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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 medium
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-hyperglycosylated, which may affect protein immunogenicity (Rasala & Mayfield, 2015). In recent years, to solve the problem of protein expression, methylotrophic yeasts such as *Hansenulla 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 Guilliermond (Zahrl, 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^S). 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^S 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 methylotrophic 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 > 50GlcNAc2) 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 mannoses (Figure 1c). It is believed that the α-1,3 glycan linkages in glycosylated proteins produced from *S. cerevisiae* are primarily responsible for the hyperantigenic 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-acetylglucosamine (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, Geysens, Vervecken, Contreras, & Callewaert, 2009). Indeed, the first step of humanizing Pichia 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 Pichia GlycoSwitch® strains (BioGrammatics, Carlsbad, CA) are SuperMan5, SuperMan5HIS$^-$, SuperMan5pep4$^-$, 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 mannose-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 (M_5GN and M_5GNGal strains). M_5GN and M_5GNGal strains (BioGrammatics) are commonly used to express vaccine antigens, cytokines, and antibodies.

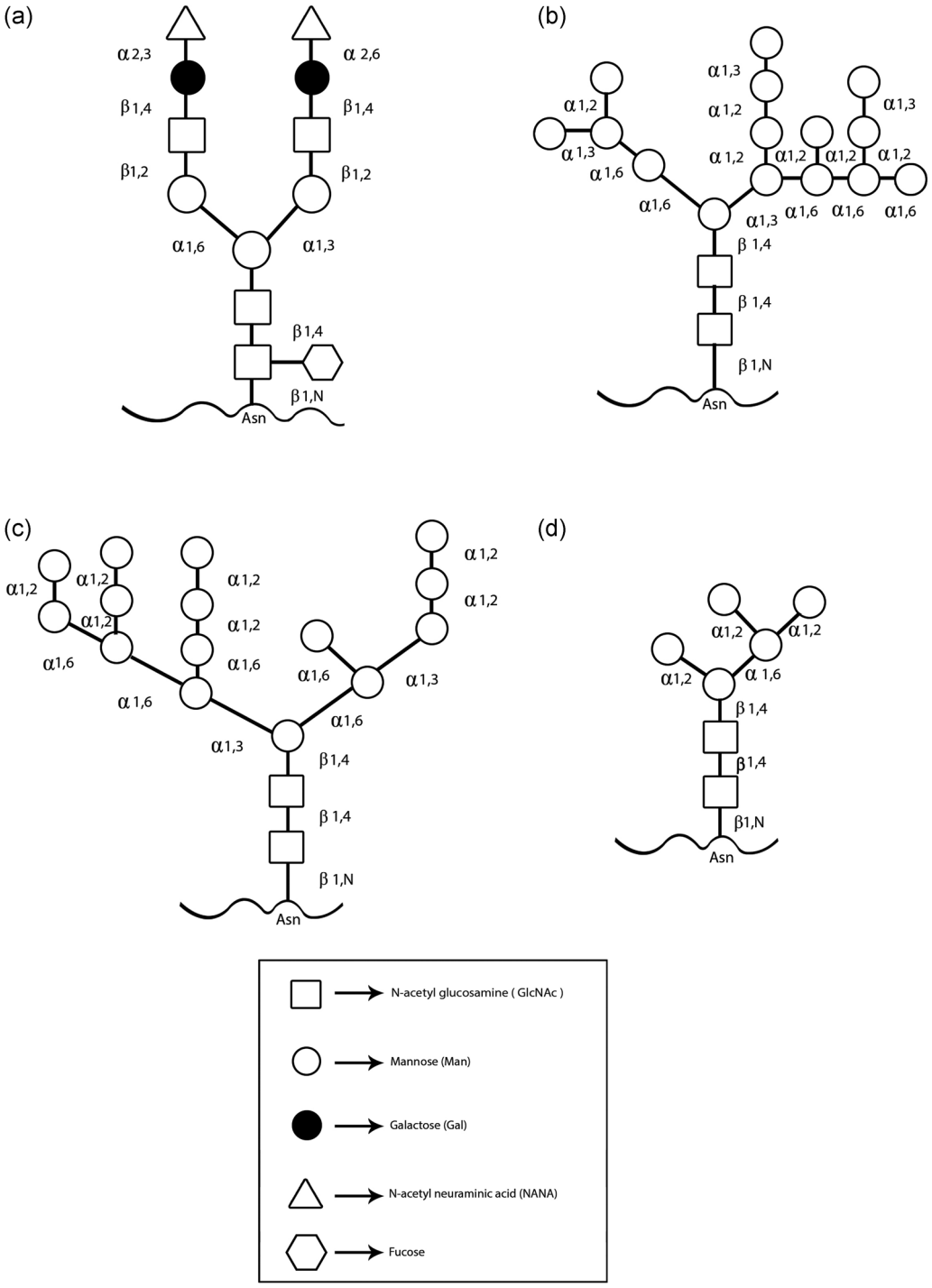

FIGURE 1 Schematic diagram of N-linked glycan structure in a mammalian cell, *Saccharomyces cerevisiae* and, *Pichia pastoris*. (a) N-linked glycan structure in mammalian cells commonly generates complex terminally sialylated structures. (b) In *S. cerevisiae*, the N-linked glycan structure is typically hypermannosylated (Man > 50GlcNAc2). Moreover, *S. cerevisiae* core oligosaccharides have terminal α-1,3 glycan linkages. (c) N-linked glycan structure in *P. pastoris* typically is of the Man8-14GlcNAc2 type with a triantennary-branched structure. Unlike *S. cerevisiae*, *P. pastoris* does not contain potentially immunogenic terminal α-1,3-linked mannoses. (d) In Pichia GlycoSwitch® strains (SuperMan5) N-linked glycan structure is typically hypomannosylated (with a mannose-5 structure)

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 (10^3–10^4) 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, & Spadiut, 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 *his4*, *arg4*, and *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 (*his4 pep4 prb1*), SMD1165 (*his4 pep4*), SMD1168 (*his4 pep4*), BG21, and *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 (*pep4*) and proteinase B (*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 *Sac* I, *Pme*I, and *Bst*X 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 *Kan*, *Shble*, *Bsd*, *Amp*, or *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.

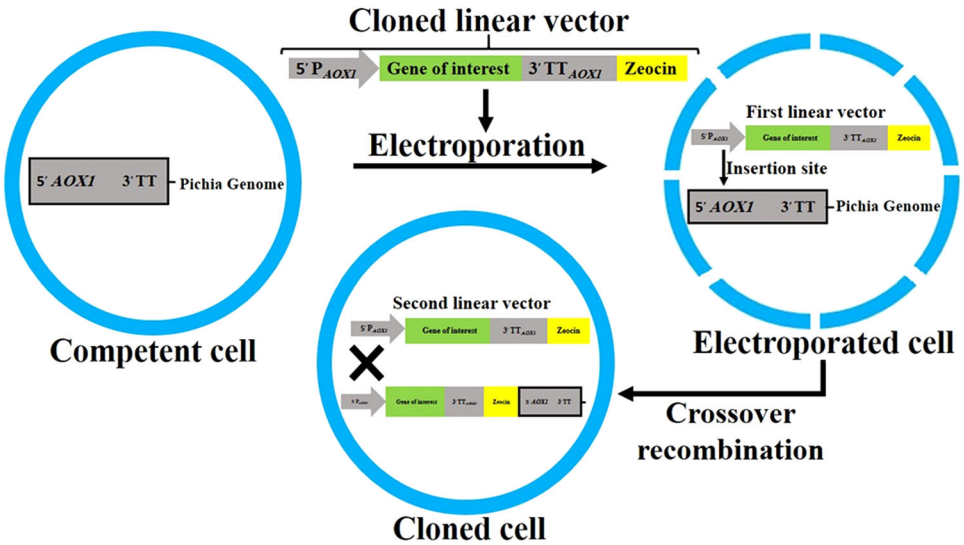

FIGURE 2 Crossover recombination phenomenon in the *Pichia pastoris* genome. Following the electroporation process of competent yeast cells, cloned linear vectors are inserted into the electroporated cells. Crossover recombination occurs between 5′ promoter (5′ P_{AOX1}) of vector and *AOX1* region of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

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-Deshpnade, 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	Baeshen et al. (2016)
		X-33	Human RNase4	Bardiya and Chang (2017)
pHIL-S1	*His4*, *Amp*	GS115	Rabies virus glycoprotein	Ben Azoun, Belhaj, Göngrich, Gasser, and Kallel (2016)
		GS115	*Rhizopus oryzae* Lipase	Satomura, Kuroda, and Ueda (2015)
		KM71	Camel lactoferricin	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)

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 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, Xu, 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.

TABLE 4 Recombinant subunit vaccine expressed in *Pichia 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	pPICZ-A	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	Kebriaei 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.

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 is thought to be linked to certain mannose-binding receptors carried by professional antigen-presenting cells, like dendritic cells and macrophages (Luong, Lam, Chen, & Levitz, 2007). Some of the new recombinant subunit vaccines that have been expressed in *P. pastoris* system are listed in Table 4.

4 | STRATEGIES TO IMPROVE PROTEIN EXPRESSION IN *P. pastoris*

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.

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^S strains with only *AOX2* gene; and Mut^- mutant strains without any *AOX* genes (Anggiani, Helianti, & Abinawanto, 2018). Therefore, the growth rate of different strains depends on their Mut forms. Mut^+ strains can grow in a wide range of micron from 0.028 to 0.154 per hour, while Mut^S 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	Mut^S	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.

heterologous proteins, which results in lower productivity (W. Zhang, Bevins, Plantz, Smith, & Meagher, 2000). The optimum methanol concentration used in some research articles is listed in Table 5.

4.2 | 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, Çalık, & 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, Naghdi, 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; Zhong et al., 2014). 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.

TABLE 6 *Pichia pastoris* as a suitable host for the production of recombinant biological molecules

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	pGAPZαB	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)
Pisum 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)
Fowlicidin-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	pPICZα, 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	Luniak, Meiser, Burkart, and Müller (2017)
Keratinocyte growth factor	X-33	pPICZαA	Epithelialization-phase of wound healing	Kalhor (2016)

(Continues)

TABLE 6 (Continued)

Product	Used strain	Used vector	Usage	Reference
DM64	X-33	pPICZαA	Anti-myotoxic	Vieira, da Rocha, da Costa Neves-Ferreira, Almeida, and Perales (2017)
Trypsin	GS115	pPIC9K	Hydrolysis of proteins in the digestive system	Y. Zhang et al. (2018)
Human sialyltransferase	KM71H	pPICZαB	Pharmacological uses	Luley-Goedl et al. (2016)
Transglutaminase	GS115	pPIC9K	Restructured meat products	X. Yang and Zhang (2019)
Streptokinase	X-33	pPICZαA	Thrombolytic medication	Dagar, Devi, and Khasa (2016)
Staphylokinase	GS115, KM71H	pPICZαA	Thrombolytic medication	Faraji et al. (2017)
TFPR1	X-33	pPICZαA	Adjuvant	Ning et al. (2016)

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

6 | CONCLUSION AND PERSPECTIVES

For the production of recombinant proteins with medical and industrial purposes, researchers should apply biological expression systems. Yeast expression systems such as *S. cerevisiae* and *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. 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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