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Anu Malik
Ph.D., Scholar, Department of Veterinary Microbiology, LUVAS, Hisar, Haryana, India

Akhil Gupta
Scientist, Department of Veterinary Microbiology, LUVAS, Hisar, Haryana, India

Swati Dahiya
Scientist, Department of Veterinary Microbiology, LUVAS, Hisar, Haryana, India

Vaishali Sehrawat
Ph.D., Scholar, Department of Veterinary Public Health & Epidemiology, LUVAS, Hisar, Haryana, India

Sangeeta Das
Ph.D., Scholar, Department of Veterinary Microbiology, LUVAS, Hisar, Haryana, India

Corresponding Author
Anu Malik
Ph.D., Scholar, Department of Veterinary Microbiology, LUVAS, Hisar, Haryana, India

A review on *Pichia pastoris*: A successful tool for expression of recombinant proteins

Anu Malik, Akhil Gupta, Swati Dahiya, Vaishali Sehrawat and Sangeeta Das

Abstract
With the expanding importance and prevalence of protein therapeutics, recombinant proteins offer different applications in diverse fields including therapeutics and research. For the efficient production of target proteins, various expression systems are available in the market including prokaryotic, eukaryotic and mammalian systems. *Pichia pastoris* (*P. pastoris*) is the most widely used novel expression cassettes with the scope of genetic engineering and cell modifications in secretion pathways. *P. pastoris* can be considered as a distinguished production system for its growth to very high cell densities, for the available strong and tightly regulated promoters, and for the options to produce gram amounts of recombinant protein per litre of culture both intracellularly and in secretory fashion. Thus, making *P. pastoris* a successful expression system for recombinant protein production with due scope of improvements for future discoveries. We review the characteristic features of *P. pastoris* as eukaryotic host system for the expression of recombinant proteins and advantages over other expression systems.

Keywords: *Pichia pastoris*, recombinant proteins, expression system, genetic engineering

1. Introduction
Nowadays, numerous approaches are available for the expression of recombinant proteins for their usage in medical as well as industrial fields. These heterologous proteins can include pharmaceutical drugs, recombinant vaccines, agricultural or industrial products [1]. Recombinant proteins have great application and utility in medical and research sphere which arises the need for the large scale production of recombinant proteins. Therefore, biological expression systems are the most favoured for heterologous protein production employing both prokaryotic and eukaryotic hosts. Some of the established expression hosts includes bacteria, yeasts, molds, mammals, plants and insects [2]. Among the prokaryotic cells, Gram negative bacteria are mostly used with *Escherichia coli* being ranked as the most important organism used in cloning experiments and recombinant protein production in the branch of genetic engineering [3].
Although, prokaryotic expression system offers the advantages of fast multiplication, simple and easy modification and cheaper system of expression in terms of nutritional requirements. Despite of such remarkable features, bacterial cells have certain demerits like improper protein folding, intracellular aggregation in form of inclusion bodies, chances of endotoxin contamination due to production of lipopolysaccharide, possibility of protein degradation and lack of posttranslational modifications [4]. Therefore, researchers experimented with other better alternatives to overcome these limitations for successful production of heterologous proteins. Eukaryotic yeast expression system like *Saccharomyces cerevisiae (S. cerevisiae)*, *P. pastoris* and mammalian systems [e.g. Chinese hamster ovary (CHO) cells] harbours the good potential for their efficient utilization as heterologous host for protein production. Out of all, *P. pastoris* is the most popular and standard tool in molecular biology for nanobody production [5, 6]. *P. pastoris* has been one of the most successful heterologous protein expression systems for production of wide variety of recombinant proteins until now [7]. This methylotrophic yeast is recently reclassified as *Komagataella pastoris* as a distinguished production system for its growth to very high cell densities, for the available strong and tightly regulated promoters, and for the options to produce gram amounts of recombinant protein per litre of culture both intracellularly and in secretory fashion [8]. Compared with bacteria, *P. pastoris* has many of the remarkable advantages of higher eukaryotic expression systems. Some of the key features and benefits of *P. pastoris* expression system include fast growth, high product titers, lower production cost, post-translational modifications, high clone stability, simplified downstream processing, low levels of endogenous protein secretion etc. These features make *P. pastoris* very useful as a protein expression system [9].

2. *P. pastoris* as an efficient heterologous protein expression system

Yeasts were more frequently used for production of heterologous proteins than bacterial systems because prokaryotes (*E. coli*) lacked the same post-translational protein folding machinery seen in higher species [10, 11]. Protein production using yeast as expression system is gaining popularity because of significant advantages like rapid proliferation in protein-free media, ability to carry out post-translational modifications, disulfide bond formation, which facilitates proper folding and capability to secrete product [12, 13]. While *P. pastoris* is the major strain used for expression of recombinant antibodies, other strains like *S. cerevisiae*, *Yarrowia lipolytica*, and *Schizosaccharomyces pombe* have also been used but to a lesser extent. Recombinant protein named (human glutamic acid decarboxylase) hGAD65 was expressed in the yeast and with yield of up to 12.16 mg/L was reported [14]. Recombinant hIL-6 was expressed successfully in large-scale cultures of the methylotrophic yeast *P. pastoris* with the yield of 280 mg/L [15]. *P. pastoris* was demonstrated as the most efficient host for remarkable production of protein with a yield of more than 18 g/L [16].

3. Historical background

The methylotrophic yeast *P. pastoris* was first isolated from a chestnut tree in France and described as Zygosaccharomyces pastori by the French mycologist and cytologist Alexandre Guilliermond Alexandre [17]. In 1950's, Herman Phaff isolated further related strains from oak trees in California and renamed the species as *P. pastoris* [18]. In 1995, the *P. pastoris* strains were recognized to a new genus named *Komagataella* and was separated into two species: *K. pastoris* and *K. phaffii*. Both the strains were used for recombinant protein production under the name *P. pastoris* [19, 20].

Koichi Ogata was the first to describe the ability of certain yeast species to utilize methanol as a sole source of carbon and energy [21]. The yeast *P. pastoris*, today allocated to the genus *Komagataella*, was introduced in field of biotechnology and it was in the era of 1970s when *P. pastoris* was evaluated for single cell protein production for food and feed applications due to the unique ability of utilizing methanol as a sole carbon source [22, 23]. The Phillips Petroleum Company was the first to develop media and protocols for the growth of *P. pastoris* on methanol in continuous culture at high cell densities (>130 g/dry cell weight [23]. However, in the upcoming years, Phillips Petroleum, together with the Salk Institute Biotechnology/Industrial Associates Inc. (SIBIA, La Jolla, CA, USA) investigated *P. pastoris* as a system for heterologous protein expression. It was SIBIA to come up as a pioneering institute for the isolation of the gene and promoter for alcohol oxidase and also evolved vectors, strains and corresponding protocols for the molecular manipulation of *P. pastoris* [24]. Research Corporation Technologies (Tucson, AZ, USA) are the current holders of the patent for the *P. pastoris* expression system, which they have held since 1993, and the *P. pastoris* expression system is now provided in the kit form that can be purchased from Invitrogen Corporation (Carlsbad, CA, USA).

4. Characteristics of *P. pastoris* as eukaryotic host system for the expression of recombinant proteins

4.1 Methanol metabolism in *P. pastoris*

The conceptual basis for the *P. pastoris* expression system stems from the observation that unique enzymes required for the metabolism of methanol are present at substantial levels only when cells are grown on the media containing ample amount of methanol [25]. *P. pastoris* was able to metabolize methanol via a novel pathway called MUT (methanol utilization) pathway that could be explained well in a step-by-step procedure involving several enzymes [26, 27]. The initial reactions occur in specialized micro-bodies, the peroxisomes, followed by subsequent metabolic steps in the cytoplasm. Peroxisomes play an indispensable role during growth, as they harbour the three main essential enzymes that were alcohol oxidase (AOX), catalase and dihydroxyacetone synthase [28]. The subsequent reactions of methanol assimilation and dissimilation were localized in the cytosol.

In the first step, AOX enzyme catalysed the oxidation of methanol to formaldehyde and hydrogen peroxide. Then, AOX was sequestered within the peroxisome along with enzyme catalase, which degrades hydrogen peroxide to oxygen and water. A portion of the formaldehyde generated by AOX leaves the peroxisome and was further oxidized to formate and carbon dioxide with help of two cytoplasmic dehydrogenases and the remaining formaldehyde was assimilated to form cellular constituents by a cyclic pathway that starts with the condensation of formaldehyde with xylulose 5-monophosphate, a reaction catalyzed by a third peroxisomal enzyme dihydroxyacetone synthase (DHAS) [29, 30]. Glyceraldehyde 3-phosphate and dihydroxyacetone are the main products of this reaction that leave the peroxisome and enter a cytoplasmic pathway that regenerates xylulose 5-monophosphate [31, 32]. Two novel enzymes namely AOX and DHAS that are essential for the methanol metabolic pathway were present at high levels only in the cells that were grown on methanol and were not found in appreciable amount in cells grown on some other carbon sources like glucose, glycerol or ethanol [33].

4.2 Plasmids and protein expression platforms

For recombinant protein expression in *P. pastoris*, it is very important to design a suitable expression machinery system that includes plasmid, promoter, selection marker, secretary signal sequence and host strain. All of these factors may be modified with the type of recombinant protein to be expressed and its final applications. The current status of the various types of promoter and plasmid systems, markers and platform strains that were commercially available is briefly explained.

4.2.1 AOX Promoters

Depending on the mode of controlled gene expression, there are different types of promoters used for the expression of recombinant proteins. The genome of *P. pastoris* comprised of two genes that encodes for the enzyme alcohol oxidase: AOX1 and AOX2; AOX1 was responsible for a vast majority of alcohol oxidase activity in the cell [23]. The AOX1 gene product was the most important protein in initializing the methanol utilization pathway and mainly used to construct expression vectors for heterologous protein expression. The AOX1 (pAOX1) promoter is having remarkable strength and constitutes up to 30% of total soluble protein in the *P. pastoris* cells grown on solely methanol [31]. The advantages of using the AOX1 promoter include that high levels of heterologous protein expression can be achieved, transcription of recombinant protein was controlled by a repression/de-repression mechanism and tightly regulated, the repression of the AOX1 gene by most carbon sources other than methanol ensures high cell growth before gene expression and induction

of transcription can be easily attained by the addition of methanol as a sole inducer [33, 34]. While on the contrary, the second alcohol oxidase AOX2 was controlled by a much weaker promoter (pAOX2) and only accounts for just 15% of the total AOX activity in the cells [35].

4.2.2 Methanol utilization phenotypes

Presently, three phenotypes of *P. pastoris* with regard to methanol utilization have been recognized: i) Mut+ (methanol utilization plus), where both AOX genes are intact and active; ii) MutS (methanol utilization slow), where AOX1 is knocked out; iii) Mut- (methanol utilization minus) where both AOX genes are knocked-out and is unable to grow on methanol as the sole carbon source [23]. It has been reported that the Mut+ strains were characterized by a higher growth rate than MutS strains and usually associated with higher productivities [36, 37]. When Mut+ and MutS strains were grown in the presence of sufficiently high concentrations of glucose or glycerol, the transcription of MUT pathway was repressed [38]. Since the rate of methanol consumption was higher for Mut+ strains, they were very sensitive to high methanol concentrations which may even cause difficulty in scaling up of bioprocesses [39]. Also sometimes it was cumbersome to meet high the demand for oxygen requirements of Mut+ phenotype in high cell density cultures [40, 41]. Moreover, the strong production of AOX1 in Mut+ strains during growth on methanol may compete with the production of recombinant proteins [42]. These drawbacks can be overcome by using *P. pastoris* strains with MutS phenotype due to the lower methanol consumption rate. However, the MutS phenotype also leads to long induction times and decreased growth rates. Commonly while using MutS phenotypes mixed feed strategies (e.g. glycerol and methanol) were employed for the induction phase [41]. Interestingly, it has been investigated that MutS phenotypes were more advantageous over Mut+ phenotypes for the production of some recombinant proteins [43, 44].

4.2.3 Host strains

All strains of *P. pastoris* yeast used for heterologous protein expression derive their origin from NRRL-Y 11430 or X-33 wild type strain. The choices of these strains depend on the desired application and their ability to metabolize methanol [45]. Mutations in the auxotrophic genes have given rise to different strains with specific growth in complex media and minimal media supplemented with His and arginine. Similarly, deletions in AOXI and AOXII genes render mutant variants of *P. pastoris* [46]. Strain GS115 (his4) has both AOX genes functional and thus metabolizes methanol at a high rate, similar to that of wild type, and were recognized as Mut+ (methanol utilization phenotype). Strain KM71 (his4 arg4 aox1D::ARG4) was generated by deleting the chromosomal AOX1 gene and replacing it with *S. cerevisiae* ARG4, it relies on activity of weaker AOXII gene and show slow growth on methanol, this phenotype was called as MutS (methanol utilization slow). The third type of expression host, MC100-3 (his4 arg4 aox1D::SARG4 aox2D::Phis4) had deletion of both AOX genes and cannot grow on methanol as carbon source; this phenotype was Mut- (methanol utilization minus). Recombinant proteins secreted by *P. pastoris* into the culture medium were rapidly degraded by yeast vacuole proteases during high-cell-density fermentations leading to lower yield [47, 48]. The use of protease-deficient strains like SMD 1163, SMD 1165 and SMD 1168 had been very well documented for the expression of certain proteins that were protein sensitive [49]. The host strains, SMD1165 (his4 prb1) and SMD1168 (his4 pep4), were developed by deleting the PEP4 gene, which encoded for proteinase A, and PRB1 encoded for proteinase B [50]. PEP4 and PRB1 protease deficient strains were commonly used in inhibiting protein degradation [7].

4.2.4 Selectable markers

Several selectable markers were available for the molecular manipulation of *P. pastoris* at genetic level. The plasmid of *P. pastoris* had selectable markers that contain antibiotic resistance genes such as Shble, bsr and nptII or nptIII which confer resistance to antibiotics such as zeocin, blasticidin and kanamycin [51]. The most commonly used antibiotic resistance genes included Shble gene derived from *Streptoalloteichus hindustanus* that confered resistance to drug Zeocin and the blasticidin S deaminase gene (BSD) from *Aspergillus terreus* which confered resistance to blasticidin [52, 53]. The auxotrophic strains contain genes that are used as selectable markers include HIS4 (histidinol dehydrogenase gene) from either *P. pastoris* or *S. cerevisiae* and ARG4 (argininosuccinate lyase gene) from *S. cerevisiae* [51]. However, there were certain limitations with the use of certain selectable marker genes for example Zeocin and blasticidin were bit expensive and the URA3 and ADE1 strains of *P. pastoris* were slow-growing but if these strains were transformed with vectors containing the complementing biosynthetic genes (i.e. ADE1 or URA3) growth rate similar to that of wild strain could be attained [51].

4.2.5 Expression vector and secretion signal

P. pastoris vector system was a typical *E. coli*-based shuttle vector system which means that they can propagate in two different host species [54]. The expression vectors in the *P. pastoris* were mainly composed of three sequences: promoter sequence (most often AOX1) in 5′ region; transcriptional termination sequence in 3′ region which was essential in the processing and polyadenylation of messenger RNAs; and one sequence that contains single or multiple cloning sites (MCS) essential for the insertion of foreign DNA/ the gene of interest [55].

The vectors also contain an origin of replication for maintenance of plasmid in *E. coli* and selectable markers for selection of transformant colonies from either organism [54]. Drug resistance genes such as Kan, Shble, Bsd, Amp, or FLD1 were present that confered resistance to geneticin, zeocin, blasticidin, ampicillin and formaldehyde respectively [54, 57]. Signal sequences derived from the *P. pastoris* acid phosphatase pho1p or the *S. cerevisiae* α-mating factor were also present to generate in-frame gene fusions in vectors used for the expression of heterologous proteins that were ultimately secreted from the host cell. It has been observed that plasmids used in the *P. pastoris* expression system can produce both extracellular and intracellular proteins [51].

4.3 Protein glycosylation in *P. pastoris*

For certain therapeutic proteins such as monoclonal antibodies, interferons and erythropoietin, glycosylation was a crucial element that palyed important role in acquiring complete biological and functional activity [58]. *P. pastoris* was an organism capable of producing a variety of active proteins with N- and/or O-linked glycans [59, 60]. The polypeptides after being translocated to the endoplasmic reticulum were either linked to asparagine residues (N-glycans) or to serine or threonine residues (O-glycans).

4.3.1 O-linked glycosylation
O-linked glycosylation in yeast was different from that of mammalian O-glycosylation and it is known that in *P. pastoris* O-linked oligosaccharides were mainly composed of mannose (Man) residues arranged in form of short linear chains of α-1, 2-linked mannans with β-1, 2-linked or phosphorylated mannose at the outer ends [60]. In mammals, O-linked oligosaccharides were composed of a variety of sugars including N-acetylglucosamine, galactose (Gal) and sialic acid (NeuAc). It had been discovered that there were five native protein O-mannosyltransferases (PMTs) that initiated O-linked glycosylation and grouped into subfamilies PMT1, PMT2 and PMT4. The PMT1 and PMT2 subfamily genes played major role in O-linked glycosylation [61].

4.3.2 N-linked glycosylation
N-linked glycosylation was an important fundamental post translational modification that played a pivotal role in protein folding, pharmacodynamics behaviour and biological functions of various therapeutic proteins. N-glycosylation was evolutionarily conserved in almost all life forms including eukaryotes, bacteria and even archaea [62, 63]. It had been observed that membrane-bound and secreted proteins with essential biological activity were mainly N-glycosylated in mammal [64]. Generally, the core structure of N-glycans consisted of two N-acetyl glucosamine and three mannose residues forming a branch [65]. Describing the classical pathway of N-linked glycosylation, the oligosaccharide core unit, Glc3Man9GlcNAc2 (Glc=glucose, GlcNAc=N-acetyl glucosamine) was transferred to the asparagine residue in the recognition sequence Asn-X-Ser/Thr in the endoplasmic reticulum and finally matured in Golgi apparatus [66]. Finally, the oligosaccharides produced in *P. pastoris* did not have any terminal α-1,3-linked mannosylation as mannosylation was found to reduce activity of therapeutic proteins and rendered them unsuitable for pharmaceutical use in humans. However, *Pichia* had been engineered to the mammalian N-glycosylation pathway [67].

5. Advantages of using *P. pastoris* as an expression host system
Yeast strain *P. pastoris* has several advantages over other eukaryotic and prokaryotic expression systems as far as recombinant protein production is concerned [68]. The distinguishing features of *P. pastoris* include rapid growth in high cell densities using minimal media, coupled with ease of high cell-density fermentation even in continuous and large scale fermenters [69]. *P. pastoris* showed high levels of productivity in an almost protein-free medium with elimination of endotoxin and there was no chance of bacteriophage contamination [70]. *P. pastoris* is capable of genetic manipulations e.g. gene targeting, high-frequency DNA transformation, cloning by functional complementation, high levels of protein expression at the intra- or extracellular level [7, 23]. This methylotropic yeast is capable of carrying out diverse post-translational modifications that included polypeptide folding, glycosylation, methylation, acylation, proteolytic adjustment, and targeting to subcellular compartments and has the ability to engineer secreted proteins that can be purified by simple processing from growth medium without harvesting the yeast cells themselves [55, 70]. Since *P. pastoris* has the unique ability of thriving in a methanol rich media, with consumption driven by the alcohol oxidase I (AOX1) promoter which is employed for methanol-dependent expression of a desired protein product and hence it can simplify the downstream processing steps for a recombinant protein production [71]. It has been observed that yeast cells may lead to hyperglycosylation of the recombinant proteins which may result in altered pharmacokinetics of the desired product. Thus to mitigate this problem, glycoengineered *P. pastoris* cells have been developed that express antibodies with superior volumetric productivity [72]. Yields of up to 1.4 g/L of humanized IgG have been reported with glycoengineered *P. pastoris* which is far superior to mammalian systems [73]. Also expression systems like bacteria, *S. cerevisiae* or the insect cell/baculovirus failed to express certain proteins so efficiently that were successfully produced in *P. pastoris* in a functionally active form [74, 75].

6. Conclusion and perspectives
Yeast expression systems namely, *S. cerevisiae* and *P. pastoris* are gaining popularity as successful biological expression systems for production of recombinant proteins for their utility in medical and research fields. For heterologous protein production, *P. pastoris* is one of the most widely used and standard tool that favours the different experiments of genetic cloning in molecular biology. Though, *P. pastoris* is the unique host with some remarkable features for recombinant protein production. However, to achieve the maximum benefit in terms of higher yield of target proteins, the transformation process can be optimized for more efficient and large scale production in large biofermentors employing minimum nutritional requirements, thus making the process inexpensive, rapid and easy to perform at industrial scale for production of suitable products.

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