Indian Journal of Biotechnology
Vol 6, April 2007, pp 175-186

Pichia pastoris: A notable heterologous expression system for the production of foreign proteins—Vaccines

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Received 16 May 2005; revised 6 March 2006; accepted 12 May 2006

Improvements in yeast expression systems, coupled with the development of more information on methylotrophic yeast are expanding the role of yeast in the process of understanding and engineering eukaryotic proteins. *Pichia pastoris* has become a highly popular expression host for the production of a wide variety of intracellular and extracellular recombinant proteins of interest. Initial success with this system was greatly facilitated by the development of versatile expression vectors that were almost exclusively based on the strong, tightly regulated promoter of the *P. pastoris* major alcohol oxidase gene. Recent advances in the development of yeast as a host for the production of heterologous proteins have provided a catalogue of new applications, methods and system components, which will help us to understand about more details and applications of the system even further. Characteristic features of *Pichia* species, which are the most advantageous and favourable, have resulted in an increasing number of biotechnological applications. During the past two decades, *P. pastoris* has been developed into a highly successful system, due to its increasing popularity, which can be attributed to several factors such as, the simplicity of techniques needed for the molecular genetic manipulation; the ability to produce foreign proteins at high level, the capability of performing many eukaryotic post-translational modifications (glycosylation, disulfide bond formation and proteolytic processing) and finally the availability of the expression system as a commercially available kit.

Keywords: *Pichia pastoris,* heterologous expression system, foreign proteins, vaccines
IPC Code: Int. Cl.[8] C12N15/09, 15/11

Introduction

Significant advances in the development of new strains and vectors, improved techniques, and the commercial availability of these tools coupled with a better understanding of the biology of *Pichia* species have led the value and power for the microbe in commercial and research laboratories[1]. In the Biotech Century, scientists are continuing to engineer yeasts for producing eukaryotic foreign proteins. Yeast offers the ease of microbial growth and gene manipulation found in bacteria along with the eukaryotic characteristics[2]. Yeast combines molecular genetic manipulations and growth characteristics of prokaryotic organisms together with the sub-cellular machinery for performing post-translational protein modifications of eukaryotes. Because of the vast knowledge in genetics and physiology of *Saccharomyces cerevisiae,* it was selected first for this purpose and accepted as safe for human use through the experience with organisms used in brewing and baking[3, 4]. However, *S. cerevisiae* has not been proved as an ideal foreign gene expression host. The methylotrophic yeast, *Pichia* species is the most highly developed one among a small group of alternative yeast species chosen for their perceived advantages over *S. cerevisiae* as a expression host[5,6]. Yeasts are economical, usually give higher yield and are less demanding in terms of time and effort relative to complex eukaryotic systems such as Chinese hamster ovary cells and baculovirus infected cell lines. Nevertheless, there are disadvantages while using them for expression of some proteins, mostly related to their inability to perform certain complex post-translational modifications, such as prolyl hydroxylation and amidation as well as some types of phosphorylation and glycosylation[7]. However, recent findings may help to alleviate some of these problems and diverse the scope of future applications of yeast in biotechnology.

At present, *P. pastoris* is one of the most heterologous systems being extensively applied for the commercial production of various foreign proteins

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in many laboratories worldwide. In this direction, authors have provided an overview of existing knowledge on the subject to highlight overall basic features of the *P. pastoris* system as well as focus on interesting aspects of new developments in the understanding and application of *Pichia* system for the production of recombinant foreign proteins with special reference to medical and veterinary vaccines.

General Characteristics of *Pichia pastoris*

As an eukaryotic organism*, P. pastoris* has many of the advantages of higher eukaryotic expression systems especially post-translational modifications such as protein processing, protein folding and protein secretion into the medium, among which the later facilitates easy purification[8] while being manipulated as easy as *Escherichia coli* or *S. cerevisiae.* It is faster, easier and less expensive to use when compared to other expression systems such as baculovirus or mammalian system, and generally gives higher expression level. As yeast, it shares the advantages of molecular and genetic manipulations with *Saccharomyces,* and it has added advantage of higher levels of expression. These features along with easy maintenance, easy scale-up, inexpensive growth requirements makes *P. pastoris* as a very useful protein expression system. The process can be scaled up to a level of expression, which is 10-100 times higher than that of *E. coli*[9-11]. In many instances, heterologous proteins were produced at much higher level when compared to their respective productivities in the traditional *S. cerevisiae* host. Their growth to high cell density provides a means to produce large quantities of antigen economically[6]. Since 1984 over 300 heterologous proteins have been well expressed in *P. pastoris*[1,12]. *P. pastoris* has gained more popularity because of several factors such as alcohol oxidase I (AOX I), which is one of the strongest and most regulated promoters, its ability to integrate expression plasmids in its own genome in one or more specific sites, its ability to culture strains in high density fermenters; and its ready availability as a kit (Invitrogen, USA). Transformation of *P. pastoris* can easily be carried out either by electroporation method or spheroplasting method, using linearized recombinant plasmids and efficiencies are usually in several orders of magnitude below those for other yeasts. Pretreatment of *P. pastoris* with 0.1 *M* lithium acetate (LiAc) and 10 m*M* dithiothreitol (DTT) before electroporation increased transformation efficiency approximately 150-fold[13].

Genotype of *Pichia* strain

The *Pichia* host strains GS115 and KM71 have a mutation in the histidinol dehydrogenase gene (his4), which prevents them from synthesizing histidine. All expression plasmids carry the *HIS4* gene which complements his4 in the host so that transformants are selected for their ability to grow on histidine-deficient medium (Invitrogen, USA). The parent strain of KM71 has a mutation in the arginosuccinate lyase gene (arg4) that prevents the strain from growing in the absence of arginine. The wild-type *ARG4* gene was used to disrupt AOX I creating KM71, a Mut^S Arg^+ His^- strain. Several wild-type genes from *Saccharomyces* complements comparable mutant genes in *Pichia*. Genes such as *HIS 4, LEU2, ARG4, TRPI,* and *URA3* all component their respective mutant genes in *Pichia* species (*Pichia* expression kit manual, Invitrogen, USA).

Alcohol Oxidase Proteins

There are two genes in *P. pastoris* that code for alcohol oxidase-AOX I and AOX II. The AOX I gene is responsible for the vast majority of AOX activity in the cell and expression of the AOX I gene is tightly regulated and induced by methanol to very high level. Separately, biochemical studies established that methanol metabolism requires induction of a unique set of pathway enzymes[14]. The most interesting of these enzymes is AOX, the first enzyme in the methanol-utilization pathway. AOX is undetectable in cells cultured in carbon sources such as glucose, glycerol or ethanol, but constitutes up to 30% of total soluble protein in methanol-grown cells[15].

The AOX I gene has been isolated and plasmid-borne version of the AOX I promoter is used to drive expression of the gene of interest encoding the desired heterologous protein[16-18]. While AOX II is about 97% homologous to AOX I, the growth of cells with AOX I in methanol is much slower than that with AOX II. This slower growth in methanol allows isolation of Mut^S (Methanol utilization slow) strain (AOX I)[18,19]. The AOX I promoter is tightly repressed by glucose and most other carbon sources but is induced more than 1000-fold in cells shifted to methanol as a sole carbon source[17]. With this promoter, expression of recombinant proteins is highly repressed while cultures are grown to high density in glucose or glycerol, which prevents selection for non-expressing mutant cells. Cultures are then shifted to a methanol medium to induce rapid high-level expression[20]. As methylotrophic yeast, it is capable of metabolizing

methanol as its sole carbon source. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde using molecular oxygen by the enzyme AOX. In addition to formaldehyde, this reaction generates hydrogen peroxide. To avoid hydrogen peroxide toxicity, methanol metabolism takes place within a specialized cell organelle, called peroxisome, which sequesters toxic by-products away from rest of the cell. AOX has poor affinity for oxygen; this difficulty is, however, compensated by generating large amount of the enzyme.

Expression of the AOX I gene is controlled at the level of transcription. In methanol-grown cells, approximately 5% of the poly A^+ RNA is from AOX I gene. The regulation of the AOX I gene is a two-step process. The first step is a repression/derepression mechanism followed by an induction mechanism (e.g. GAL 1 gene in *Saccharomyces*)[21]. Briefly, growth in glucose represses transcription, even in the presence of the inducer methanol. For this reason, growth in glycerol is recommended for the optimal induction with methanol. It is important to remember that growth in glycerol (derepression) is not sufficient to generate even minute level of expression from the AOX-I[16-18]. Loss of the AOX I gene and eventually, a loss of most of cell's AOX activity results in a strain which is phenotypically Mut^S. This results in the reduction of cell's ability to metabolize methanol and exhibits poor growth in methanol medium. Mut^+ (Methanol utilization plus) refers to the ability of wild type strains to metabolize methanol. These two phenotypes are used while evaluating *Pichia* transformants for integration of gene of interest. The strong and highly regulated promoter of the AOX gene will become an attractive control element for heterologous gene expression [8].

Intracellular and Extracellular/Secretory Protein Expression

Heterologous protein expression in *P. pastoris* can either be intracellular or secretory. Secretion requires the presence of a signal sequence in the expressed protein to target it to the secretory pathway. Several different secretion signal sequences including the native secretion signal, which is present in some heterologous proteins, have been used successfully with varying success levels. The most successfully used secretion signal sequences are from the *S. cerevisiae* such as ∝ factor prepro peptide[4,22]. The major advantage is that *P. pastoris* secretes very low level of native proteins. Thus, very low amount of native protein in the minimal *Pichia* growth medium means that secreted heterologous protein comprises the vast majority of the total protein in the medium and serves as the first step in purification of the protein[23]. However, if there are recognized glycosylation sites (Asn-X-Ser/Thr) in our protein's primary sequence, glycosylation may occur at these sites.

Post-translational Modifications

Pichia species may have advantages in the glycosylation of secreted proteins because it may not hyperglycosylate. Both *S. cerevisiae* and *P. pastoris* have a majority of N-linked glycosylation of high-mannose type, however, the oligosaccharide chains added post-translationally to proteins in *P. pastoris* (average 8-14 mannose residues per side chain) are much shorter than those added to proteins in *S. cerevisiae* (50-150 mannose residues) and very little O-linked glycosylation has been observed in *Pichia*[17,24]. In addition, *S. cerevisiae* core oligosaccharides have terminal ∝ 1,3 glycan linkages whereas *P. pastoris* does not. It is believed that ∝ 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. Although it is not yet proven, this problem is predicted less for glycoproteins generated in *P. pastoris*, because it may resemble the glycoprotein structure of higher eukaryotes [4].

Technical Advantages

Comprehensive catalogues of methods and procedures dealing with methylotrophs as host for heterologous gene expression have been provided in detail for *P. pastoris*[25]. Technical advantages in yeast include site-specific integration, increase in copy number, leader sequence for the secretion of heterologous protein and post-translational modifications[8]. Intact protein production and secretion into the medium could be an efficient method for production and purification of the expressed protein.

1. Host Cells, Vector Design and Elements

The generation of *P. pastoris* gene expression relies on auxotrophic host cells of which the established *his4* host cell has been developed for the applications in the heterologous gene expression. Accordingly, the transformation vectors harbour the respective genes for complementation. New examples for G418 selection due to the presence of a dominant selection

marker are also described for *P. pastoris*[26]. For *P. pastoris,* a new generation of expression vectors is commercially available (Invitrogen, USA). These vectors harbour a Zeocin resistance gene, selective in both bacterial and yeast hosts. As a result, shuttle vectors of reduced size are obtained. Since this compound is a strong mutagen, cells challenged with Zeocin may acquire undesirable mutations[11]. A novel selectable marker, the *P. pastoris* formaldehyde dehydrogenase gene (*FLD1*) was described for selection of recombinant clones on plates containing methylamine and also for DNA-mediated transformations of this yeast[27]. Three new biosynthetic genes such as *ARG4*, *ADE1*, and *URA3*- from *P. pastoris* have been characterized. The predicted products of the genes share significant sequence similarity to *S. cerevisiae*, namely argininosuccinate lyase, PR-aminoimidazolesuccino-carboxamide synthase, and orotidine-5'-phosphate decarboxylase, respectively. Along with the previously described *HIS4* gene, each gene was incorporated as the yeast selectable marker into a set of shuttle vectors designed to express foreign genes in *P. pastoris*. In addition, a series of host strains containing all possible combinations of ade1, arg4, his4, and ura3 auxotrophies to be used with these new vectors have also been constructed[28]. Gene replacement in yeast is often accomplished by using a counter selectable marker such as URA3. Although ura3 strains of *P. pastoris* are generated, these strains are inconvenient to work with because they grow slowly even in the presence of uracil. An alternative marker is T-urf13 gene from the mitochondrial genome of male-sterile maize, which can be used in any *P. pastoris* strain. T-urf13 is incorporated into a vector that also contains ARG4 marker for positive selection. The resulting plasmid allows for pop-in/pop-out gene replacement in *P. pastoris*[29].

With the development and application of *P. pastoris*, these anticipated advantages have been realized along with others that were unanticipated. Several new *Pichia* systems have been reported (Table 1) including *P. methanolica*, which touts many attributes of *P. pastoris* and *Hansenula polymorpha* including the ability to grow rapidly to very high cell densities and the availability of expression vectors that contain the tightly regulated AOX promoter to control expression of foreign genes[30]. A new species, *Pichia* (*Komagataella*) *pseudopastoris* was recently isolated from rotten willow samples in Hungary[31]. The *P. pastoris* system has now been successfully utilized to produce a number of heterologous proteins at concentrations creating commercial interest (Table 2).

2. Site-Specific Integration

P. pastoris vectors are designed for homologous integration into either AOX I locus, one of the two homologous AOX I genes present in this species or *his4 locus*. Like *S. cerevisiae,* linear DNA can generate stable transformants of *P. pastoris* via integration or homologous recombination between the transforming DNA and regions of homology within the genome[19,32]. Such integrants show extreme stability in the absence of selective pressure even when present as multiple copies. The most commonly used expression vector carry the *HIS4* gene for selection. Note that single crossover events (insertions) are much more likely to happen than double crossover events (replacement).

a. Gene Insertion at AOX I or aoxI:: ARG4

Table 1 — Features of *Pichia* expression system

Species Name	Promoter	Regulation	References
P. pastoris	AOX 1	Methanol induced	7
	FLD 1	Methanol or methylamine	36
	GAP	induced	35
	PEX 8	Strong constitutive	39
	YPT1	Moderate methanol induced	40
		Moderate constitutive	
P. methanolica	AUG 1	Methanol induced	31
P. stipitis	XYL1	Xylose induced	85

Table 2 — Some of the recombinant proteins produced in *Pichia pastosris*

Protein	Expression levels g/L	Nature/Mode of expression*	References
Vaccines /Antigens			
Hepatitis B surface antigen	0.4	I	64
Pertussis Ag (Pertactin) P69	3.0	I	53
Tetanus toxin fragment C	12.0	I	9
HIV-1 gp120	1.25	I	22
Tick anticoagulant protein	1.7	S	50
Bm86 tick gut glycoprotein	1.5	S	49
Bovine herpes virus-1	0.01	S	54
Dengue viral envelope proteins	0.1	S	60,62
FMD viral proteins	0.15-2	S	81,82
Pseudorabies virus gE	10	S	86
P. falciparum antigens	0.5-2.6	I/S	72,77

*I — Intracellular, S — secreted

Gene insertion events at the AOX I (GS115) or aox I:: ARG4 (KM71) loci arise from a single crossover events between the loci and any of the three AOX I regions in the vector, the AOX I promoter, the AOX I transcription termination region (TT), or sequences even further downstream of AOX I (3′ AOX I). This results in the insertion of one or more copies of the vector upstream or downstream of the AOX I or aox I::ARG4 genes. The phenotype of such a transformant is His^+Mut^+ (GS115) or His^+Mut^S (KM71). Fig. 1 shows the result of insertion of the plasmid 3′ AOX to the intact AOX I locus (Mut^+) and the gain of $P_{AOX\ I}$, your gene of interest, and HIS4 (expression cassette). This event occurs with non-linearized plasmid and plasmid that religates, although at a lower frequency (*Pichia* Expression kit Manual, Invitrogen, USA).

b. Gene Insertion at Events at HIS 4

In either GS115 (Mut^+) or KM71 ((Mut^s), gene insertion events at his4 locus arise from a single crossover event between his4 locus in the chromosome and *His4* gene of the vector. This results in the insertion of one or more copies of the vector in his4 locus. Since the genomic AOX I or aoxI::ARG4 loci are not involved in this recombinant event, the phenotype of such a his^+ transformant has the same Mut phenotype as the parent strain. Fig. 2 shows the result of insertion of the plasmid between duplicated copies of the *HIS4*/his4 genes, one is still mutant but the other is wild type (*Pichia* expression kit manual, Invitrogen, USA).

c. Multiple Gene Insertion Events

Multiple gene insertion events in a single locus in a cell do occur spontaneously with a low but detectable frequency-between 1 and 10% of all selected His^+ transformants. Multi-copy events can occur as gene insertions either in AOX I, aox I::ARG4, or in his4 loci. Multiple gene insertion events can be detected by quantitative dot-blot analysis, PCR, Southern blot analysis and differential hybridization[31] .

d. Gene Replacement at AOX I in GS115

In a his4 strain such as GS115, a gene replacement (omega insertion) event arises from a double crossover event between the AOX I promoter and 3′ AOX I regions of the vector and the genome. This results in the complete removal of the AOX I coding region (i.e. gene replacement). The resulting phenotype is His^+Mut^s transformants can be readily and easily screened for their Mut phenotype, with Mut^s serving as a phenotypic indicator of integration via gene replacement in the AOX I locus. The net result is the loss of AOX I locus (Mut^s) and the gain of an expression cassette containing $P_{AOX\ I}$, your gene of interest, and His 4. Fig 3. shows a gene replacement event in AOX I locus (*Pichia* expression kit manual, Invitrogen, USA).

3. Increase in Copy Numbers

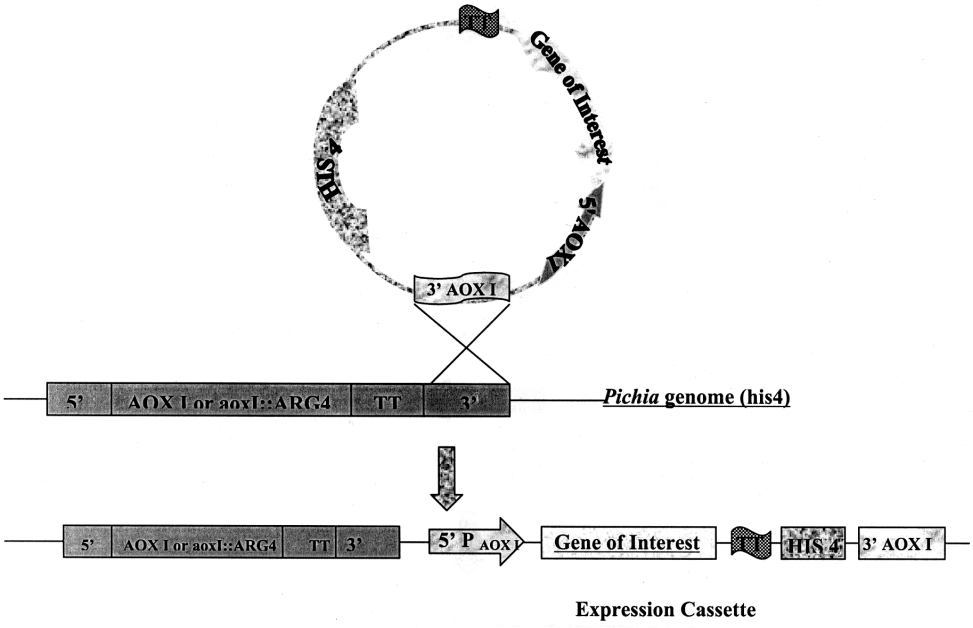

Fig. 1 — Schematic diagram showing gene insertion event at AOX I locus

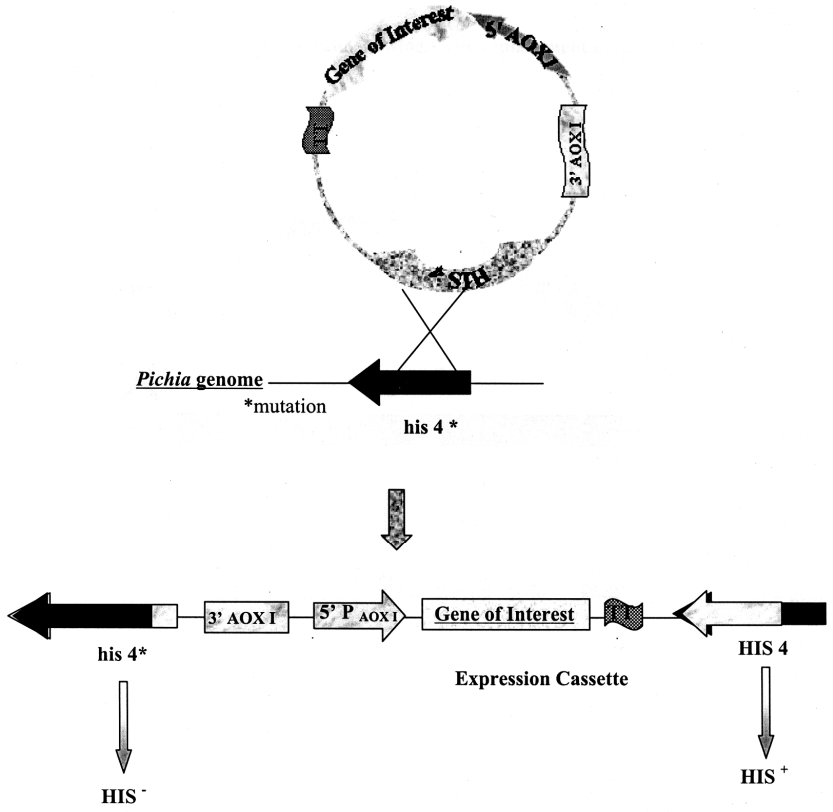

Fig. 2 — Schematic diagram showing gene insertion event at HIS4 locus

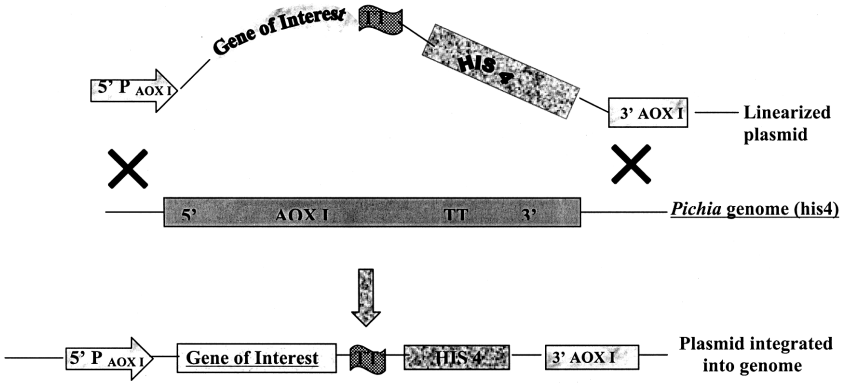

Fig. 3 — Schematic diagram showing gene replacement event at AOX I locus

High copy number integrants can be identified by PCR screening for heterologous DNA content. A simple PCR method for analyzing clones from a transformation plate has been published[33,34]. A collection of recombinant *P. pastoris* strains generated by targeted integration mostly consists of single-copy integrants. By screening for high productivity, integrants with four, eight or even more copies can be identified.

4. Promoter Elements

The established production systems based on methylotrophic yeast rely on strong inducible promoter (AOX) elements that are derived from genes of the methanol metabolism pathway and leader sequences for fusion proteins. A range of new leader sequences has been applied for secretion of heterologous compounds in addition to widely used *S. cerevisiae*-derived MFα1 prepro-sequences[8]. Among others, the leader sequences from several genuine

metalloproteases are functional in the *P. pastoris*. Vectors containing the constructive glyceraldehydes-3-phosphate dehydrogenase promoter are available for *P. pastoris* (Invitrogen, USA).

Limitations

Nevertheless, there are limitations to this system, some of which have been remedied recently. Although it is essential for maximum induction of the AOX I promoter, methanol is a potential fire hazard and may not be suitable for the production of food products. The glyceroldehyde-3-phosphate dehydrogenase (GAP) promoter provides constitutively high level of expression on glycerol, glucose, and methanol media but it cannot repress expression of recombinant proteins, which limits its usage[35]. The promoter *FLD 1* gene, whose product is a glutathione-dependent formaldehyde dehydrogenase, can be induced either by methanol or methylamine in glucose containing media with an expression level comparable to those expressed from AOX I promoter in methanol[36].

P. pastoris lacks promoters for moderate expression. The high level of expression provided by the AOX I, FLD1 and GAP promoters is toxic in some cases and may overwhelm the protein-handling machinery of the cell, through misfolding or unprocessing of significanct portion of the cellular protein[37]. The availability of the promoters would also facilitate the simultaneous expression of multiple genes, each at an optimal level, which may be important for the production of multisubunit proteins[38]. The promoter of the PEX 8 (peroxin) gene, which encodes a peroxisomal biogenesis protein gives low-level expression in glucose and is induced modestly when cells are shifted to methanol[39]. *YPT1* (GTPase involved in secretion) gene, which is also a moderate promoter, provides a low but constitutive expression level in glucose, methanol, or mannitol containing media[40].

There are only few selectable markers available for *P. pastoris* transformation. Till now, only *HIS4, ARG4* and *Sh ble* (Zeocin antibiotic resistance) selection markers are available. To alleviate this problem, a series of expression vectors with new biosynthetic marker genes such as *ADE1* and *URA3*, have been constructed along with respective auxotrophically marked strains[41].

Production and Purification of Proteins

A relatively simple combination of chromatographic procedure is usually being employed for the downstream processing i.e., purification of recombinant proteins. Yeast systems are particularly valued for their ability to secrete heterologous protein products since passage of proteins through the secretory pathway permits post-translational events such as proteolytic maturation, glycosylation and disulfide bond formation to occur. A specific advantage of secretion in *P. pastoris* is that since the organism secretes only very low levels of native proteins, secretion of a foreign protein becomes an effective purification step that separates products from most other cellular components[4]. Despite the apparent purity of many foreign proteins secreted from *P. pastoris*, further purification is almost always necessary. The structure of carbohydrate added to secrete proteins is known to be very organism specific[42,43]. Comparison of *S. cerevisiae* and *P. pastoris* has revealed distinct differences between N-linked oligosaccharide chains added to proteins secreted from these yeasts[23,42]. However, oligosaccharide chains added to *P. pastoris*-secreted *S. cerevisiae* invertase (*P. pastoris* invertase) are much shorter than those added to invertase secreted from *S. cerevisiae* [24,43].

P. pastoris has demonstrated its most powerful success as a large-scale (fermentation) recombinant protein production tool[1]. Using this system, recent advances have been made in improving the quality of recombinant proteins in fermenter culture and also in improving the quality of secreted protein products through improved secretion signals and glycosylation patterns[44]. Recent studies have also shown the versatility and utility of the *P. pastoris* system. Improvements in strains have boosted the yield of proteins and peptides to the commercially feasible range. The *P. pastoris* system will soon be used to manufacture proteins for human clinical trials[45]. The use of methylotrophic yeasts for the production of single-cell-protein (SCP), AOX and fine chemicals has been proposed. Fermentation technology developed for the growth of these yeasts on methanol at high cell densities has also been commercialized. However, it is the production of heterologous recombinant proteins by *P. pastoris* that is emerging as the most significant application of the methylotrophic yeasts[46].

Research Developments and Application for Human and Animal Vaccines

The technology of using *P. pastoris* has essentially matured now and being complemented with attractive new applications, methods and components, adding to the versatility of gene expression systems. Range of *P. pastoris*-derived proteins have been generated, including those for detailed structural studies[8]. Yeast is capable of secreting the protein in a form that mirrored its native conformational structure and immunogenicity, a state in which majority of the antigenic epitopes engender neutralization activity with the antibodies[47,48].

The Mb86 antigen from the cattle tick, *Boophilus microplus* produced as a recombinant protein in *P. pastoris,* forms highly immunogenic particles of 17-45 nm, thereby providing significant protection against ticks[49]. The yield of secreted protein was about 1.5-2.0 g/L, which was higher than that obtained in *E. coli* [50,51]. A production and downstream process has also been described to manufacture a vaccine based on this protein, which fulfills both safety and good manufacturing practices (GMP) guideline requirements[49]. The large-scale production and purification of Bm95 antigen from *B. microplus* following a simple and cost-effective process (yield 0.55 g/L) in *P. pastoris* has induced protection in cattle against infestations by *B. microplus* under controlled and production conditions[52]. A high level expression (12 g/L) of tetanus toxin fragment C in *P. pastoris* has also been reported[9].

Recombinant *Bordetella pertusis* pertactin (P69) an outer membrane protein having immunological property, was produced in high levels in *P. pastoris*[53]. Truncated bovine herpes virus-1 (BHV-1) gD gene, expressed in *P. pastoris* as a secretary protein while maintaining its native conformation, can serve as a subunit vaccine against BHV-1[54]. Structural conversion of the serotype-A recombinant botulinum neurotoxin heavy chain fragment (rBoNTA(Hc), which is being produced intracellularly in *P. pastoris*[55], was observed and characterized during purification development efforts. The preliminary scale-up and potency data showed scalability and robustness in the production of an active disulfide-bonded form of a recombinant botulism vaccine candidate. The presence of the disulfide bond did not affect the vaccine potency and it enhanced molecule's thermal stability[55].

Human papilloma virus (HPV) type 6 L1 protein expressed in *P. pastoris* has facilitated the HPV vaccine development and structure-function study[56]. Synthetic genes encoding the carboxyl-terminal region of toxin types A, B, C1, E, and F were expressed in *P. pastoris* and the manufacturing processes developed for producing highly purified vaccines. These vaccines were shown to be safe, highly efficacious, stable, and amenable for high-level industrial production[57]. The expressed recombinant equine herpes virus-1 (EHV-1) gD protein in *P. pastoris,* while using in a DNA prime/protein boost inoculation schedule, induced high EHV-1 ELISA and virus neutralizing antibodies and also provided protection from challenge infection in BALB/c mice[58].

The success of trials on *P. pastoris* expressed recombinant polio and dengue viral vaccines have also been reported[59,60]. The recombinant truncated dengue 4 virus envelope glycoprotein (E4rec) expressed in *P. pastoris* elicited both DEN 4 neutralizing antibody response and haemagglutination inhibition antibodies as well as specific memory T cell response when it was administered to BALB/c mice. Immunized mice were also significantly protected against lethal DEN 4 virus challenge[61]. The dengue virus envelope protein having immunogenic nature of recombinant E protein was also expressed as secretory form (GST fusion protein) in *P. pastoris* up to the concentration of 100 mg/L[62]. In another study, *P. pastoris* expressed envelope (E) gene of dengue virus type 4 recombinant vaccine provided immunogenicity and partial protection against *Macaca fascicularis*. However, the results were not effective enough to use it as a vaccine candidate. Further work is required to improve the quality of the immunogen[63].

An efficient production process that is developed using *P. pastoris*[64] and *H. polymorpha*[65] for particles containing hepatitis B surface antigen (HBsAg) subtypes is available commercially. The first report of antibody elicited by *P. pastoris* expressed recombinant hepatitis E virus (HEV) ORF2 protein, recognizes native HEV. High immunogenicity of this kind of ORF2 was also demonstrated by inducing strong immune response in mice[66]. The novel fusion protein incorporating HBsAg with neutralization epitope-containing HEnAg was expressed successfully in *P. pastoris*. Chimeric HBV/HEV virus like particles produced in this system may have

potential to be used as a recombinant HBV/HEV bivalent vaccine candidate[67]. The sequence of Somatostatin gene (*S14*) and HBsAg (ss/HbsAg) chimera was constructed and expressed in *P. pastoris*. Expressed protein was proved with high specificity and may be useful as engineering vaccine for developing the diagnosis and cure tumours in clinical medicine and promoting the growth of animal husbandry production[68]. A novel chimeric protein that was derived by fusion of surface protein of HBV with envelope protein of type-2 dengue virus was expressed in *Pichia* system. This hybrid protein retains the inherent property of both components and neutralizing antibodies specific to both of them recognize it, which has important implications for multivalent vaccine development[69]. Many studies have suggested that HBsAg with PreS sequences could be an ideal candidate for highly effective HBV vaccine. Modified with PreS epitopes surface antigens such as S1S, SS1 and S2S were expressed in this system, was more efficient than that in *S. cerevisiae*[70]. The purification of recombinant SS1 protein, expressed in *P. pastoris*, and the investigation of its physiochemical characters and immunogenicity were also described. The simultaneous injection of a CpG adjuvant induced a Th1-like immune response against both HBs and PreS1 epitopes[71].

Plasmodium falciparum chimeric protein 2.9 (PfCP-2.9) was constructed and produced by *P. pastoris* in secreted form with a yield of 2.6 g/L. The combination of extremely high yield of the protein and the enhancement of its immune response provides a basis to develop an effective and affordable malaria vaccine[72]. Designed synthetic gene based on *Pichia* codon usage was used for expression of recombinant F2 in *P. pastoris*. *Pichia*-based EBA-F2 malaria vaccine construct has further potential to be developed for clinical use[73]. A candidate trans-mission-blocking malaria vaccine antigen Pfs25 against *P. falciparum* was expressed in *P. pastoris* to address the low yield seen with *S. cerevisiae*. *P. pastoris* is apparently better than *S. cerevisiae* in producing biologically more active, immunologically more potent Pfs25H-A with higher yield[74]. Efficacy of a form of *P. falciparum* AMA1 produced in *P. pastoris* was evaluated in *Aotus vociferans* monkeys and the results showed significant protection against lethal challenge with *P. falciparum*[75]. The study of immunogenic properties of *P. pastoris* expressed in *Plasmodium vivax* MSP1(19) epitope (PvMSP1(19)) and the Pan-Allelic DR epitope (PADRE) deserves further evaluation in pre-clinical immunizations against *P. vivax* in non-human primates[76]. The purified form of P30P2MSP1(19) antigen produced as secretory protein in *P. pastoris* in large scale (500 mg/L), high-density fermentation, may prove to be a more efficacious malarial vaccine than that produced in *Saccharomyces.* Moreover, *Pichia* will be useful as a system for the cost-effective production of such vaccine[77].

Recently, the first report has been reported from our laboratory on the production of foot and mouth disease virus (FMDV) structural proteins in *P. pastoris* and their further applications as vaccine. The capsid precursor (P1-2A) proteins of FMDV serotypes Asia1[78] serotype O, A_{22} and C[79,80] were expressed as secretory protein in *P. pastoris* using yeast transfer vector pPIC-9K. The expressed structural proteins type 'O'[81] and other serotypes such as Asia1, A_{22} and C[80,82] were found to induce neutralizing antibodies and protective response, while challenging with virulent virus in guinea pigs after 28th day post vaccination. The yield of proteins varies between 120-150 mg/L of culture supernatants. The non-structural (3AB) proteins of FMDV serotype A_{22} were also expressed as secretory proteins and used as antigens in ELISA for differentiation of vaccinated and infected animals[83]. Our studies showed that, FMDV proteins can efficiently be produced in native form in *Pichia* system and can potentially be used as diagnostics and/or vaccines. The expressed recombinant VP1 protein of bovine FMDV type O in *P. pastoris* using pSuperY-vector can elicite similar humoral and cellular immune responses in mice to traditional FMDV killed vaccine[84]. These preliminary studies lay the foundation for further FMDV vaccine research.

Concluding Remarks

P. pastoris has proven to be one of the powerful and standard tools used in molecular biology for the generation of recombinant protein of commercial interest. Advantages of the system include the AOX1 promoter, which has transcription characteristics useful for regulating heterologous protein expression, well-developed methods for classical and molecular-genetic manipulation of the organism and technology for the growth of expression strain in large high-density fermentor cultures. These, along with improvements in its manipulation for expression purpose, have moved *P. pastoris* into the main stream

of foreign gene expression systems especially for prophylactics. An increasing number of publications attest to the favourable properties of *P. pastoris* as host for heterologous systems. As a consequence, methylotrophs have become the preferred option among various yeast expression systems. Safe and convenient systems provide competitive and reproducible processes for producing relevant components in both a laboratory and an industrial scale. A comprehensive catalogue of methods and components including a commercial kit based on *P. pastoris*, is now available and subject to further improvements and expansion/extensions.

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