations (above 5%) are very toxic for cell viability and can stop the production process (Santoso, Herawati, & Rubiana, 2012). Another limitation in the Pichia system is the presence of a few selectable markers for \( P.\ pastoris \) transformation. Selectable marker genes include \( \text{his4} \), \( \text{arg4} \), and \( \text{Shble} \) (needed to resist against Zeocin antibiotic) (Cereghino & Cregg, 1999). A common occurrence in this system is the contamination of expressive broth culture with saprophytic bacteria and fungi. The secreted proteases of these microorganisms potentially hydrolyze the secreted proteins to the supernatant (Stewart, 2015). Indeed, one of the problems in the \( P.\ pastoris \) expression system is the destruction of proteins produced by proteases. New \( P.\ pastoris \) strains such as SMD1163 (\( \text{his4 pep4 prb1} \)), SMD1165 (\( \text{his4 pep4} \)), SMD1168 (\( \text{his4 pep4} \)), BG21, and \( \text{Pichia pink} \) have no protease; therefore, the degradation of a secreted protein is prevented. In these strains, to achieve high product yields and the quality of recombinant proteins, the genes encoding of proteinase A (\( \text{pep4} \)) and proteinase B (\( \text{prb1} \)) have been disrupted (Safder, Khan, Islam, & Kazim, 2018).

3.2 | General topics for cloning and expression in \( P.\ pastoris \)

The expression of any recombinant gene in \( P.\ pastoris \) has three phase: (a) Cloning of a new gene into a suitable expression vector, (b) insertion of the cloned vector into \( P.\ pastoris \) host genome; and (c) trial of the potential different strains for the expression of the recombinant integrated gene (Macauley-Patrick, Fazenda, McNeil, & Harvey, 2005). Based on EasySelect™ Pichia Expression Kit (Invitrogen), to increase the efficacy of external DNA integration into the \( Pichia \) genome, first, the cloned vector should be linear with restriction enzymes such as \( \text{SacI} \), \( \text{Pmel} \), and \( \text{BstX I} \). Then, linear DNA should be inserted into the competent cells by electroporation. The entered gene is integrated into the cell genome via the crossover recombination phenomenon (Figure 2), and consequently, recombinant cells are formed. In most cases, a single crossover occurs in the genome, but multiple insertions occur in 1–10% of the cases (Kit).

The expression vectors in the \( P.\ pastoris \) expression system are one of the major components of this system. These vectors are composed of three sequences: Promoter sequence (most often \( AOX1 \)) in 5′ region; transcriptional termination sequence in 3′ region which is essential in the processing and polyadenylation of messenger RNAs; and one sequence that contains single or multiple cloning sites, necessary for the insertion of the gene of interest. The episomal vectors can replicate either autonomously in the cytoplasm or as part of a chromosome. But the vectors used in \( P.\ pastoris \) have no stable episomal status; therefore, they should first be linearized with enzymes and then be integrated into the \( P.\ pastoris \) chromosome (Li et al., 2007). Like \( E.\ coli \), expression vectors in \( P.\ pastoris \) are shuttle vectors that is they can propagate in two different host species. The vectors also contain one drug resistance genes such as \( \text{Kan} \), \( \text{Shble} \), \( \text{Bsd} \), \( \text{Amp} \), or \( \text{FLD1} \), which are resistant to geneticin, zeocin, blasticidin, ampicillin, and formaldehyde, respectively (Ilgen, Lin-Cereghino, & Cregg, 2005). Common plasmids used in the \( P.\ pastoris \) expression system to produce extracellular and intracellular proteins are listed in Tables 2 and 3, respectively.

**FIGURE 2** Crossover recombination phenomenon in the \( Pichia\ pastoris \) genome. Following the electroporation process of competent yeast cells, cloned linear vectors are inserted into the electroporated cells. Crossover recombination occurs between 5′ promoter (5′ \( P_{AOX1} \)) of vector and \( AOX1 \) region of \( P.\ pastoris \) genome. Consequently, cloned cells with a recombinant genome are formed. \( AOX1 \), alcohol oxidase 1; \( TT \), transcription termination region.
3.3 Subunit vaccines expressed in Pichia pastoris

Recombinant protein therapeutics is a growing market within the human medical biotechnology industry. The majority of all approved biopharmaceuticals are protein-based and include blood factors, anticoagulants, hormones, hematopoietic growth factors, interferons, interleukins, vaccines, and monoclonal antibodies. Vaccine production is one of the major protective strategies against infectious diseases. In general, vaccines used for all pathogens are divided into three groups: live attenuated, inactivated/killed, and subunit vaccines (Schiller & Lowy, 2014). After the injection of live attenuated vaccines, microorganisms start to replicate in the injection area and could induce strong immune responses. However, reversion of attenuated pathogen to its wild-type strain especially in immunocompromised individuals has restricted the application of these vaccines (Minor, 2015). Although, inactivated/killed vaccines are safe, they are relatively less effective in the induction of strong immune responses. Therefore, these vaccines should be injected in multiple doses along with suitable adjuvants (S. Lee & Nguyen, 2015). Subunit vaccines consist of one or more immunodominant antigens of pathogens that can be produced in eukaryotic and prokaryotic systems. Unlike live attenuated and killed/inactivated vaccines, subunit vaccines are completely safe and cost-effective. Recently, subunit vaccines have been replaced with other forms of vaccines.

### Table 2: Common Pichia pastoris expression vectors for the production of secretory proteins

| Vector name | Marker gene | Used strain | Recombinant protein | Reference |
|-------------|-------------|-------------|---------------------|-----------|
| pPIC9K      | His4, Kan, Amp | GS115       | Xylanase            | Fu, Zhao, Xiong, Tian, and Peng (2011) |
|             |             | GS115       | Porcine circovirus type 2 | Tu et al. (2013) |
|             |             | GS115       | Endo-1,3(4)-b-d-glucanase | X. Chen et al. (2012) |
|             |             | GS115       | Staphylokinase      | Apte-Deshpande, Mandal, Soorapaneni, Prasad, Kumar, and Padmanabhan (2009) |
| pPICZα      | Shble       | SMD1168     | Human chitinase     | Goodrick et al. (2001) |
|             |             | GS115       | Human topoisomerase I | Chan et al. (2018) |
|             |             | GS115       | Human interferon gamma | Prabhu, Veeranki, and Dsilva (2016) |
|             |             | X-33        | C-reactive protein  | J. Li et al. (2017) |
|             |             | SuperMan5   | Insulin             | Baishen et al. (2016) |
|             |             | X-33        | Human RNase4        | Birdiya and Chang (2017) |
| pHIL-S1     | His4, Amp   | GS115       | Rabies virus glycoprotein | Ben Azoun, Belhaj, Géorgin, Gasser, and Kallel (2016) |
|             |             | GS115       | Rhizopus oryzae Lipase | Satomura, Kuroda, and Ueda (2015) |
|             |             | KM71        | Camel lactoferrin   | Chahardooli, Niazi, Aram, and Sohrabi (2016) |
| pGAPZα      | Shble       | GS115       | Acyl homoserine lactonase | J. Wu et al. (2016) |
|             |             | SMD1168     | Variable lymphocyte receptor B | J. S. Lee et al. (2018) |
|             |             | X-33        | Human gastric lipase | Sams et al. (2017) |
| pJL-SX      | FLD1, Amp   | MS105       | Formaldehyde dehydrogenase | Sunga and Cregg (2004) |
| pBLHIS-SX   | His4, Amp   | JC100       | Leukocyte protease inhibitor | Li et al. (2010) |

### Table 3: Common Pichia pastoris expression vectors for the production of intracellular proteins

| Vector name | Marker gene | Used strain | Recombinant protein | Reference |
|-------------|-------------|-------------|---------------------|-----------|
| pPIC3.5K    | His4, Kan, Amp | KM71       | Maltooligosyltrehalose synthase | Han, Su, Hong, Wu, and Wu (2017) |
|             |             | SMD1168     | Camellia sinensis heat shock protein | Wang, Zou et al. (2017) |
|             |             | GS115       | Pleurotus ostreatus laccases | Zhuo et al. (2018) |
|             |             | GS115       | Rhizopus oryzae Lipase | Jiao, Zhou, Su, X, and Yan (2018) |
|             |             | GS115       | HSA/GH fusion protein | M. Wu et al. (2014) |
| pPICZ       | Shble       | X-33        | Aquaporin            | Nordén et al. (2011) |
|             |             | KM71        | Membrane protein     | J. Y. Lee, Chen, Liu, Alba, and Lim (2017) |
|             |             | KM71        | Dengue virus envelope glycoprotein | Khetarpal et al. (2017) |
| pHIL-D2     | His4, Amp   | GS115       | Prostaglandin H synthase-2 | Kukk and Samel (2016) |
|             |             | GS115       | CatA1 and SODC       | Mina et al. (2017) |
|             |             | KM71        | Rhodococcus nitrile hydratase | Pratush, Seth, and Bhalla (2017) |
|             |             | GS115       | Feline serum albumin | Yokomaku, Akiyama, Morita, Kihira, and Komatsu (2018) |
| pGAPZ       | GS115       | GTPase RabA4c | Glöckner and Voigt (2015) |
|             | GS115       | Xylose isomerase | Li, Sun, Chen, Li, and Zhu (2015) |
|             | GS115       | β-Galactosidase | H. Sun et al. (2017) |
| pJL-IX      | FLD1, Amp   | MS105       | Formaldehyde dehydrogenase | Sunga and Cregg (2004) |
| pBLHIS-IX   | His4, Amp   | KM71        | L1-L2 proteins of HPV virus type 16 | Bredell, Smith, Görgens, and van Zyl (2018) |

Abbreviation: HPV, human papillomavirus.
Also, for the production of recombinant subunit vaccines, *P. pastoris* is more famous than other expression systems (Wang, Jiang, & Wang, 2016). Subunit vaccines often suffer from poor immunogenicity and require certain helper molecules known as adjuvants to induce or enhance an appropriate immune response to the antigen. T-cell activation is crucial in inducing protective immune responses (Todryk, 2018). Antigens mannosylated by *P. pastoris* have shown to have enhanced antigen presentation and T-cell activation properties compared with their nonglycosylated counterparts. Therefore, glycoproteins derived from *P. pastoris* have the potential to function as adjuvants. The increased immunogenicity of mannosylated glycoproteins derived from *P. pastoris* have shown to have enhanced antigen presentation and T-cell activation properties compared with their nonglycosylated counterparts. Some of the new recombinant subunit vaccines that have been expressed in *P. pastoris* are listed in Table 4.

### Table 4: Recombinant subunit vaccine expressed in *P. pastoris*

| Construct name | Used strain | Used vector | Targeted disease | Reference |
|----------------|-------------|-------------|------------------|-----------|
| PIMP-V1 and PIMP-V2 | KM71 | pPICZαA | Malaria | Spiegel et al. (2015) |
| P1-3CD | PichiaPink | pPink-HC | Hand-foot-mouth disease | C. Zhang et al. (2015) |
| DENV-3 E | KM71 | pPIC-Zα | Dengue | Tripathi et al. (2015) |
| CHIKV-C-E3-E2-6K-E1 | GS115 | pPIC9K | Chikungunya | Saraswat et al. (2016) |
| Gp350 | GS115 | pPICZαA | EBV infection | Wang et al. (2016) |
| RBD219-N1 | X-33 | pPICZαA | SARS | W.-H. Chen et al. (2017) |
| VP2-VP5-Fc | GS115 | pPIC9K | Infectious bursal | H. Wang et al. (2017) |
| F protein | GS115 | pPICZαA | Newcastle | Kang et al. (2016) |
| OmpA | GS115 | pPIC9K | *P. mirabilis* infection | Y. Zhang et al. (2015) |
| BoNT Hc | X-33 | pPICZ-A | Botulism | Webb et al. (2017) |
| Tc52 | GS115 | pPICZαA | Chagas | Matos, Alberti, Morales, Cazorla, and Malchiodi (2016) |
| Apa | GS115 | pPIC9K | Tuberculosis | S. Wang, Wang, Wang, Chen, and Kong (2018) |
| HBHA | GS115 | pPIC9K | Tuberculosis | Teng, Chen, Zhu, and Xu (2018) |
| CFP10-Fcγ2a | GS115 | pPICZαA | Tuberculosis | Baghani et al. (2017) |
| ESAT6-CFP10-Fcγ2a | GS115 | pPICZαA | Tuberculosis | Farsiani et al. (2016) |
| ESAT6-Fcγ2a | GS115 | pPICZαA | Tuberculosis | Kebrinaei et al. (2016) |
| CFP10-HspX-Fcγ2a | GS115 | pPICZαA | Tuberculosis | Mosavat et al. (2016) |
| ESAT6-HspX-Fcγ2a | GS115 | pPICZαA | Tuberculosis | Soleimanpour et al. (2015) |
| Glycoprotein D | GS115 | pPIC9K | HSV-2 infection | Wang, Jiang et al. (2017) |
| OmpA-Fc | GS115 | pPIC9K | Bordetellosis | Dong et al. (2016) |

Abbreviations: EBV, Epstein-Barr virus; SARS, severe acute respiratory syndrome.

### 4.1 The impact of Mut forms and methanol on protein concentration

Methanol is the principal carbon source and gene expression induction agent in most *P. pastoris* fermentation strategies. Monitoring methanol levels during fermentation enables cell growth and optimizes productivity, whilst methanol toxicity is avoided. Methanol is used for both biomass growth and the production of protein in *P. pastoris*. Based on the utilization of methanol, *P. pastoris* strains are divided into three phenotypes: Mut⁺ strains with both AOX1 and AOX2 genes in their chromosomes; Mut⁻ strains with only AOX2 gene; and Mut⁺ mutant strains without any AOX genes (Anggiani, Helianti, & Abinawan-to, 2018). Therefore, the growth rate of different strains depends on their Mut forms. Mut⁺ strains can grow in a wide range of microm from 0.028 to 0.154 per hour, while Mut⁻ strains at a narrow range of 0.011–0.035 per hour (Looser et al., 2015). As noted above, based on EasySelect™ Pichia Expression Kit, at least 0.5% concentration of methanol is necessary for the production of recombinant protein, and to fully express the protein, the concentration must be at most 2–2.5% (wt/vol). Besides, high levels of methanol (concentrations above 5%) are toxic to the cells, leading to the accumulation of formaldehyde and hydrogen peroxide, and consequently the death of the cells. On the other hand, low levels of methanol trigger proteolytic degradation of
TABLE 5 Optimum concentration of methanol for the expression of recombinant protein

| Used strain | Mut form | Optimum methanol concentration (%) | Reference |
|-------------|----------|-------------------------------------|-----------|
| GS115       | Mut+ 2   | Z. Wang et al. (2010)               |           |
| X-33        | Mut+ 2.5 | Santoso et al. (2012)               |           |
| GS115       | Mut+ 3   | Anggiani et al. (2018)              |           |
| GS115       | Mut+ 2   | Farsiani et al. (2016)              |           |
| GS115       | Mut+ 2.5 | Mosavat et al. (2016)               |           |
| GS115       | Mut+ 2   | Soleimanpour et al. (2015)          |           |
| X-33        | Mut+ 1   | Tyagi et al. (2016)                 |           |
| X-33        | Mut+ 0.5 | T. Zhao et al. (2018)               |           |
| GS115       | Mut+ 2   | Cunha, Gama, Cintra, Bataus, and Ulhoa (2018) | |
| GS115       | Mut+ 1   | Camattari et al. (2016)             |           |
| GS115       | Mut+ 0.5 | J. Wang et al. (2017)               |           |
| KM71        | MutS 1   | Han et al. (2017)                   |           |
| GS115       | Mut+ 0.5 | Apte-Deshpnade et al. (2009)        |           |
| GS115       | Mut+ 1   | Dehnavi, Siadat, Roudsari, and Khajeh (2016) | |
| X-33        | Mut+ 0.5 | Jain, Kumar, Bhardwaj, and Kuhad (2018) | |
| GS115       | Mut+ 2   | Farsiani et al. (2016)              |           |
| GS115       | Mut+ 2.5 | Soleimanpour et al. (2015)          |           |
| GS115       | Mut+ 2   | Mosavat et al. (2016)               |           |

Abbreviation: Mut+, methanol utilizing plus.

The impact of sorbitol and temperature on protein concentration

In the P. pastoris expression system, one of the most well-known carbon sources which can be used with methanol is sorbitol. Sorbitol does not induce or repress AOX promoters, hence using sorbitol instead of glycerol in mixed substrate methods could reduce cell growth rate, and increase specific product formation rates. The use of mixed substrates increases productivity and cell density and reduces induction time (Orman, Calik, & Ozdamar, 2009). Moreover, cofeeding of sorbitol with methanol reduces the toxic effects of intermediate metabolites and oxygen consumption. However, sorbitol has a negative impact on the specific activity of the AOX1 promoter (Çelik, Çalik, & Oliver, 2009). Several studies revealed that methanol/sorbitol cofeeding increases the expression of the recombinant proteins (Gao et al., 2012; Z. Wang et al., 2010; T. Zhu et al., 2011). The results obtained by Azadi, Mahboubi, Naghdil, Solaimanian, and Mortazavi (2017) showed that sorbitol at a concentration of 50 g/L (5%) could significantly increase the expression of recombinant protein. Moreover, according to the results of other studies, 2.5% methanol with 1% sorbitol (Mosavat et al., 2016), 2% methanol with 0.5% sorbitol (Farsiani et al., 2016), and 2% methanol with 1% sorbitol (Soleimanpour et al., 2015) were considered optimal for the highest recombinant protein production in shake flask culture. Therefore, the presence of other carbon sources such as glycerol and sorbitol can be beneficial to increase protein production. Since the AOX1 promoter is repressed severely by glycerol, sorbitol is considered an appropriate replacement (M. Gao & Shi, 2013).

The required growth temperature for P. pastoris is 28–30°C. Temperatures above 32°C could be detrimental to protein expression induction and can even cause cell death. Several studies revealed that by lowering the cultivation temperature from 30°C to 20°C, protein production can be improved according to higher yeast cell viability, decreased folding stress and lower proteolytic activity against the target protein (Dragosits et al., 2009; Gao et al., 2015; Gasser et al., 2007; Li et al., 2001; Santoso et al., 2012). The decreased synthetic rate of target protein in low-temperature cultivation causes ER stress reduction, preserves the folding capacity of the ER and enhances cell viability (Zhong et al., 2014).

4.3 | The impact of expression time on protein concentration

Incubation time is one of the most critical factors for acquiring the highest protein expression level in the P. pastoris expression system. In the P. pastoris expression system, production time is relatively long (about 100 hr). The incubation time is related to the number of yeast cells and the degree of target protein degradation. Santoso et al. (2012) showed that the highest growth of P. pastoris cells was at 96 hr incubation time, whereas the highest protein expression occurred at 48 hr. This suggests that it is very possible that longer incubation time may cause more proteolytic digestion of the expressed protein. Moreover, other studies discussed that optimal protein expression occurred at 72 to 96 hr incubation time (Farsiani et al., 2016; Soleimanpour et al., 2015).

5 | OTHER BIOMOLECULES PRODUCED BY P. pastoris

The P. pastoris has also been established as a versatile cell factory for the production of thousands of biomolecules both on a laboratory and industrial scale. Some of the new recombinant biological molecules that have been expressed in the P. pastoris system are listed in Table 6.

...
| Product                        | Used strain | Used vector | Usage                           | Reference                                                                 |
|-------------------------------|-------------|-------------|---------------------------------|---------------------------------------------------------------------------|
| Lycopene and β-carotene       | X-33        | pGAPZA      | Feed supplements                | Araya-Garay, Feijoo-Siota, Rosa-dos-Santos, Veiga-Crespo, and Villa (2012) |
| Plectasin                     | X-33        | pPICZαA     | Antibacterial peptide           | J. Zhang et al. (2011)                                                    |
| Bovine lactoferrin            | KM71H       | pJ902       | Transferrin and antibacterial protein | Iglesias-Figueroa et al. (2016)                                            |
| Bovine IFN-α                   | GS115       | pPIC9K      | Prevention and therapy of viral diseases | Tu et al. (2016)                                                          |
| Apidaecin                     | SMD1168     | pPIC9K      | Antibacterial peptide           | X. Chen et al. (2017)                                                     |
| hPAB-β                        | GS115       | pPIC9K      | Antibacterial peptide           | Z. Chen et al. (2011)                                                     |
| Tachyplesin I                 | GS115       | pGAPZaB     | Antibacterial peptide           | H. Li et al. (2019)                                                       |
| Snakin-1                      | GS11        | pPIC9       | Antimicrobial peptide           | Kuddus et al. (2016)                                                      |
| PAF102                        | X-33        | pGAPZA      | Antifungal peptide              | Popa, Shi, Ruiz, Ferrer, and Coca (2019)                                  |
| P. sativum defensin 1         | GS115       | pPIC9K      | Antifungal peptide              | Cabral, Almeida, Valente, Almeida, and Kurtenbach (2003)                  |
| Class I chitinase             | KM71H       | pPICZαA     | Antifungal peptide              | Landim et al. (2017)                                                      |
| Ch-penaeidin                  | KM71H       | pPIC9K      | Antimicrobial peptide           | L. Li et al. (2005)                                                      |
| Hispidalin                    | GS115       | pPICZαA     | Antimicrobial peptide           | Meng et al. (2019)                                                       |
| Fowlidicin-2                  | X-33        | pPICZαA     | Antimicrobial peptide           | Xing et al. (2016)                                                       |
| Parasin I                     | X-33        | pPICZαA     | Antimicrobial peptide           | H. Zhao et al. (2015)                                                     |
| CecropinA-thanatin            | X-33        | pPICZαA     | Antimicrobial peptide           | Z. Liu et al. (2018)                                                     |
| Type I collagen               |              |             | Connective tissue              | Nokelainen et al. (2001)                                                  |
| Human serum albumin           | GS115       | pPIC9K      | Maintaining osmolarity and carrier in blood | W. Zhu et al. (2018)                                                     |
| Legumain                      | X-33        | pPICZαA     | Lysosomal protease              | T. Zhao et al. (2018)                                                     |
| Goat chymosin                 | X-33        | pPICZαA     | Hydrolysis of κ-casein          | Tyagi et al. (2016)                                                      |
| Carrot antifreeze protein     | GS115       | pPIC9K      | Inhibition of gluten deterioration | M. Liu et al. (2018)                                                     |
| Proinsulin                    | SuperMan5   | pPICZαA     | Treatment of diabetes mellitus  | Baeshen et al. (2016)                                                    |
| hIFN-γ                       | X-33, GS115, KM71H, CBS7435 | pPICZαA, pPIC9, pPpT4aS | Critical cytokine for innate and adaptive immunity | Razaghi et al. (2017)                                                    |
| IL-1β                        | GS115, SMD1168, X-33 | pPICZ-A | Proinflammatory cytokine       | Li et al. (2016)                                                          |
| IL-3                         | X-33        | pPICZαA     | Multipotent hematopoietic cytokine | Dagar and Khasa (2018)                                                   |
| IL-11                        | GS115       | pPINKαHC    | Thrombopoietic growth factor    | Yu et al. (2018)                                                          |
| IL-15                        | X-33        | pPICZαA     | Differentiation and proliferation of T, B, and NK cells | W. Sun et al. (2016)                                                     |
| Cyanate hydratase             | GS115       | pPICZαA     | Detoxification of cyanate and cyanide | Ranjan, Pillai, Permaul, and Singh (2017)                                |
| Human antiplatelet scFv antibody | X-33        | pPICZαA     | Treatment of atherosclerosis    | Vallet-Courbin et al. (2017)                                              |
| α-Amylase                     | X-33        | pPICZαA     | Starch saccharification         | Parashar and Satyanarayana (2017)                                        |
| Human epidermal growth factor | GS115       | pPIC9K      | Generation of new epithelial and endothelial cells | Eissazadeh et al. (2017)                                                 |
| Bromelain                     | KM71H       | pPICZαA     | Oedematous swellings            | Luniai, Meiser, Burkart, and Müller (2017)                                |
| Keratinocyte growth factor    | X-33        | pPICZαA     | Epithelialization-phase of wound healing | Kalhor (2016)                                                            |

(Continues)
Eventually, optimal conditions for the production of a recombinant protein are more popular. *P. pastoris* expression system is one of the most popular and standard tools for the production of recombinant protein in molecular biology. It is also considered a unique host for the expression of subunit vaccines which could significantly affect the growing market of medical biotechnology. Although *P. pastoris* expression systems are impressive and easy to use with well-defined process protocols, some degree of process optimization is required to achieve maximum production of the target proteins. Eventually, optimal conditions for the production of a recombinant protein in *P. pastoris* expression system differ according to the target protein.

### CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

### DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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### TABLE 6 (Continued)

| Product       | Used strain | Used vector | Usage                        | Reference                                                                 |
|---------------|-------------|-------------|------------------------------|---------------------------------------------------------------------------|
| Trypsin       | GS115       | pPIC9K      | Hydrolysis of proteins in the digestive system | Y. Zhang et al. (2018)                                                     |
| Human sialyltransferase | KM71H       | pPICZaB     | Pharmacological uses          | Luley-Goedl et al. (2016)                                                 |
| Transglutaminase | GS115       | pPIC9K      | Restructured meat products    | X. Yang and Zhang (2019)                                                  |
| Streptokinase  | X-33        | pPICZaA     | Thrombolytic medication       | Dagar, Devi, and Khasa (2016)                                             |
| Staphylokinase | GS115, KM71H | pPICZaA     | Thrombolytic medication       | Faraji et al. (2017)                                                      |
| TFPR1         | X-33        | pPICZaA     | Adjuvant                     | Ning et al. (2016)                                                        |

Abbreviations: hIFN-γ, human interferon γ; IL, interleukin; NK, natural killer.
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