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Pichia pastoris expression system: a potential candidate to express protein in industrial and biopharmaceutical domains

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Abstract

The methylotrophic yeast Pichia pastoris is a widely used, well established protein expression system for the production of biopharmaceutical and industrial enzymes. It is by far one of the most commonly utilized microbial species due to its high cell density growth, ease to use, rapid expression time with low cost and post-translation modification system. Moreover, its strong regulated promoters, the capacity to meet high production yield even of proteins of large molecular weight, simple recovery and purification steps makes it superior in contrast to other expression systems. Recent advancements in high-throughput genomics and development of humanized glycosylated Pichia pastoris strain will increase its importance in biopharmaceutical production. This review aims to highlight Pichia pastoris expression system as a potential candidate to express industrial and biopharmaceutical proteins alongside bioprocess design and expression optimization strategies to increase product yield.

Introduction

Yeast *Pichia pastoris* is successfully used as an expression host for the production of various recombinant proteins[1]. It is used as an industrial host for heterologous proteins and metabolic production, with the capacity to meet target production levels [2]. In addition, the expression system has also proven to be an economical and effective source for the enhanced synthesis of different industrially valuable proteins [3, 4]. *P. pastoris* was initially used by the Philips petroleum in 1973, for the production of single-cell protein (SCP) to fulfill the demand of animal feed.Since then, it is being investigated extensively to express variety of recombinant proteins for commercial use [3]. Over 500 proteins including the industrial enzymes and biopharmaceuticals have been expressed in this expression system, with an increasing number of commercial products reached the market in recent years [5]. It was developed as a protein expression system in the 1980s using the strong and highly regulated promoter AOXI to metabolize methanol as an inducer for protein expression [3].In 1990, the plant derived hydroxy nitrile lyase was the first enzyme that was produced on large-scale in *Pichia* with more than 20 g protein per liter of culture volume [6]. Host strains having their genome sequenced initially were the GS115[7], DSMZ 70382 [8] and the strain CBS7435 [9]. Advancement in *Pichia* strain comprised the development of genetically modified strains to produce humanized glycoprotein [10], synthetic promoters to precisely control gene expression [11] and the expression systems having modified transcription, translation, Post-translational modification and producing synthetic regulatory networks [10]. Notable breakthroughs on the commercial side include the GRAS (generally recognized as safe) status given by FDA (Food and Drug Administration), and the FDA approval to market initial biopharmaceutical products, Jetrea® and Kalbitor® [12, 13].

Pichia pastoris provides the potential to synthesize soluble, correctly folded recombinant proteins [2]. It has several advantages over other eukaryotic and prokaryotic expression systems, for instance : (i) rapid growth rate with high cell density fermentation (ii) do not require complex medium or culture conditions (iii) easy to manipulate genetically (iv) diverse post-translational modification, afterwards, targeting to subcellular compartments (v) less extensive glycosylation as compared to other yeasts such as *S. cerevisiae* (vi) negligible levels of host protein secretion [14,15].

In this review, the salient features of *Pichia pastoris* to be used as a prospective expression host in the recombinant biotechnology industry are focused. The first part refers to the established Pichia vectors, host strains used in routine and the development in its genomics. In the second part, the expression optimization strategies, and recombinant proteins of industrial and biopharmaceutical importance expressed in *Pichia pastoris* are described.

Established systems for protein expression

Host strains

Commercial Pichia pastoris strains used in academic research and industry are GS115, X-33, PichiaPinkTM and KM71. Commonly employed protease deficient strains are SMD1168 and SMD1168H. Humanized glycoengineered strains provided by Biogrammatics are Super Man5 (AOXI-, Muts), SuperMan5 (pep4-, prb1-) and SuperMan5 (pep4-,Sub2-)(**Table 1**). The choices of these strains depend on the desired application and their ability to metabolize methanol. Pichia strains are categorized according to their ability to utilize methanol as wild type methanol utilization (Mut+ phenotype), carrying the alcohol oxidase1 gene (AOXI) [16], methanol utilization slow (Muts), due to nonfunctional AOXI gene, the alcohol oxidase is produced by AOX2 gene. AOX2 has the same specificity as AOXI but expression level is low, and lastly, methanol utilization minus (Mut-), both AOXI and AOX2 genes are deleted [4]. Additionally, Pichia strains carry modifications, including protease deficiency and auxotrophic mutants to allow recombinant selection, where the recombinant vectors harbor the deficient gene as selectable marker. Several auxotrophic mutants are used such as (His4, Arg4, Ade1, Ura3), with mutant strains carrying one or two auxotrophies, some strains even carry 4 auxotrophies such as JCDO8 (His4, Arg4 Ade1, Ura3). Furthermore, this expression system carries protease deficient strains to minimize undesired proteolysis, which in turn effects downstream processing. PEP4 and PRB1 protease deficient strains are commonly used in inhibiting protein degradation [17].

Expression vectors

At commercial scale, Thermo Fisher Scientific (USA) markets Pichia expression vectors including constitutive (pGAP) glyceraldehydes dehydrogenase

Table 1: *P. pastoris* host strains used for transformation and protein expression in commercial and research labs.

Strain	Genotype	Reference/Supplier
Glyco-engineered strains		
SuperMan5	HIS4^{+}, Och1-disruption	BioGrammatics
SuperMan5	HIS4^{-}, Och1 disruption	BioGrammatics
SuperMan5	HIS4^{+},pep4,Och1 disruption	BioGrammatics
Wild type strain		
BG10	Willd type	BioGrammatics
X-33	Wild type	Thermo Fisher Scientific
CBS 704 (DSMZ 70382)	Wild type	CentraalbureauvoorSchimmelcultures
CBS7435(NRRLY-11430)	Wild type	CentraalbureauvoorSchimmelcultures
pps-9010	Wild type	DNA 2.0
Protease deficient strain		
SMD1163	his4,pep4,prb1	2
SMD1165	his4,prb1	2
SMD1168	his4,pep4,::URA3,ura3	Thermo Fisher Scientific
SMD1168H	pep4	Thermo Fisher Scientific
BG21	sub2	BioGrammatics
PichiaPink™	ade2,pep4	Thermo Fisher Scientific
PichiaPink™	ade,prb1	Thermo Fisher Scientific
PichiaPink™	ade2,prb1,pep4	Thermo Fisher Scientific
pps-9016	pep4Δ,prb1Δ	DNA 2.0
Auxotrophic strain		
PichiaPink™	ade2	Thermo Fisher Scientific
GS115	his4	Thermo Fisher Scientific
BG12	his4	BioGrammatics
KM71	his4,aox1::ARG4,arg4	Thermo Fisher Scientific
BG09	arg4::nourseoR Δlys2::hygR	BioGrammatics

Table 2: Commercial *Pichia pastoris expression vectors.*

Supplier/Reference	Vector names	Promoter	Signal sequence	Selection
BioGrammatics	pJAN–s1, pJAZ–s1,	AOXI	*S. cerevisiae* α-MF	nourseothricin
	pJAG–s1 ,pJAN, pJAZ, pJAG			zeocin G418 selection
BioGrammatics-GlycoSwitch®	pGlycoSwitch®–GnT–I pGlycoSwitch®–GnT–I–HIS pGlycoSwitch®–ManII/1 pGlycoSwitch®–GnT–II pGlycoSwitch®–GalT/1	GAP		Zeocin, Histidine, kanamycin, Hygromycin, Nourseothricin
Thermo Fisher – Scientific	pPICZ pGAPZ and pGAPZα pAO815/ pPIC3.5K, pPIC9K pPIC6 and pPIC6α	AOXI GAP AOXI AOXI	S. cerevisiae α-MF	Zeocin™, Ampicillin, Gentamicin, Blasticidin
Thermo Fisher Scientific - Pichia pink™	pPINK-HC pPINKα-HC	AOXI	S. cerevisiae α-MF; set of 8 different secretion signal sequences	Ampicillin
DNA2.0	pD902,pD905 pD912,pD915	AOXI, GAP	11 different signal sequences	Ampicillin, Zeocin™

promoter and inducible promoter (pAOXI), expressed by methanol. In addition, Biogrammatics (USA), market Glycoswitch® vectors for humanized glycosylation protein including pIAZa and PJAGa. **Table 2** summarizes the available commercial P. pastoris expression vectors. These expression vectors contain an origin of replication to propagate. Most of the Pichia vectors carry multiple cloning sites (MCS) to insert the desired coding sequence flanked by the promoter and terminal sequence [16]. For secretory expression, these vectors comprise of *S. cerevisiae* alpha-MF secretion signal to target desired protein to secretory pathway [18]. Typically, Pichia pastoris system exhibit stable integration into the host

genome for protein expression. Another selection system reported is Pichiapinkn TM expression that offers an efficient way to screen high-expression transformants based on white and pink colonies. The color of colonies indicates the level of protein expressed due to the copy number of the plasmid. Pink color expresses very low construct integrated into Pichia strains, whereas white colonies reflect higher transformed gene construct via stable integration of the recombinant plasmid into the host genome [19, 20].

Genomics of *Pichia pastoris*

Efforts have been made in the past to explore and elucidate *P. pastorisgenome*, however, despite its demonstrated utility and commercial success to date, relatively little strain engineering has been done. The prime reason is the limited number and inconsistent frameworks of reported genomes [21]. The first assembled P. pastoris genome with annotated genes of *K. phaffi* GS115 strain was published in 2009 [7]. A similar approach for wild type *K. phaffi* (NRRL Y-11430; CB 57435) refined the assembly of GS115 [9]. These studies demonstrated that Pichia pastoris genome is organized in four chromosomes, and they were estimated to be 2.88(2.8+0.08), 2.39, 2.24 and 1.8(1.78+0.017) Mbp in size after assembly, with an estimated size of 9.4 Mbp of GS115 strain. Moreover, manually created annotation of 5313 protein-coding genes contain 41.6% GC content and protein coding genes occupy 80% of the genome sequence [7].

Recent studies have explored other functional elements within the Pichia pastoris genome including autonomously replicating sequences (ARS) and two internal ribosome entry site IRES elements [22], although these functional elements may not impact gene expression directly but they could have a regulation role in nearby functional elements [22]. A detailed comparative analysis of genomic features of common *P. pastoris* strains including *P. pastoris* wild-type, *K.phaffi* wild type and *K.phaffi* GS115 strains reveal 35 non-synonymous coding mutations that infer potential influence on protein function. Additionally, transcriptomic profiles of gene expression during cultivation stages using different carbon sources including methanol, glycerol, and glucose reveal that most highly transcribed genes were consistent in all these carbon sources [23].

Use of Pichia pastoris as a model organism in the industry and its future development is impeded by shortcomings in the functional annotation of its genome. There are important gaps in our understandings of its molecular cell biology and physiology which impedes its rational design to accommodate biopharmaceutical and biotechnology industry [21]. There is also limited knowledge on how gene expression of null growth strains compare during growth on relevant carbon sources [22]. Despite these limitations, more consistent and common genomic data will help in facilitating strain engineering to utilize the host effectively in future, to overcome secretory capacity limitations and to improve metabolic pathways. Further insight into transcriptional activity will help in host engineering and process engineering as well. Similarly, there are interesting opportunities to explore in future like the activity of promoters gene expression under different carbon sources. Discovery of telomeric and linear plasmid sequence will facilitate the engineering of new plasmids and artificial chromosomes [23].

Application of *Pichia pastoris*

Yeasts are feasible to produce heterologous recombinant proteins because of their ease of genetic manipulation, high yield with rapid growth rate, ability to perform post-translation modification in contrast to bacterial cells [24]. Therefore, these important features have been exploited in biotechnology, particularly, in the brewing industry in baking yeast where yeast Candida grew together with *Saccharomyces cerevisiae*. One of their important roles is in the supply of ethanol as a renewable energy source, which has substantial potential in future due to the dearth of major economic recourses globally [25]. Pichia pastoris presented as one of the key expression systems among these yeasts. Its success story has to line with its ability to grow in inexpensive media with high cell density that makes it favorable for industrial scale [26]. This part describes the expression optimization and bioprocess operations for Pichia and its beneficial outcomes in recombinant products.

Expression optimization and bioprocess operations

Prior to expression yield, Pichia pastoris recombinant products need to meet three pre-conditions to successfully develop an optimal multistage production process. These pre-conditions include (i) approaches to fully perform automated and observable processes, (ii) identification of the host-cell distinct reaction parameters to perform an adapted process layout for aeration and feeding strategies, and lastly (iii) Information about favorable optimal operation parameter conditions including; pH, temperature, induction and feeding strategies,

media compositions, in order to maximize quantitative and qualitative productivity of desired protein[16, 27]. Although, these parameters slightly differ but can be modified according to the genotype of *Pichia* strain, and desired protein for high productive yield.

The culture media affects the product yield because it directly alters the growth rate and viability of microbial cells. Pichia strains grow in specific media with additional supplements included for respective strains [28]. For instance, minimal media is required for strains X-33, GS115, and KM71H, along with supplements including arginine and histidine [19]. Complex media including YPDS, BMGY, BMMY are also used for protein expression and selecting zeotransformants. Initially, most commonly used media was Basal salt medium (BSM), comprising of Biotin, ammonium hydroxide, methanol or glycerol as the carbon source with trace elements (ferrous sulpfate, heptahydrate, zinc chloride). However, precipitation of salts and turbidity was a major issue, therefore alternative media were developed with trace salt guidelines [15]. Although many proteins have been expressed using the standard protocols but there are some proteins which don't follow these standard guidelines and protocols. Invitrogen (Thermo Fisher) has provided general guidelines regarding culture conditions, to achieve an increased recombinant protein yield, particularly helpful in proteins where conditions are difficult to optimize. The temperature range in this regard for bioreactor is usually between 28-30°C [29]. For higher secretory expression, it is better to use an antifoam agent. Routledge et al. found that presence of antifoam in shake flask cultures enhanced the total yield, also few antifoams enhanced the density of culture subsequently increasing the yield [30]. pH plays a crucial role in enzyme stability and growth rate, and Pichia pastoris relatively confers a broad pH range from 3.0 to 7.0 but usually, the strain is cultured at pH 5-6 [29, 31]. Agitation speed for bioreactors is suggested between ranges of 500 and 1500 rpm, for shake flask range of 250-300 rpm is proposed. Aeration is also an important factor as it influences high cell density, therefore, a range of 0.1-1.0 vvm (in order to achieve maximum oxygen concentration in the medium) is used [15].

Generally, a three-step scheme is deployed in the fed-batch mode as a standard fermentation procedure [24]. The first stage (growth phase) can produce wet cell weight up to 100g/L. The second stage (termed as transition stage) is a fed-batch phase, glycerol is further added in culture media to produce more biomass, also, to transition successfully from glycerol to methanol metabolism phase[3, 29]. During this transition phase, glycerol is utilized completely. This step involves either the sole supply of glycerol or mixed feed of methanol and glycerol. The last stage (production phase) marked with the addition of methanol to induce protein expression. AOXI promoter is used commonly since it is repressed by glucose; hence glycerol is used as a carbon source to generate biomass while repressing gene expression [3, 29]. Fermentation strategy is also utilized employing pGAP promoter (promoter for constitutive expression using glucose). Without having transition and production stages required using pGAP promoter, a protein produced depends on cell mass. It substitutes the need for methanol as carbon source having longer production periods during the continuous fermentation phase, along with higher protein yield presents an alternative option in large-scale fermentation processes using Pichia pastoris. The overall efficiency of protein expression using GAP promoter depends on the target protein to express [20, 32].

Besides, success in achieving high cell densities in *P. pastoris*, sometimes the secreted protein is proteolytically degraded by host strain specific proteases, because of the environmental stress during high-density processes and proteases released in the culture medium. A number of strategies have been used to overcome this issue including; the addition of amino acid supplements and organic nitrogen sources in culture media, increase inpH, addition of synthetic inhibitors into the culture broth [33] and lastly using protease-deficient *P. pastoris* strains including SMD 1168, BG 21, SMD163 and SMD1165 [34].

Cultivation strategies and biopharmaceutical design as discussed above focused entirely on finding optimum conditions to increase biomass growth and proliferate product yield by optimizing pH, temperature, nutrient supply, oxygen and media compositions. At the same time, a recent trend in literature also suggests that there is a shift from standard protocols (i.e. growth-based production kinetics) towards a specific process approach; relative to the product, genetic construct, and features of specific bioprocess design [35-37]. The relationship between biomass growth (expressed in specific growth rate u) and product formation (qp) values, which are strain specific with different bioreactors, can be used to compare system and strain designs not aligned to specific operation settings as reactor volume or biomass concentration.

This customizable approach which has carried physiological characterization can be adapted to any industrial strain or bioreactor and it will help in exploring their difference with standard protocols[38]. However, there is still need of efforts in this process development approach, to incorporate further methods in upgrading the productivity of process control at large scale. These processes, for instance, includes: using software-based sensors; to reduce experimental load wasted in time-consuming physiological cell characterization; screening single cell to indicate product formation and to improve the quality of proteins by boosting growth and process conditions [39, 40]. To attain optimum process operation associated with the specific product, the relationship between protein production and biomass is crucial. Therefore, further understanding would encourage the concept of process characteristics. The process comparison and development is more significant now, with the advent of new genetic constructs [38].

Recombinant products of industrial importance expressed in *Pichia pastoris*

Pichia pastoris is widely used to produce a large number of important proteins and enzymes of industrial importance (**Table 3**). Recent expanding developments reflect a substantial interest in the application of this system for its use in industrial and pharmaceutical products in contrast to other expression systems [41]. Monogastric animals are incapable of digesting phytate phosphorus which is a major form of phosphorus in cereals, legumes and this ingested phytate is largely excreted causing nutritional deficiencies, so these animals require extrinsic supplementation of phytase in their feed to increase phosphorus availability [42]. Most phytases are produced in bacteria and fungi. However, only a few expressed at high level. In addition, the high cost of phytase is a barrier to use it in industrial applications. To facilitate phytase usage in industry, an industrial grade host having high expression level is necessary. *Pichia pastoris* provides improved phytase expression levels for industrial application. Several studies have been conducted indicating high expression of phytase in *P.pastoris* that can be applied in different feed additive applications. A synthetic gene encoding Peniophoralyciiphytase incorporated in Pichia by codon usage optimization and α-factor modification expressed 12.2 g/L in a 5-L fermenter and 10540 U/mL phytaseactivity. Similarly, in a 10-L scaled-up fed-batch fermenter, the phytase gene from C. amalonaticus CGMCC achieved 9.58 g/L yield with an activity of 35,032 U/mL [43].

Amylase, another important industrial enzyme, is utilized in a broad range of industries including starch hydrolysis, textile, food, and brewing industries. Most of the commercial requirement of amylases could meet only through fungal amylases. A pertinent issue regarding amylase usage is their low thermal and acid stability and thermal stabilities of most fungal amylases is not satisfactory. A promising way proposed in this regard is to clone thermostable fungal amylase and subsequently expressing it heterologously in a high-level expression system. This promising way could accelerate the utility of thermostable enzyme and reveal its further application in starch hydrolysis industry. In a study, a novel fungal glucoamylase and α-amylase genes cloned from Rhizomucorpusillus, and then expressed successfully in Pichia pastoris significantly improved amylase activity and much higher yield was obtained in contrast to other glucoamylases and α-amylases [44].

Lytic enzymes are widely used in different industries such as food, pharmaceutical, paper, textile, detergent, and beverage industry. Pectinases are applied in extracting and clarification of juices in the food industry[45]. A high expression level pectinase, polygalacturonase expressed in Pichia pastoris obtained, showed a maximal activity of 2408.70 ± 26.50 U/mL in the culture supernatant with 4.8 times higher yield than that from shake flask. This increased expression level and homogeneity can be achieved in the bench-scale bioreactor[46].

Lipase, a vital enzyme widely used in medical and therapeutic fields, food processing industries, biodiesel production, ester synthesis and waste water treatment because of its catalytic properties. Lipases from microorganism have been used substantially particularly from *Yarrowialipolytica*. Though the production has improved by increasing copy number in *Y. lipolytica* but the use of long chain fatty acids as its carbon source and inducer in fermentation, bind lipase and cause difficulty in downstream processing, purification in *Y. lipolytica*, and require organic solvents to remove fatty acids or oil from lipase. Heterologous expressions of YlLip2 in P. pastoris have been used to overcome this problem; as a result, increased lipase yield can be expressed into culture medium during fermentation. High yield of Lipase in P. pastoris with similar characteristics to the native lipase in *Y. lipolytica* could decrease its production cost [47].

Table 3: Examples of enzymes of industrial importance expressed in *Pichia pastoris*

Enzyme	Origin	Pichia strain	Yield	Reference
Xylanase				
Alkaline Xylanase	*Bacillus pumilus*	GS115	6,403 U/ml	[67]
Xylanase XYN10B	*Thermotogamaritima*	GS115	375 IU/mg	[68]
Protease				
Neutral protease I	*Aspergillus oryzae*	GS115	12.87 mg/mL	[69]
Serine protease	*Trichoderma koningii*	GS115	3.2g/l	[70]
Alkaline Protease	*Aspergillus oryzae*	GS115	513 mg/L	[71]
Amylase				
α –Amylase	*Bacillus licheniformis*	GS115	8.3 g/l	[72]
α-Amylase	*G. stearothermophilus*	GS115	28.6 U mL−1	[73]
Glucoamylase RpGla	*Rhizomucor pusillus*	KM71/9KGla	1237 U ml-1	[44]
α –Amylase	*Barley*	X-33	68U/ml, 0.125mg/L	[74]
Lipase				
Lipase	*Aureobasidium melanogenum*	X-33	3.8 U/mL	[75]
Lipase	*Yarrowiali polytica*	GS115	2.82g/L	[76]
Lipase	*B. cepacia*	GS115	184.3 U/mL	[77]
Phytase				
phytase	*P. lycii*	KM71	12.2g/L	[78]
Phytase	*Neosartoryas pinosa*	X33	30.95 U/mg	[79]
Phytase	*Citrobacteram alonaticus*	GS115	9.58 g/L	[43]
Laccase				
Laccase	*Trametes versicolor*	GS115	11.972 U/L	[80]
Laccase	*Ganoderma lucidum*	CBS7435	685.8 U L^{-1}	[81]
Laccase	*Botrytis aclada*	X33	495 mgL-1	[50]
Cellulase	*Trichoderma reesei*	GS115	6.55g/L	[82]
β-glucosidase	*Paecilomyces thermophila*	GS115	274.4 U/ml	[83]
β-glucosidase	*Trichoderma reesei*	GS115	60 U/ml	[84]
Trypsin	*porcine*	GS115	0.48mg/ml	[85]
Trypsin	*Streptomyces griseus*	GS115	14.4 U ml^{-1}	[86]
Trypsin	*Streptomyces griseus*	GS115	47.4U/ml	[87]
Lysozyme	human leukocytes	GS115	533 U/ml	[88]
Pectate lyase	*Bacillus subtilis168*	GS115	271 U/mL	[89]
Hydrolase	Commercial encoded gene	GS115	4.1g/L	[90]
Peroxidase	*Trachycarpus fortune*	GS115	112 U/mg	[91]
Galactosidase	*A. niger*	KM71H	1299 U/ml	[92]
Aminopeptidases	*Aspergilus oryzae*	GS115	61.26 U mL	[93]
Bovine Lactoferrin	Optimized codon	KM7H1	3.5 g/L	[94]

Gelatin (denatured collagen), a gelling agent and versatile biomaterial, have medical and technical applications. Traditional animal gelatin and their extraction procedures limit its application in industry. The high gelling temperature of traditional gelatin is another loophole to use it in low-temperature processes. Gelatin synthesized from microbial systems provides additional benefit to having well defined, free of animal derived contagious agents and can be modified for specific needs. Pichia is able to produce non-gelling mammalian gelatin to cope thermal and stability issues. In fact, utilizing the α-factor pre-prosequece in *P. pastoris*, secretion yield can be reached to ∼15 g/liter in clarified fermentation broths as a substitute for animal-derived gelatin with improved features [45, 48].

Trypsin, a serine protease, is used in food processing, leather batting, pharmaceuticals, clinical diagnosis and various other biochemical applications. This commercially important enzyme is currently extracted from bovine pancreas, but this conventional approach is unable to meet its demand. This extraction process also gets contaminated with pathogens that can cause immunogenic issues in the pharmaceutical industry. To circumvent cell toxicity of intracellular trypsin and to enhance secretory expression, trypsin gene is expressed in Pichia pastoris from different sources. Recently Streptomyces griseus trypsin (SGT), a potential alternative candidate of bovine trypsin is expressed in Pichia by engineering the N-terminus of mature SGT that significantly improved the extracellular expression to 47.4 ± 1.2 Uml−1, 329% higher than parent Pichia pastoris GS115-SGT strain [49]. Recombinant trypsin produced in P. pastoris is commercially marketed by Roche, is used as a

protease in research [45]. Heterologous expression of enzyme laccases is important for their large-scale production that is a prerequisite for their commercial application. They act as promising biocatalysts for a broad range of applications including bleaching, dye decolorization, stabilizing beverages and bioremediation. P. pastoris is the preferred system for fungal laccases, and expression can reach up to 495 mgL-1 in the culture supernatant with higher specific activity, using GAP promoter resulting in a shorter fermentation time [50]. In addition with *S. cerevisiae* system, which is considerably used in protein expression processes over the years, *P. pastoris* is increasingly becoming a versatile expression system in the industry to scale up heterologous proteins due to its high-cell-density fermentation (HCDF) with as much as >100 g/L dry cell weight or >400 g/L wet cell weight and an increasing number of protein products are hitting the market[29].

Pichia pastoris and Biopharmaceutical Industry

Biopharmaceuticals are markedly important sector of modern medicine. These include therapeutic proteins including hormones, vaccines, interferon, growth hormones and antibodies, specifically categorized as protein biopharmaceuticals [51]. Their estimated market share is 25% in commercial pharmaceuticals, with global sales exceeding US$ 100 billion in 2010 [52], growing at an annual rate between 7 and 15%. In 2012, the biopharmaceutical market turnover was between 100–120 billion US dollars per year and it's expected to reach 278 billion US$ by 2020 at an annual growth rate of 9.4% [53]. P. pastoris system is used successfully to produce high titers of numerous recombinant therapeutic protein, as the system is capable of synthesizing complex proteins requiring; post-translational modifications, high yield volume of active functional protein, simple medium formulation requirement, fermentation easily scale up to commercial scale [22, 52]. Among biopharmaceuticals, Insulin is by far the leading recombinant protein produced in yeast, and the growth market is anticipated to reach more than USD32 billion by 2018, due to the continuous growth of global insulin market [51]. Currently, Insulin is produced either through the synthesis of Insulin precursor synthesized as inclusion bodies in the E. coli as expression host with further solubilization and refolding procedures. The second route harness yeast expression system particularly *S. cerevisiae* and *P. Pastoris*, with the formation of insulin precursor (IP) into the culture supernatant, afterward subsequent insulin purification steps. Several reports have suggested *P. pastoris* application for insulin production, and comparative studies suggested an equal or even better performance in contrast to S. cerevisiae. The expression yields reported varies and production up to 4 gram per liter of cell-free culture supernatant [54] has been reported.

P. pastoris system is also used to express a number of subunit vaccines, particularly against human viruses. These recombinant protein antigens used in vaccines include; Epstein-Barr virus (EBV), Dengue virus (DENV-3) envelope domain-III (EDIII), Avian influenza virus (H5N1) neuraminidase (NA), Hepatitis B virus surface antigen (HBsAg), Human papillomavirus type 16 major capsid protein L1 and *Human enterovirus* 71 capsid protein VP1. Though P. pastoris system elements are well developed now, there is still room to further optimize protein expression in the context of antigenic vaccine production, for instance, gene dosage, codon bias, culture conditions and endoplasmic reticulum protein folding [55]. Antibodies are one of the largest group and rapidly growing class of protein biopharmaceutical market. Even though the first full-length IgG was reported in 1980 in Pichia pastoris, but humanized N-linked glycan was not feasible until recently due to glycoengineering. Literature data shows that Pichia produces similar stable IgG as expressed in Chinese hamster ovary (CHO) cells. Currently full-length antibodies are produced in mammalian cells, but they have severe limitations in scaling up and long processing time at industrial level, whereas Pichia allows high cell densities, providing higher yield than mammalian cells, therefore, humanized glycol-engineered *Pichia* strain is increasingly becoming an important system to produce human glycosylated full-length antibodies[56, 57]. A list of proteins of biopharmaceutical importance expressed in Pichia pastoris is given in **Table 4**.

Pichia pastoris is also in the quest to produce antimicrobial peptides (AMPs) that are generated in response to innate immune system and provide immunity against foreign microorganisms. The consistent resistance to conventional antibiotics increased the need for new antibiotics that has triggered researcher's interest in developing AMPs. Pichia has been a promising candidate to produce AMPs derived from different sources including bacteria and humans and many AMPs have been produced using the AOX1 promoter system reaching over 1 g g^{-1} [58]. Novozymecompany has licensed Sanofi-Aventis to market an AMP, Plectasin peptide derivative NZ2144 in Pichia pastoris. NZ2114 has

Table 4: Heterologous proteins of biopharmaceutical importance expressed in *Pichia pastoris*

Protein	*Pichia* strain	Yield	Reference
Human Insulin			
Human Insulin	X-33	4 g/L	[54]
Human Insulin	GS115	2.6 g/L	[95]
Human Insulin	SuperMan5	5 mg/L	[49]
Antibodies			
IgG (ScFv fragment)	x-33	60mg/L	[96]
anti-CTLA4 scFv Ab	EasySelect™ Pichia Expression Kit	16mg/L	[97]
human anti-αIIbβ3 antibody (HuAb)	X-33	30 mg/L	[98]
Monoclonal antibodies (mAbs)	Glycoengineered Pichia strain	1g/L	[99]
IgG	YGLY27435/YGLY27431	148 mg/L/227 mg/L	[100]
Full length mAb	Glycoengineered strain YGLY18483	0.5g/L	[101]
Vaccine			
HBsAg	GS115	50mg/L	[102]
HBsAg	GS115	6–7 g L-1	[103]
DENV-3 E VLPs(Dengue)	KM71H	15 mg/L	[104]
HCV E1E2 protein	SMD 1168	35 mg/L	[105]
Recombinant influenza HINI	His+ Pichia strain	60 mg/L	[106]
Cytokines			
recombinant human interleukin-6	X-33	280mg/L	[107]
recombinant human interleukin-3	X-33	26mg/L	[108]
Recombinant Bovine Interferon α1	GS115	0.2 mg/ml	[109]
Hormones			
Human parathyroid hormone	Methylotrophic Pichia strain	0.3g/L	[110]
Other Proteins			
human plasminogen	GS115	510.1 mg/L	[111]
Recombinant human erythropoietin (rhEPO)	X-33	150 mg/l	[112]
Recombinant human adiponectin	X-33	0.111 mg/mL	[113]
Human Albumin fusion protein	Methylotropic Pichia pastoris	275 mg/L	[114]
Angiogenin	Gs115	30 mg/L	[115]

potent activity against staphylococci and streptococci, works on bacterial infections resistant to conventional antibiotics [17].

More than 300 biotechnology and pharmaceutical companies have been licensed to utilize this expression system and over 5000 proteins have been expressed using this expression system till to date according to the pichia.com web page, by Research corporation technologies (RCT- rctech.com). Since the purchase of Pichia expression system by the RCT from the Philips petroleum in 1993, more than 70 therapeutic and industrial products are approved by FDA and are in the market or in the last stages of development. Some of the biopharmaceutical products licensed by RCT in healthcare market as listed by pichia.com web page is Kalbitor® (ecallantide, a recombinant kallikrein inhibitor protein), produced by (Dyax, Cambridge) used in hereditary angioedema treatment. It was the first FDA approved biopharmaceutical synthesized in Pichia [83]. Insugen® (recombinant human insulin), produced (Biocon, India) to treat diabetes mellitus. Medway (a recombinant serum albumin) by Mitsubishi Tanabe Pharma, Japan for blood volume and expansion. Shanvac® (recombinant Hepatitis B vaccine) and ShanferonTM (recombinant interferon alpha 2b), both provided by (Shantha/Sanofi, India). Ocriplasmin (recombinant microplasmin) by Thrombogenesis (Belgium), used in vitreomacular adhesion. Nanobody R ALX-0061(anti IL-6 receptor in rheumatoid arthritis) and Nanobody® ALX00171 (anti-RSV single domain Ab fragment against Respiratory syncytial virus infection) both produced by Ablynx, Belgium. Heparin- binding EGF-like growth factor provided by (Trillium, Canada), used in interstitial cystitis. Numerous products expressed in Pichia, not yet approved to use for therapeutic purpose are available in the market such as stem cellfactors, human serum albumin, interferons, tumor necrosis factor etc.

Membrane protein expression

Membrane proteins provide important cellular functions, particularly in signaling, transport and energy generation. They constitute 30% of theproteome. Misfunction of many of these proteins has an important role in the etiology of many human diseases, which increases their importance as targets for many drugs. *Pichia pastoris* is used successfully as a useful expression system for the production of membrane proteins including transporters, channels, and receptors for structural studies [59]. A number of high-resolution structures expressed employing Pichia pastoris is briefly described in a review by Ana Ramon, that discussed all sort of membrane

spanning topologies; ion channels, enzymes, and aquaporins have been produced [60]. Few expression strains have been used sharing methanol utilization (Mut) phenotype. Wild-type X-33 strain used successfully to express SoPIP2; 1aquaporin, GS115 (his4), SMD1163 and KM71 strains were used to express a number of proteins. Although, the SMD1163 strain was used most successfully to produce proteins for structural studies, but now it is no longer used commercially[61].

In order to increase gene expression levels of membrane proteins, genetic modification strategies have been utilized. Codon optimized genes were used to express stable proteins. G-protein-coupled receptors (GPCR) are modified at C-terminus, this truncation results in increased expression and crystallization. Removal of N-linked glycosylation sites through mutagenesis is considered to be handy in crystallization [60]. In Pichia, untagged version of membrane proteins have been produced, however, the isolation of tagged proteins would be more useful to detect and isolate target molecule. As Pichia pastoris is compatible with tags, therefore a number of tags have been used. His-tagged construct is aggressively used. Green fluorescent protein (GFP) is additionally used to incorporate in protein constructs to analyze the optimal expression and to assess solubilization[61]. *S. cerevisiae* alpha mating factor incorporated in *Pichia pastoris* is used to target secretory pathway. It needs to be cleaved by Kex2 protease during protein expression. A high-level expression sometimes requires an increase KeX2 copy number to counter this issue. Recently, a novel strain to optimize Kex2 PI site is produced, that will facilitate membrane protein production [62]. In addition, to hydrophobic nature of membrane proteins, it is also more difficult to handle them as compared to soluble protein, a major reason why there is a reduced number of membrane protein structure determination [63]. Moreover, there remain clear guidelines for researchers to exploit membrane proteins successfully. Though, it is expected that with expanded series of more strains and vectors, the production of more complex proteins will be improved in future[61].

Humanized glycol-engineered *P. pastoris* strain

A large number of biopharmaceutical proteins require post-translational modifications (PTM). Glycosylation is the most common and complex among these PTM [27]. Although yeast performs PTM, however, the final pattern differs resulting in hyper mannosylation and additional terminal alpha-1,3mannose linkages with glycoproteins in *S. cerevisisae* cause immunogenic effects. On the other hand, hyper mannosylation and alpha-1, 3 mannose linkages are not observed in Pichia, therefore, efforts have been made to humanize glycosylation in*P. pastoris* strain [64, 65]. To achieve humanized glycosylated proteins in Pichia, synthetic biology approaches were used to induct; tailor-made glycosyltransferases and glycosidases with specific localized characteristics, six endogenous Pichia genes were deleted [13]. Moreover, the processes have also been optimized to generate humanized glycoform with improved yields. RCT has improved its classical Pichia expression system and assembled its PichiaGlycoswitch® in collaboration with Biogrammatics, Inc. Strain has been engineered to produce human complex type N-glycans [66]. Advancement in implementing synthetic biology will increase commercial success of Pichia system, substantially paving the way to further optimize further and express proteins having homogenous humanized glycoforms, more efficient than produced in traditional *P. pastoris* strains in various therapeutic areas such as vaccines, enzymes, antibodies, fusion proteins, and binding domains.

Conclusions

Pichia pastoris expression system has been extensively used in the biopharmaceutical industry and academic research due to its low cost, high yield and proves a viable alternative to mammalian and bacterial cell systems for large-scale production. Many industrial enzymes, proteins, and biopharmaceuticals have been produced, numerous products have approved by the FDA. It is an attractive host for humanized glycoforms and antimicrobial peptides. Site-directed integrations, new cloning strategies, and marker recycling will strengthen gene expression and gene deletion constructs in future. Further insights in telomeric and linear plasmid sequences will provide a way to engineer new constructs. In addition, growing interest in synthetic promoter variants in contrast to their natural promoters can be used to increase expression levels having tailor-made regulatory profiles. Advancement in genomics will facilitate to explore Pichia strain providing the basis for host reengineering, improved strains having customized properties using synthetic parts and biochemical pathways, promises improvement in industrial and biopharmaceutical production.

Declaration

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