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Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*

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Abstract

During the past 15 years, the methylotrophic yeast *Pichia pastoris* has developed into a highly successful system for the production of a variety of heterologous proteins. The increasing popularity of this particular expression system can be attributed to several factors, most importantly: (1) the simplicity of techniques needed for the molecular genetic manipulation of *P. pastoris* and their similarity to those of *Saccharomyces cerevisiae*, one of the most well-characterized experimental systems in modern biology; (2) the ability of *P. pastoris* to produce foreign proteins at high levels, either intracellularly or extracellularly; (3) the capability of performing many eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation and proteolytic processing; and (4) the availability of the expression system as a commercially available kit. In this paper, we review the *P. pastoris* expression system: how it was developed, how it works, and what proteins have been produced. We also describe new promoters and auxotrophic marker/host strain combinations which extend the usefulness of the system. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Foreign gene expression; Heterologous protein production; Methylotrophic yeast; *Pichia pastoris*; Alcohol oxidase 1 gene promoter; Protein secretion

Contents

1. Introduction . . . 46
   1.1. *Pichia pastoris* as an experimental organism . . . 46
   1.2. Methanol metabolism . . . 46
   1.3. *AOX1* promoter . . . 46
   1.4. Molecular genetic manipulation . . . 47
2. Construction of expression strains . . . 47
   2.1. Expression vectors . . . 48
   2.2. Alternative promoters . . . 48
   2.3. Selectable markers . . . 48
   2.4. Host strains . . . 49
   2.5. Integration of expression vectors into the *P. pastoris* genome . . . 50
   2.6. Generating multicopy strains . . . 50
   2.7. High cell density growth in fermenter cultures . . . 50
3. Post-translational modification of secreted proteins . . . 52
   3.1. Secretion signal selection . . . 52
   3.2. *O*-Linked glycosylation . . . 53
   3.3. *N*-Linked glycosylation . . . 53
4. Conclusions . . . 53

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Case No.: IPR2023-00322
U.S. Patent No. 10,273,492

Acknowledgements 58

References 58

1. Introduction

1.1. Pichia pastoris as an experimental organism

Thirty years ago, Koichi Ogata first described the ability of certain yeast species to utilize methanol as a sole source of carbon and energy [1]. The methylotrophs attracted immediate attention as potential sources of single-cell protein (SCP) to be marketed primarily as high-protein animal feed. During the 1970s, Phillips Petroleum Company developed media and protocols for growing *Pichia pastoris* on methanol in continuous culture at high cell densities (>130 g l^{-1} dry cell weight, Fig. 1) [2]. Unfortunately, the oil crisis of the 1970s caused a dramatic increase in the cost of methane. Concomitantly, the price of soybeans, the major alternative source of animal feed, fell. As a result, the economics of SCP production from methanol were never favorable.

In the following decade, Phillips Petroleum contracted with the Salk Institute Biotechnology/Industrial Associates, Inc. (SIBIA, La Jolla, CA) to develop *P. pastoris* as an organism for heterologous protein expression. Researchers at SIBIA isolated the gene and promoter for alcohol oxidase, and generated vectors, strains, and corresponding protocols for the molecular genetic manipulation of *P. pastoris*. The combination of the fermentation methods developed for the SCP process and the alcohol oxidase promoter's strong, regulated expression effected surprisingly high levels of foreign protein expression. In 1993, Phillips Petroleum sold its *P. pastoris* expression system patent position to Research Corporation Technologies (Tucson, AZ), the current patent holder. In addition, Phillips Petroleum licensed Invitrogen Corporation (Carlsbad, CA) to sell components of the system, an arrangement that continues under Research Corporation Technologies.

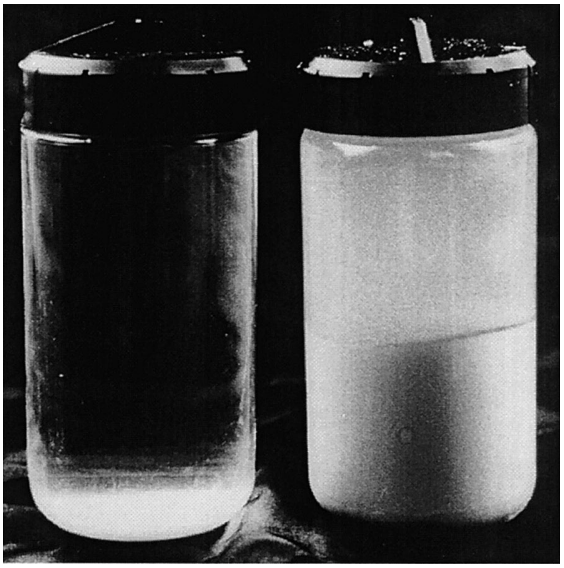

Fig. 1. High cell density culture of *P. pastoris*. The centrifuge bottle on the left shows a *P. pastoris* culture grown in a flask to a density of 1 OD_{600} unit. The bottle on the right contains a sample of the strain grown in a fermenter to a density of 130 g l^{-1} dry cell weight ($\sim$500 OD_{600} units).

1.2. Methanol metabolism

The conceptual basis for the *P. pastoris* expression system stems from the observation that some of the enzymes required for methanol metabolism are present at substantial levels only when cells are grown on methanol [3,4]. Biochemical studies showed that methanol utilization requires a novel metabolic pathway involving several unique enzymes [3]. The enzyme alcohol oxidase (AOX) catalyzes the first step in the methanol utilization pathway, the oxidation of methanol to formaldehyde and hydrogen peroxide (Fig. 2). AOX is sequestered within the peroxisome along with catalase, which degrades hydrogen peroxide to oxygen and water. A portion of the formaldehyde generated by AOX leaves the peroxisome and is further oxidized to formate and carbon dioxide by two cytoplasmic dehydrogenases, reactions that are a source of energy for cells growing on methanol.

The remaining formaldehyde is 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). The products of this reaction, glyceraldehyde 3-phosphate and dihydroxyacetone, leave the peroxisome and enter a cytoplasmic pathway that regenerates xylulose 5-monophosphate and, for every three cycles, one net molecule of glyceraldehyde 3-phosphate. Two of the methanol pathway enzymes, AOX and DHAS, are present at high levels in cells grown on methanol but are not detectable in cells grown on most other carbon sources (e.g., glucose, glycerol, or ethanol). In cells fed methanol at growth-limiting rates in fermenter cultures, AOX levels are dramatically induced, constituting $>30\%$ of total soluble protein [5,6].

1.3. AOX1 promoter

There are two genes that encode alcohol oxidase in *P. pastoris*: *AOX1* and *AOX2*; *AOX1* is responsible for a vast majority of alcohol oxidase activity in the cell [7–9].

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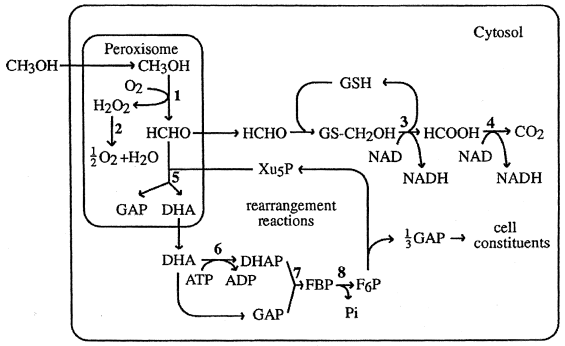

Fig. 2. The methanol pathway in *P. pastoris*. 1, alcohol oxidase; 2, catalase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase, 5, dihydroxyacetone synthase; 6, dihydroxyacetone kinase; 7, fructose 1,6-biphosphate aldolase; 8, fructose 1,6-bisphosphatase.

Expression of the *AOX1* gene is controlled at the level of transcription [7–9]. In methanol-grown cells, ~5% of poly(A)$^+$ RNA is from *AOX1*; however, in cells grown on most other carbon sources, *AOX1* message is undetectable [10]. The regulation of the *AOX1* gene appears to involve two mechanisms: a repression/derepression mechanism plus an induction mechanism, similar to the regulation of the *Saccharomyces cerevisiae GAL1* gene. Unlike *GAL1* regulation, the absence of a repressing carbon source, such as glucose in the medium, does not result in substantial transcription of *AOX1*. The presence of methanol is essential to induce high levels of transcription [7].

1.4. Molecular genetic manipulation

Techniques required for the molecular genetic manipulation of *P. pastoris*, such as DNA-mediated transformation, gene targeting, gene replacement, and cloning by functional complementation, are similar to those described for *S. cerevisiae*. *P. pastoris* can be transformed by electroporation, a spheroplast generation method, or whole cell methods such as those involving lithium chloride and polyethylene glycol$_{1000}$ [11–14]. As in *S. cerevisiae*, *P. pastoris* exhibits a propensity for homologous recombination between genomic and artificially introduced DNAs. Cleavage of a *P. pastoris* vector within a sequence shared by the host genome stimulates homologous recombination events that efficiently target integration of the vector to that genomic locus [15]. Gene replacements occur at lower frequencies than those observed in *S. cerevisiae* and appear to require longer terminal flanking sequences to efficiently direct integration [14].

P. pastoris is a homothallic ascomycetous yeast that can also be manipulated by classical genetic methods [10,16]. Unlike homothallic strains of *S. cerevisiae*, which are diploid, *P. pastoris* remains haploid unless forced to mate. Strains with complementary markers can be mated by subjecting them to a nitrogen-limited medium. After 1 day on this medium, cells are shifted to a standard minimal medium supplemented with nutrients designed to select for complementing diploid cells (not self-mated or non-mated parental cells). The resulting diploids are stable as long as they are not subjected to nutritional stress. To obtain spore products, diploids are returned to the nitrogen-limited medium, which stimulates them to proceed through meiosis and sporulation. Spore products are handled by random spore techniques rather than micromanipulation, since *P. pastoris* asci are small and difficult to dissect. Yet most standard classical genetic manipulations, including mutant isolation, complementation analysis, backcrossing, strain construction, and spore analysis, can be accomplished.

2. Construction of expression strains

Expression of any foreign gene in *P. pastoris* requires three basic steps: (1) the insertion of the gene into an expression vector; (2) introduction of the expression vector into the *P. pastoris* genome; and (3) examination of potential expression strains for the foreign gene product. A variety of *P. pastoris* expression vectors and host strains are available. A generalized diagram of an expression vector and a list of possible vector components are shown in Fig. 3 and Table 1, respectively. More detailed information on vectors and strains can be found elsewhere [17,18]. In addition, the DNA sequence of many vectors can be found at the Invitrogen website (www.invitrogen.com). Table 2 shows a list of commonly used *P. pastoris* host strains.

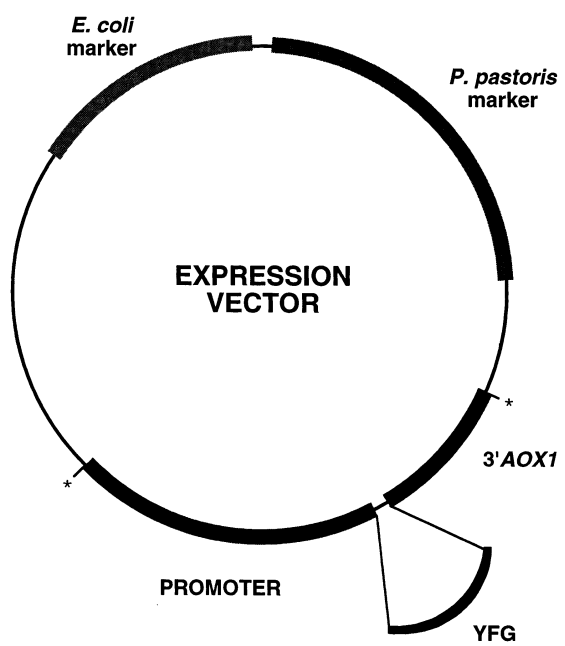

Fig. 3. General diagram of a *P. pastoris* expression vector. YFG, 'Your Favorite Gene;' *, sites for cassette amplification.

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Case No.: IPR2023-00322
U.S. Patent No. 10,273,492

Table 1
Relevant components of vectors used for protein expression in *P. pastoris*

Secretion signals	none, PHO1, α-MF, SUC2, PHA-E
Marker genes	ADE1, ARG4, G418, HIS4, URA3, Zeo^r
Promoters	AOX1, GAP, FLD1, PEX8, YPT1

See text for explanation of different elements.

2.1. Expression vectors

All expression vectors have been designed as *Escherichia coli*/*P. pastoris* shuttle vectors, containing an origin of replication for plasmid maintenance in *E. coli* and markers functional in one or both organisms. Most expression vectors have an expression cassette composed of a 0.9-kb fragment from *AOX1* composed of the 5′ promoter sequences and a second short *AOX1*-derived fragment with sequences required for transcription termination [19]. Between the promoter and terminator sequences is a site or multiple cloning site (MCS) for insertion of the foreign coding sequence. In the native *AOX1* gene, the alcohol oxidase open reading frame (ORF) is preceded by an unusually long 5′ untranslated region (116 nt) [8]. Generally, the best expression results are obtained when the first ATG of the heterologous coding sequence is inserted as close as possible to the position of the *AOX1* ATG. This position coincides with the first restriction site in most MCSs. In addition, for secretion of foreign proteins, vectors are available where in-frame fusions of foreign proteins and the secretion signals of *P. pastoris* acid phosphatase (*PHO1*) or *S. cerevisiae* α-mating factor (α-MF) can be generated.

2.2. Alternative promoters

Although the *AOX1* promoter has been successfully used to express numerous foreign genes, there are circumstances in which this promoter may not be suitable. For example, the use of methanol to induce gene expression may not be appropriate for the production of food products since methane, a petroleum-related compound, is one source of methanol. Also, methanol is a potential fire hazard, especially in quantities needed for large-scale fermentations. Therefore, promoters that are not induced by methanol are attractive for expression of certain genes. Alternative promoters to the *AOX1* promoter are the *P. pastoris GAP*, *FLD1*, *PEX8*, and *YPT1* promoters.

2.2.1. P_{GAP}

Both northern and reporter activation results indicate that the *P. pastoris* glyceraldehyde 3-phosphate dehydrogenase (*GAP*) gene promoter provides strong constitutive expression on glucose at a level comparable to that seen with the *AOX1* promoter [20]. *GAP* promoter activity levels in glycerol- and methanol-grown cells are approximately two-thirds and one-third of the level observed for glucose, respectively. The advantage of using the *GAP* promoter is that methanol is not required for induction, nor is it necessary to shift cultures from one carbon source to another, making strain growth more straightforward. However, since the *GAP* promoter is constitutively expressed, it is not a good choice for the production of proteins that are toxic to the yeast.

2.2.2. P_{FLD1}

The *FLD1* gene encodes a glutathione-dependent formaldehyde dehydrogenase, a key enzyme required for the metabolism of certain methylated amines as nitrogen sources and methanol as a carbon source [21]. The *FLD1* promoter can be induced with either methanol as a sole carbon source (and ammonium sulfate as a nitrogen source) or methylamine as a sole nitrogen source (and glucose as a carbon source). After induction with either methanol or methylamine, P_{FLD1} is able to express levels of a β-lactamase reporter gene similar to those obtained with methanol induction from the *AOX1* promoter. The *FLD1* promoter offers the flexibility to induce high levels of expression using either methanol or methylamine, an inexpensive nontoxic nitrogen source.

2.2.3. P_{PEX8}, P_{YPT1}

For some applications, the *AOX1*, *GAP*, and *FLD1* promoters may be too strong, expressing genes at too high a level. There is evidence that, for certain foreign genes, the high level of expression from P_{AOX1} may overwhelm the post-translational machinery of the cell, causing a significant proportion of foreign protein to be misfolded, unprocessed, or mislocalized [22,23]. For these and other applications, moderately expressing promoters are desirable. Toward this end, the *P. pastoris PEX8* and *YPT1* promoters may be of use. The *PEX8* gene encodes a peroxisomal matrix protein that is essential for peroxisome biogenesis [24]. It is expressed at a low but significant level on glucose and is induced modestly when cells are shifted to methanol. The *YPT1* gene encodes a GTPase involved in secretion, and its promoter provides a low but constitutive level of expression in media containing either glucose, methanol, or mannitol as carbon sources [25].

2.3. Selectable markers

Although classical and molecular genetic techniques are generally well-developed for *P. pastoris*, few selectable marker genes have been described for the molecular genetic manipulation of the yeast. Existing markers are limited to the biosynthetic pathway genes *HIS4* from either *P. pastoris* or *S. cerevisiae*, *ARG4* from *S. cerevisiae*, and the *Sh ble* gene from *Streptoalloteichus hindustanus* which confers resistance to the bleomycin-related drug zeocin [11,26,27]. The stable expression of human type III collagen illustrates the need for multiple selectable markers in

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Motif Exhibit 1015, Page 4 of 22

Case No.: IPR2023-00322
U.S. Patent No. 10,273,492

Table 2
P. pastoris host strains

Strain	Genotype	Reference
Auxotrophic strains		
Y-11430	wild-type	NRRL[a]
GS115	*his4*	[11]
GS190	*arg4*	[16]
JC220	*ade1*	[16]
JC254	*ura3*	[16]
GS200	*arg4 his4*	[11]
JC227	*ade1 arg4*	[29]
JC304	*ade1 his4*	[29]
JC305	*ade1 ura3*	[29]
JC306	*arg4 ura3*	[29]
JC307	*his4 ura3*	[29]
JC300	*ade1 arg4 his4*	[29]
JC301	*ade1 his4 ura3*	[29]
JC302	*ade1 arg4 ura3*	[29]
JC303	*arg4 his4 ura3*	[29]
JC308	*ade1 arg4 his4 ura3*	[29]
Protease-deficient strains		
KM71	*Δaox1::SARG4 his4 arg4*	[7]
MC100-3	*Δaox1::SARG4Δaox2::Phis4 his4 arg4*	[9]
SMD1168	*Δpep4::URA3 his4 ura3*	[38]
SMD1165	*prb1 his4*	[38]
SMD1163	*pep4 prb1 his4*	[38]
SMD1168 kex1::SUC2	*Δpep4::URA3 Δkex1::SUC2 his4 ura3*	[34]

[a]Northern Regional Research Laboratories, Peoria, IL.

P. pastoris [28]. The production of collagen requires the coexpression of prolyl 4-hydroxylase, a central enzyme in the synthesis and assembly of trimeric collagen. Since prolyl 4-hydroxylase is an $\alpha_2\beta_2$ tetramer, the β subunit of which is protein disulfide isomerase (PDI), three markers – Arg, His, and zeocin resistance – were necessary to coexpress all three polypeptides in the same *P. pastoris* strain.

Recently, a new set of biosynthetic markers has been isolated and characterized: the *P. pastoris ADE1* (PR-amidoimidazolesuccinocarboxamide synthase), *ARG4* (argininosuccinate lyase), and *URA3* (orotidine 5′-phosphate decarboxylase) genes [29]. Each of these selectable markers has been incorporated into expression vectors. In addition, a series of host strains containing all possible combinations of *ade1*, *arg4*, *his4*, and *ura3* auxotrophies has been generated (Table 2).

2.4. Host strains

All *P. pastoris* expression strains are derived from NRRL-Y 11430 (Northern Regional Research Laboratories, Peoria, IL). Most have one or more auxotrophic mutations which allow for selection of expression vectors containing the appropriate selectable marker gene upon transformation. Prior to transformation, all of these strains grow on complex media but require supplementation with the appropriate nutrient(s) for growth on minimal media.

2.4.1. Methanol utilization phenotype

Most *P. pastoris* host strains grow on methanol at the wild-type rate (Mut^+, methanol utilization plus phenotype). However, two other types of host strains are available which vary with regard to their ability to utilize methanol because of deletions in one or both *AOX* genes. Strains with *AOX* mutations are sometimes better producers of foreign proteins than wild-type strains [30–32]. Additionally, these strains do not require the large amounts of methanol routinely used for large-scale fermentations of Mut^+ strains. KM71 (*his4 arg4 aox1Δ::SARG4*) is a strain where *AOX1* has been partially deleted and replaced with the *S. cerevisiae ARG4* gene [15]. Since the strain must rely on the weaker *AOX2* for methanol metabolism, it grows slowly on this carbon source (Mut^s, methanol utilization slow phenotype). Another strain, MC100-3 (*his4 arg4 aox1Δ::SARG4 aox2Δ::Phis4*), is deleted for both *AOX* genes and is totally unable to grow on methanol (Mut^-, methanol utilization minus phenotype) [9]. All of these strains, even the Mut^- strain, retain the ability to induce expression at high levels from the *AOX1* promoter [32].

2.4.2. Protease-deficient host strains

Several protease-deficient strains – SMD1163 (*his4 pep4 prb1*), SMD1165 (*his4 prb1*), and SMD1168 (*his4 pep4*) – have been shown to be effective in reducing degradation of some foreign proteins [23,33]. This is especially noticeable in fermenter cultures, because the combination of high cell

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Case No.: IPR2023-00322
U.S. Patent No. 10,273,492

density and lysis of a small percentage of cells results in a relatively high concentration of these vacuolar proteases. An additional protease-deficient strain SMD1168 Δ*pep4::URA3* Δ*kex1::SUC2 his4 ura3* was recently developed to inhibit proteolysis of murine and human endostatin. Kex1 protease can cleave carboxy-terminal lysines and arginines. Therefore, the deletion strain was generated to inhibit carboxy-terminal proteolysis. After 40 h of fermentation, purification of intact endostatin was achieved [34].

Unfortunately, these protease-deficient cells are not as vigorous as wild-type strains with respect to *PEP4*. In addition to lower viability, they possess a slower growth rate and are more difficult to transform. Therefore, the use of protease-deficient strains is only recommended in situations where other measures to reduce proteolysis have yielded unsatisfactory results.

2.5. Integration of expression vectors into the P. pastoris genome

Expression vectors are integrated into the *P. pastoris* genome to maximize the stability of expression strains. This can be done in two ways. The simplest way is to restrict the vector at a unique site in either the marker gene (e.g., *HIS4*) or the *AOX1* promoter fragment and then to transform it into the appropriate auxotrophic mutant. The free DNA termini stimulate homologous recombination events that result in single crossover-type integration events into these loci at high frequencies (50–80% of His^+ transformants). The remaining transformants have undergone gene conversion events in which only the marker gene from the vector has integrated into the mutant host locus without other vector sequences.

Alternatively, certain *P. pastoris* expression vectors can be digested in such a way that the expression cassette and marker gene are released, flanked by 5′ and 3′ *AOX1* sequences. Approximately 10–20% of transformation events are the result of a gene replacement event in which the *AOX1* gene is deleted and replaced by the expression cassette and marker gene. This disruption of the *AOX1* gene forces these strains to rely on the transcriptionally weaker *AOX2* gene for growth on methanol [31], and, as a result, these strains have a Mut^s phenotype. These gene replacement strains are easily identified among transformed colonies by replica-plating them to methanol and selecting those with reduced ability to grow on methanol. As mentioned previously, the potential advantage of Mut^s strains is that they utilize less methanol and sometimes express higher levels of foreign protein than wild-type (Mut^+) strains, especially in shake-flask cultures [15].

2.6. Generating multicopy strains

Optimization of protein expression often, but not always, includes the isolation of multicopy expression strains. A strain that contains multiple integrated copies of an expression cassette can sometimes yield more heterologous protein than single-copy strains [22,35].

Three approaches lead reliably to multicopy expression strains in *P. pastoris*. As shown in Fig. 4, the first approach involves constructing a vector with multiple head-to-tail copies of an expression cassette [23]. The key to generating this construction is a vector which has an expression cassette flanked by restriction sites which have complementary termini (e.g., *Bam*HI-*Bgl*II, *Sal*I-*Xho*I combinations). The process of repeated cleavage and reinsertion results in the generation of a series of vectors that contain increasing numbers of expression cassettes. A particular advantage to this approach, especially in the production of human pharmaceuticals, is that the precise number of expression cassettes is known and can be recovered for direct verification by DNA sequencing.

A second method utilizes expression vectors that contain the *P. pastoris HIS4* and the bacterial *Tn903kan*^{r} genes. The bacterial kanamycin resistance gene also confers resistance to the related eukaryotic antibiotic G418 [36]. The level of G418 resistance can be roughly correlated to vector copy number. *P. pastoris* must first be transformed to His^+ prototrophy; then multicopy transformants are screened by replica-plating to plates containing G418. This method results in a subset of colonies enriched for those containing multiple expression vector copies. However, the vector copy number varies greatly; thus, a significant number (50–100) of transformants must be subjected to further analysis of copy number and expression level. By this approach, strains carrying up to 30 copies of an expression cassette have been isolated [35].

A third approach to constructing multicopy strains involves the use of a vector with the bacterial *Sh ble* gene, which confers resistance to the antibiotic zeocin [27]. Unlike G418 selection, strains transformed with expression cassettes containing the zeocin marker can be selected directly by resistance to the drug. Additionally, populations of transformants can be enriched for multicopy expression cassette strains simply by plating on increased concentrations of zeocin in the selection plates. Also, because the *Sh ble* gene can serve as a selectable marker in both bacteria and yeast, these expression vectors are compact and convenient to use. However, as with the G418 selection, most transformants resistant to high levels of zeocin do not contain multiple vector copies, and numerous transformants must be screened for ones that do.

2.7. High cell density growth in fermenter cultures

P. pastoris is a poor fermenter, a major advantage relative to *S. cerevisiae*. In high cell density cultures, ethanol (the product of *S. cerevisiae* fermentation) rapidly builds to toxic levels which limit further growth and foreign protein production. With its preference for respiratory growth, *P. pastoris* can be cultured at extremely high den-

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Motif Exhibit 1015, Page 6 of 22

Case No.: IPR2023-00322
U.S. Patent No. 10,273,492

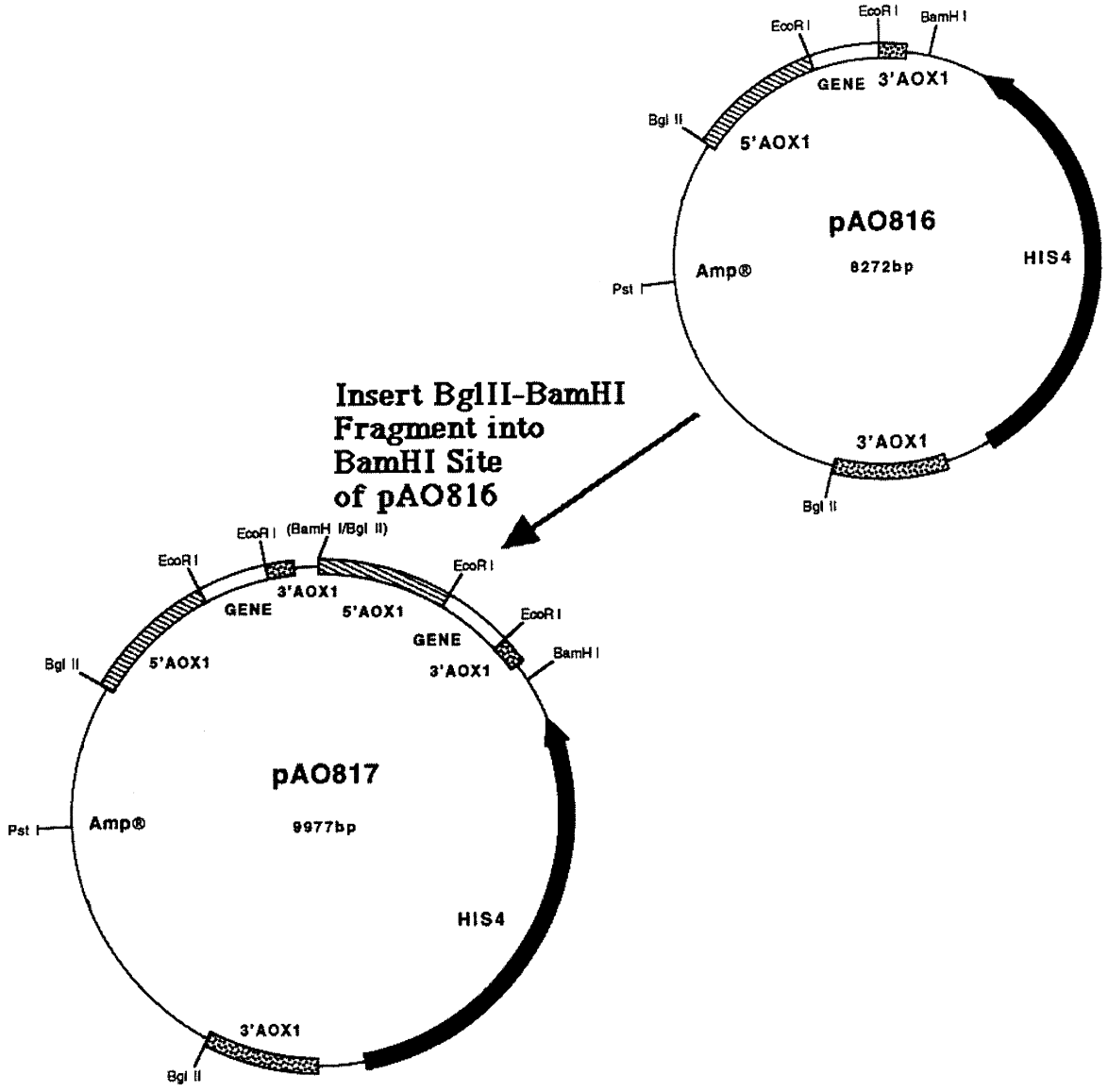

Fig. 4. Scheme for construction of vectors with multiple copies of a foreign gene expression cassette (from [22]).

sities (500 OD_{600} U ml^{-1}) in the controlled environment of the fermenter with little risk of 'pickling' itself. Fermentation growth is especially important for secreted proteins, as the concentration of product in the medium is roughly proportional to the concentration of cells in culture. Another positive aspect of growing *P. pastoris* in fermenter cultures is that the level of transcription initiated from the *AOX1* promoter can be 3–5 times greater in cells fed methanol at growth-limiting rates compared to cells grown in excess methanol. Thus, even for intracellularly expressed proteins, product yields are significantly higher from fermenter cultured cells. Also, methanol metabolism utilizes oxygen at a high rate, and expression of foreign genes is negatively affected by oxygen limitation. Only in the controlled environment of a fermenter is it feasible to monitor and adjust oxygen levels in the culture medium.

A hallmark of the *P. pastoris* system is the ease with which expression strains scale-up from shake-flask to high-density fermenter cultures. Although some foreign proteins have expressed well in shake-flask cultures, expression levels are typically low compared to fermenter cultures. Considerable effort has gone into the optimization of heterologous protein expression techniques, and detailed fed-batch and continuous culture protocols are available [23,37–39]. In general, strains are grown initially in a defined medium containing glycerol as its carbon source. During this time, biomass accumulates but heterologous gene expression is fully repressed. Upon depletion of glycerol, a transition phase is initiated in which additional glycerol is fed to the culture at a growth-limiting rate. Finally, methanol or a mixture of glycerol and methanol is fed to the culture to induce expression. The con-

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U.S. Patent No. 10,273,492

centration of foreign protein is monitored in the culture to determine time of harvest.

The growth conditions for *P. pastoris* are ideal for large-scale production of heterologous protein, because the medium components are inexpensive and defined, consisting of pure carbon sources (glycerol and methanol), biotin, salts, trace elements, and water. This medium is free of undefined ingredients that can be sources of pyrogens or toxins and is therefore compatible with the production of human pharmaceuticals. Also, since *P. pastoris* is cultured in media with a relatively low pH and methanol, it is less likely to become contaminated by most other microorganisms.

3. Post-translational modification of secreted proteins

A major advantage of *P. pastoris* over bacterial expression systems is that the yeast has the potential to perform many of the post-translational modifications typically associated with higher eukaryotes, such as processing of signal sequences (both pre and prepro type), folding, disulfide bridge formation, certain types of lipid addition, and *O*- and *N*-linked glycosylation.

3.1. Secretion signal selection

Foreign proteins expressed in *P. pastoris* can be produced either intracellularly or extracellularly. Because this yeast secretes only low levels of endogenous proteins, the secreted heterologous protein constitutes the vast majority of total protein in the medium (Fig. 5). Therefore, directing a heterologous protein to the culture medium can serve as a substantial first step in purification. However, due to protein stability and folding requirements, the option of secretion is usually reserved for foreign proteins that are normally secreted by their native hosts. In many cases, researchers simply need to take advantage of the pre-made expression cassettes available from Invitrogen. Using selected *P. pastoris* vectors, researchers can clone a foreign gene in frame with sequences encoding either the native signal, the *S. cerevisiae* α-factor prepro peptide, or the *P. pastoris* acid phosphatase (*PHO1*) signal.

Although several different secretion signal sequences, including the native secretion signal present on heterologous proteins, have been used successfully, results have been variable. The *S. cerevisiae* α-factor prepro peptide has been used with the most success. This signal sequence consists of a 19-amino acid signal (pre) sequence followed by a 66-residue (pro) sequence containing three consensus *N*-linked glycosylation sites and a dibasic Kex2 endopeptidase processing site [40]. The processing of this signal sequence involves three steps. The first is the removal of the pre signal by signal peptidase in the endoplasmic reticulum. Second, Kex2 endopeptidase cleaves between Arg-Lys of the pro leader sequence. This is rapidly followed by cleavage of Glu-Ala repeats by the Ste13 protein [41]. The efficiency of this process can be affected by the surrounding amino acid sequence. For instance, the cleavage efficiencies of both Kex2 and Ste13 proteins can be influenced by the close proximity of proline residues. In addition, the tertiary structure formed by a foreign protein may protect cleavage sites from their respective proteases.

The *S. cerevisiae* α-MF prepro signal sequence is the classical and most widely used secretion signal (see Table 3, expressed proteins). In some cases, it is a better secretion signal for expression in *P. pastoris* than the leader sequence of the native heterologous protein. In a study concerning the expression of the industrial lipase Lip1 from *Candida rugosa*, the effect of heterologous leader sequences on expression and secretion was investigated [42]. It was found that the native Lip1p leader sequence allowed for secretion but somehow hampered expression. Either the α-factor pre or prepro signal was adequate for both secretion and expression, but the highest level of lipase secretion was from a clone with the full prepro sequence. This clone produced two species of secreted protein. A small percentage was correctly processed to the mature protein. However, a majority of the product contained four additional N-terminal amino acids. Variability in the amino terminus is commonly seen with heterologous proteins secreted by *P. pastoris* using the α-factor prepro leader.

In some cases, the standard α-MF or *PHO1* secretion signals have not worked, so synthetic leaders have been created. Martinez-Ruiz et al. [43] made mutations in the native leader to reconstruct a more efficient Kex2p recognition motif (Lys-Arg). This aided in secretion of the ribosome-inactivation protein α-sarcin from the mold *Aspergillus giganteus*. Another more drastic solution was to create an entirely synthetic prepro leader. For the expression of human insulin, a synthetic leader and spacer sequence was found to improve secretion and protein yield [44].

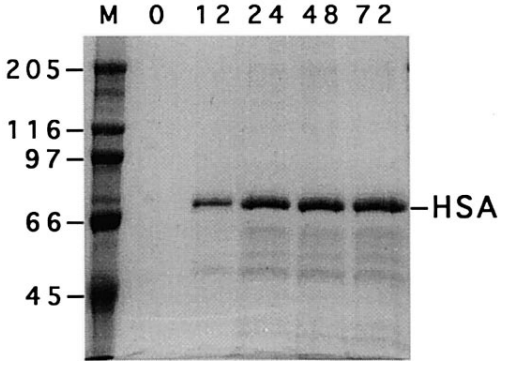

Fig. 5. Secreted expression of human serum albumin. 7.5% SDS-PAGE of 25-μl sample of culture supernatant from a *P. pastoris* strain (GS-HSA #4141) expressing human serum albumin. Cells were induced in BMMY (buffered methanol-complex medium) for 0, 12, 24, 48, and 72 h. Lane M contains molecular mass markers (kDa).

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Case No.: IPR2023-00322
U.S. Patent No. 10,273,492

Recently, yet another signal peptide – PHA-E from the plant lectin *Phaseolus vulgaris* agglutinin – was found to be effective for the secreted expression of two plant lectins and green fluorescent protein. Additionally, it was found that proteins fused to the PHA-E signal peptide were correctly processed at the amino-termini, whereas the same proteins secreted under the control of the *S. cerevisiae* α-MF signal had heterogeneous amino-terminal extensions [45]. It remains to be seen whether the PHA-E signal sequence works as well in the secretion and processing of other foreign proteins.

3.2. O-Linked glycosylation

P. pastoris is capable of adding both *O*- and *N*-linked carbohydrate moieties to secreted proteins [46]. Eukaryotic cells assemble *O*-linked saccharide onto the hydroxyl groups of serine and threonine. In mammals, *O*-linked oligosaccharides are composed of a variety of sugars, including *N*-acetylgalactosamine, galactose (Gal), and sialic acid (NeuAc). In contrast, lower eukaryotes such as *P. pastoris* add *O*-oligosaccharides composed solely of mannose (Man) residues. No consensus primary amino acid sequence for *O*-glycosylation appears to exist. Additionally, different hosts may add *O*-linked sugars on different residues in the same protein. Consequently, it should not be assumed that *P. pastoris* will not glycosylate a heterologous protein even if that protein is not glycosylated by its native host. For instance, although insulin-like growth factor I (IGF-I) is not glycosylated in humans, *P. pastoris* was found to add *O*-linked mannose to 15% of expressed IGF-I product [23]. It should also not be assumed that the specific Ser and Thr residues selected for *O*-glycosylation by *P. pastoris* will be the same as the original host.

Although there is little information concerning the mechanism and specificity of *O*-glycosylation in *P. pastoris*, the presence of *O*-glycosylation has been reported in some heterologous proteins, such as the *Aspergillus awamori* glucoamylase catalytic domain [47], human IGF-1 [23], barley α-amylases 1 and 2 [48], and human single-chain urokinase-type plasminogen activator [49].

Duman et al. [50] used a variety of chromatographic procedures [phenol/sulfuric acid colorimetric assay, Dionex high-pH anion-exchange chromatography (HPAEC)] and exoglycosidases (jack bean α-mannosidase, *Aspergillus saitoi* α-1,2-mannosidase, *Xanthomonas manihotis* α-1,2/1,3-mannosidase) to study endogenous cellular proteins and recombinant human plasminogen produced in *P. pastoris*. The study revealed the presence of *O*-linked α-1,2-mannans containing dimeric, trimeric, tetrameric, and pentameric oligosaccharides. No α-1,3 linkages were detected. Also, the majority of oligosaccharides was equally distributed between α-1,2-linked dimers and trimers [50].

3.3. N-Linked glycosylation

In all eukaryotes, *N*-glycosylation begins in the endoplasmic reticulum with the transfer of a lipid-linked oligosaccharide unit, $Glc_3Man_9GlcNAc_2$ (Glc = glucose; GlcNAc = *N*-acetylglucosamine), to asparagine at the recognition sequence Asn-X-Ser/Thr. This oligosaccharide core is then trimmed to $Man_8GlcNAc_2$. At this point, glycosylation patterns of lower (such as *P. pastoris* and other fungi) and higher eukaryotes begin to differ. The mammalian Golgi apparatus performs a series of trimming and addition reactions that generate oligosaccharides composed of $Man_{5-6}GlcNAc_2$ (high-mannose type), a mixture of several different sugars (complex type), or a combination of both (hybrid type) [46]. In *S. cerevisiae*, *N*-linked core units are elongated in the Golgi through the addition of mannose outer chains. Since these outer chains vary in length, endogenous and heterologous secreted proteins from *S. cerevisiae* are heterogeneous in size. These chains are typically 50–150 mannose residues in length, a condition referred to as hyperglycosylation.

Some foreign proteins secreted in *P. pastoris* appear to be hyperglycosylated similar to those observed in *S. cerevisiae*. *N*-Linked high-mannose oligosaccharides added to proteins by yeast secretory systems represent a significant problem in the use of foreign-secreted proteins by the pharmaceutical industry. They can be exceedingly antigenic when introduced intravenously into mammals and are rapidly cleared from the blood by the liver. An additional problem caused by the differences between yeast and mammalian *N*-linked glycosylation patterns is that the long outer chains can potentially interfere with the folding or function of a foreign protein.

Relative to the oligosaccharide structures on *S. cerevisiae*-secreted proteins, at least three differences are apparent in *P. pastoris*-produced proteins. First, and perhaps most importantly, is the frequent absence of hyperglycosylation. Using oligosaccharide profiling techniques, it has been shown that the typical outer chain on *P. pastoris*-secreted proteins is $Man_8GlcNAc_2$ or $Man_9GlcNAc_2$ [51]. Another difference is the presence of α-1,6-linked mannose on core-related structures reported in *P. pastoris*-secreted invertase [52], and the kringle-2 domain of tissue-type plasminogen activator [53] and other proteins [54]. Finally, *P. pastoris* oligosaccharides appear not to have any terminal α-1,3-linked mannosylation [51,55]. These linkages make many yeast-produced recombinant proteins unsuitable for human pharmaceutical uses [56].

4. Conclusions

The *P. pastoris* expression system has gained acceptance as an important host organism for the production of foreign proteins as illustrated by the fact that a number of proteins synthesized in *P. pastoris* are being tested for use

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Case No.: IPR2023-00322
U.S. Patent No. 10,273,492

Table 3
Heterologous proteins expressed in *P. pastoris*

Protein	Comments: mode, amount, signal sequence	Reference
Bacteria		
Bacillus licheniformis α-amylase	S, 2.5 g l^{-1}, SUC2	[51,60]
Bacillus stearothermophilus D-alanine carboxypeptidase	S, 100 mg l^{-1}, native	[61]
Bordetella pertussis pertussis pertactin (P69)	I, 3 g l^{-1}	[62]
Clostridium botulinum neurotoxin (BoNT) serotype A and B	I, 78 mg l^{-1}	[63]
Clostridium botulinum neurotoxin heavy chain fragment, serotype B	I, 390 μg g^{-1}	[64]
Clostridium botulinum neurotoxin serotype A binding domain	I, 2.4 mg total	[65]
Clostridium tetani tetanus toxin fragment C	I, 12 g l^{-1}	[66]
Escherichia coli acid phosphatase/phytase (appA2)	S, 28.9 U mg^{-1}	[67]
Escherichia coli β-galactosidase	I, 2.0×10^3 U mg^{-1}	[7]
Escherichia coli β-lactamase	I	[20]
Leishmania major cathepsin B-like protease	S, α-MF	[68]
Staphylococcus aureus staphylokinase	S, 50 mg l^{-1}, α-MF	[69]
Streptococcus equisimilis streptokinase	I, 77 mg l^{-1}	[70]
Streptomyces subtilisin inhibitor	S	[71]
Streptomyces viridosporus T7A peroxidase, endoglucanase	S, 2.47 g l^{-1} total protein, α-MF	[72]
Toxoplasma gondii SAG1 antigen	S, 12 mg l^{-1}, α-MF	[73]
Vibrio cholerae accessory cholera enterotoxin (Acc)	S, 7 mg l^{-1}, α-MF	[74]
Fungi		
Alternaria Alt 1 allergen	S, α-MF	[75]
Aspergillus awamori glucoamylase	S, 400 mg l^{-1}, native	[76]
Aspergillus awamori glucoamylase catalytic domain	S, 400 mg l^{-1}, PHO1	[47]
Aspergillus fumigatus catalase L	S, 2.3 g l^{-1}, PHO1	[77]
Aspergillus fumigatus dipeptidyl peptidase IV (DPP IV)	S, PHO1	[78]
Aspergillus fumigatus dipeptidyl peptidase V (DPP V)	S, 0.15 mg l^{-1}, PHO1	[79]
Aspergillus giganteus α-sarcin ribotoxin	S, 1 mg l^{-1}, synthetic native, PHO1	[43]
Aspergillus niger phytase (phyA)	S, 65 U ml^{-1}, α-MF	[80]
Candida guilliermondii xylose reductase gene (xylI)	I, 0.65 U mg^{-1}; S, 0.18 U mg^{-1}, α-MF	[81]
Candida rugosa lipase 1 (CRL)	S, 150 U ml^{-1}, α-MF	[42]
Fusarium solani pectate lyase (pelC)	S, 1 mg l^{-1}, PHO1	[82]
Fusarium solani pectate lyase (pelD)	S, native	[83]
Geotrichum candidum lipase isoenzymes	S, 60 mg l^{-1}, α-MF	[84]
Phytophthora cryptogea β-cryptogein	S, 45 mg l^{-1}, PHO1	[85]
Rhizopus oryzae lipase	S, 60 mg l^{-1}, α-MF	[86]
Saccharomyces cerevisiae invertase	S, 2.5 g l^{-1}, native	[30]
Saccharomyces cerevisiae Ktr1p	S, 400 mg l^{-1}, PHO1	[87]
Saccharomyces cerevisiae (α-1,2-mannosyltransferase)	S, 40 mg l^{-1}, PHO1	[87]
Schizophyllum commune vitamin B2-aldehyde-forming enzyme	S, 120 mg l^{-1}, α-MF	[88]
Trametes versicolor (white rot fungus) laccase (lccI)	S, native and α-MF	[89]
Trichoderma harzianum β-(1–6)-glucanase	S, 9.3 mg l^{-1}	[90]
Protists		
Chondrus crispus red alga hexose oxidase	I	[91]
Gracilariopsis lemaneiformis red alga α-1,4-glucan lyase (GLq1)	I	[92]
Plasmodium falciparum merozoite surface protein 1 (MSP-1)	S, 24 mg l^{-1}, α-MF	[93]
Plasmodium vivax apical membrane antigen I (AMA-1)	S, 50 mg l^{-1}, PHO1	[94]
Reticulomyxa filosa (giant freshwater ameba) α2, β2 tubulin isoforms	I, 400 μg g^{-1}	[95]
Trypanosoma cruzi acid α-mannosidase	S, 11.5 μg l^{-1}, native	[96]
Plants		
Allium sativum (garlic) alliin lyase	I, 2.167 U g^{-1}	[97]
Arabidopsis thaliana NADH:nitrate reductase	I, 18 μg g^{-1}	[98,99]
Barley (*Hordeum vulgare*) sucrose fructan 6-fructosyl transferase	S, α-MF	[100]
Barley α-amylase 1	S, 50 mg l^{-1}, native	[48]
Barley α-amylase 2	S, 1 mg l^{-1}, native	[48]
Barley aleurone tissue α-glucosidase	S, α-MF	[101]
Coffee bean α-galactosidase	S, 400 mg l^{-1}, α-MF	[102]
Cynara cardunculus (cardoon) cyprosin	S, 1 mg l^{-1}, native	[103]
Cynodon dactylon (Bermuda grass) Cyn d 1	S, 1.5 g l^{-1}, PHO1	[104,105]
Galanthus nivalis agglutinin	S, PHA-E	[45]
Hevea brasiliensis hydroxynitrile lyase	I, 22 g l^{-1}	[106]
Hevea brasiliensis Hev b 7 patatin-like allergen	S, 10 mg l^{-1}, α-MF	[107,108]
Maize cytokinin oxidase	S, native	[109]
Oat phytochrome A, phA	I, 30 μg g^{-1}	[110,111]
Oat phytochrome A, phyA65 apoprotein	I, 20 μg g^{-1}	[112]

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Case No.: IPR2023-00322
U.S. Patent No. 10,273,492

Table 3 (*continued*)

Protein	Comments: mode, amount, signal sequence	Reference
Olea europaea (olive tree) aeroallergen Ole e 1	S, 60 mg l^{-1}, α-MF	[113]
Pepper endo-β-1,4-glucanase cCel1	S, α-MF	[114]
Pepper endo-β-1,4-glucanase cCel2	S, native	[114]
Persea americana (avocado) prs a 1 major allergen	S, 50 mg l^{-1}, α-MF	[115]
Phaseolus vulgaris agglutinin (phytohemagglutinin)	S, native	[45]
Potato phytochrome B	I, 25 μg g^{-1}	[116]
Ragweed allergen Amb a 6	S, 1 mg l^{-1}, α-MF	[117]
Soybean root nodule acid phosphatase	S, 10 mg l^{-1}, α-MF	[118]
Spinach glycolate oxidase	I, 250 U g^{-1}	[119,120]
Spinach phosphoribulokinase	I, 0.5 mg g^{-1}	[121]
Timothy grass group I allergen	S, α-MF	[122]
Tomato *Lycopersicon esculentum* Mill. LeMir (*L. esculentum* miraculin)	S, PHO1	[123]
Wheat lipid transfer protein	S, 720 mg l^{-1}, PHO1	[124]
Invertebrates		
Achacina fulica Ferussac (giant African snail) achacin	S, 0.2 mg l^{-1}, native	[125]
Aplysia californica (marine invertebrate) ADP ribosyl cyclase	S, 300 mg l^{-1}, α-MF	[126]
Aequorea victoria (jellyfish) green fluorescent protein	I, S, PHA-E	[45,127]
Boophilus microplus (cattle tick) Bm86	I, S*, 1.5 g l^{-1}, SUC2	[128–131]
Cockroach allergen, Bla g 4	S, 50 mg l^{-1}	[132]
Drosophila melanogaster angiotensin I-converting enzyme	S, 160 mg l^{-1}, α-MF	[133]
Firefly luciferase	I (peroxisome)	[134]
GAVAC™ vaccine against cattle tick	S, 2.0 g l^{-1}	[135]
Haementeria ghilanii (South American leech) ghilanten	S, 10 mg l^{-1}, α-MF	[136]
Hirudo medicinalis (leech) hirudin	S, 1.5 g l^{-1}, α-MF	[137]
Honey bee odorant-binding protein (ASP2)	S, 150 mg l^{-1}, native	[138]
Nippostrongylus brasiliensis (parasitic nematode) non-neuronal secreted acetylcholine sterase	S, 27 mg l^{-1}, α-MF	[139]
Spider dragline silk protein	I, 663 mg l^{-1}	[140]
Tick anticoagulant peptide	S, 1.7 g l^{-1}	[141]
Vertebrates (non-human)		
Bovine enterokinase catalytic domain	S, 6.3 mg l^{-1}, α-MF	[142]
Bovine follicle-stimulating hormone β-subunit	S, 4 μg ml^{-1}, α-MF	[143]
Bovine IFN-omega 1	S, 4 mg l^{-1}, SUC2	[144]
Bovine lysozyme c2	S, 550 mg l^{-1}, native	[145]
Bovine opsin	S*, 0.3 mg l^{-1}, PHO1	[146]
Bovine pancreatic trypsin inhibitor (aprotinin)	S, 930 mg l^{-1}, α-MF	[147]
Bovine β-casein	I, 1 g l^{-1}	[148]
Bovine β-lactoglobulin	S, >1 g l^{-1}, α-MF	[149–151]
Bovine tissue-type plasminogen activator (tPA)	S, 1.1 mg l^{-1}, α-MF	[152]
Brushtail possum TNFα	S, α-MF	[153]
Bungarus fasciatus (snake) venom gland acetylcholinesterase	S, 2 mg l^{-1}, native	[154]
Chicken liver α-*N*-acetylgalactosaminidase	S, 11.6 mg l^{-1}, α-MF, PHO1	[155]
Electrophorus electricus acetylcholinesterase AChE type T	S, native	[156]
Hen lysozyme	S, 20 mg l^{-1}, α-MF	[157]
Mammalian lipocalin allergen Bos d2	S, mg amounts, native	[158]
Mouse 5HT5A 5-tryptamine receptor	S*, 40 pmol mg^{-1}, α-MF	[159]
Mouse epidermal growth factor	S, 450 mg l^{-1}, α-MF	[35]
Mouse gelatinase B	S, 10 mg l^{-1}, α-MF	[160]
Mouse lysosomal acid α-mannosidase	S, native	[161]
Mouse major urinary protein complex (MUP)	S, 270 mg l^{-1}, native	[162]
Mouse Mdr3 P-glycoprotein	I (membrane-bound), 6 μg mg^{-1}	[163–165]
Mouse single-chain Fv fragments (sFv)	S, 250 mg l^{-1}, α-MF, PHO1	[166]
Murine endostatin	S, 200 mg l^{-1}, α-MF	[34]
Murine Golgi mannosidase IA	S, PHO1	[167]
Murine macrophage inflammatory protein-2 (MIP-2)	S, 40 mg l^{-1}, α-MF	[168]
Ovine follicle-stimulating hormone (oFSH)	S, 22 mg l^{-1}, α-MF	[169]
Porcine follicle-stimulating hormone	S, 10 mg l^{-1}, PHO1	[170]
Porcine inhibitor of carbonic anhydrase (transferrin family)	S, 5 mg l^{-1}, α-MF	[171]
Porcine leukocyte 12-lipoxygenase	I	[172]
Rabbit intestinal peptide transporter (PEPT1)	I	[173]
Rabbit intestinal peptide transporter (PEPT2)	I	[174]
Rabbit monoclonal single-chain Fv specific for recombinant human leukemia inhibitory factor	S, 100 mg l^{-1}, α-MF	[175]

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Case No.: IPR2023-00322
U.S. Patent No. 10,273,492

Table 3 (*continued*)

Protein	Comments: mode, amount, signal sequence	Reference
Rabbit plasma cholesteryl ester transfer protein	S, PHO1	[176]
Rabbit testicular angiotensin-converting enzyme	S, PHO1, native	[177]
Rat acetylcholinesterase	S, 1 mg l^{-1}, native	[154]
Rat brain acetylcholinesterase T subunit	S, 100 U l^{-1} α-MF	[178]
Rat complement regulator, crry	S, α-MF	[179]
Rat Golgi sialoglycoprotein MG160	S, 10 mg l^{-1}, α-MF	[180]
Rat high-mobility group 1 (HMG 1)	S, 50 mg l^{-1}, α-MF	[181]
Rat liver mitochondrial carnitine palmitoyl transferases I and II (CPTI and II)	I (mitochondria)	[182,183]
Rat NO synthase reductase domain	I, 25 mg l^{-1}	[184]
Rat peroxisomal multifunctional enzyme (perMFE-II)	I	[185]
Rat procathepsin B	S, 100 mg l^{-1}, α-MF	[186,187]
Sea raven type II antifreeze protein (SRAFP)	S, 30 mg l^{-1}, α-MF	[188,189]
Shark 17α-hydroxylase/C17,20-lyase	I	[190]
Syrian golden hamster prion protein PrP^c	I, <0.1 mg l^{-1}	[191]
Humans		
α(1,3/4)-Fucosyltransferase	S, 30 mg l^{-1}, α-MF	[128]
α-1,2-Mannosidase 1B w/o TM domain	S, α-MF	[192]
α-*N*-Acetylgalactosaminidase (α-NAGAL)	S, 11.6 mg l^{-1}, α-MF	[193]
α1-Antitrypsin (α1-AT)	S, inulinase signal sequence	[194]
β2-Adrenergic receptor	S*, 25 nmol g^{-1}, α-MF	[159]
μ-Opioid receptor	S*, α-MF	[195]
ADAR1, ADAR2, ds-RNA-specific adenosine deaminases	I, 1 mg l^{-1}	[196]
Alzheimer's disease amyloid precursor protein α, β, and γ-secretase products	S, PHO1	[197]
Alzheimer's disease amyloid precursor protein, 2 domains	S, 24 mg l^{-1}, 0.1 mg l^{-1}, α-MF	[198]
Amyloid precursor-like protein 2 (APLP2)	S, 40 mg l^{-1}, α-MF	[199]
Amyloid precursor protein (APP)	S, 24 mg l^{-1}, PHO1	[200,201]
Amyloid precursor proteins, rAPP695, rAPP770	S, 4.5+1 mg l^{-1}, native	[202]
Bile salt-stimulated lipase	S, 300 mg l^{-1}, native, INV	[203]
Bivalent diabody against carcinoembryonic antigen (CEA), T-cell coreceptor CD2	S, 1 mg l^{-1}, α-MF	[204]
c-Kit receptor kinase domain	I, 0.2 mg l^{-1}	[205,206]
Carcinoembryonic antigen	S, 20 mg l^{-1}, α-MF	[207]
Caspase-3	I, 1 μg g^{-1}	[208]
Cathepsin K	S, 38 mg l^{-1}, α-MF	[209,210]
Cathepsin L propeptide	S, 10 mg l^{-1}, α-MF	[211,212]
Cathepsin V	S, α-MF	[213]
CD38	S, 455 mg l^{-1}, α-MF	[214]
CD40 ligand soluble form	S, 255 mg l^{-1}	[215]
Chimeric B7-2 antibody fusion protein	S, 15 mg l^{-1}, α-MF	[216]
Chorionic gonadotropin α subunit, β subunit, and αβ heterodimer	S, 24 mg l^{-1} (α), 3 mg l^{-1} (β), 16 mg l^{-1} (αβ), α-MF	[217]
Cromer blood group antigen decay-accelerating factor	S, α-MF	[218]
Cytomegalovirus ppUL44 antigen	I, 0.1 mg ml^{-1}	[219]
Decay-accelerating factor DAF (CD55)-Echovirus-7 receptor	S, 6 mg l^{-1}, α-MF	[220]
Double-stranded RNA-specific editase I (hREDI)	I, 1 mg l^{-1}	[221]
Endostatin	S, 20 mg l^{-1}, α-MF	[34,222]
Fas ligand	S, 100 mg l^{-1}, α-MF	[223]
Fibrinogen, 143–411, 143–427	S, 100 mg l^{-1}, 75 mg l^{-1}, α-MF	[224]
Fibroblast collagenase (proMMP-1)	S, 2.3 mg l^{-1}, α-MF	[225]
Fibrinogen-420 αEC domain	S, α-MF	[226]
Gastric cathepsin E	S, 0.6 mg l^{-1}, native	[227]
Heart muscle carnitine palmitoyltransferase I (M-CPTI)	I (mitochondria)	[228]
Insulin	S, synthetic signal	[44]
Insulin-like growth factor-1 (IGF-1)	S, 600 mg l^{-1}, α-MF	[23]
Interferon-γ receptor cytoplasmic domain	I	[229]
Interleukin-17 (hIL-17)	S, 0.35 mg l^{-1}, α-MF	[230]
Intracellular proteinase inhibitor (PI-6)	I, 50 mg l^{-1}	[231]
Kunitz-type protease inhibitor domain of protease nexin-2/amyloid β-protein precursor	S, 1.0 g l^{-1}, α-MF	[232]
Leukemia inhibitory factor (LIF)	S, 17 mg l^{-1}, α-MF	[233]
Lymphocyte surface antigen CD38	S, 400 mg l^{-1}, PHO1	[234]
Lysosomal α-mannosidase	S, 83 μg l^{-1}, native	[235]
Mast cell tryptase	S, 6.5 mg l^{-1}, α-MF	[236,237]

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Motif Exhibit 1015, Page 12 of 22

Case No.: IPR2023-00322
U.S. Patent No. 10,273,492

Table 3 (*continued*)

Protein	Comments: mode, amount, signal sequence	Reference
MHC class II heterodimers (soluble form/HLA-DR2)	S, 400 μg l^{-1}, α-MF	[238]
Monoclonal single-chain Fv	S, 50 mg l^{-1}, α-MF	[239]
Monocyte chemoattractant protein-1 (MCP-1)	S, 100 mg l^{-1}, native and α-MF	[240]
Monocyte chemotactic protein 3 (hMCP-3)	S, 1 mg l^{-1}, PHO1	[241]
Neural cell adhesion molecule (NCAM)	S, 50 mg l^{-1}, PHO1	[242]
NonO nucleic acid binding protein	I (endoplasmic reticulum)	[243]
Pancreatic α-amylase	S, 20 mg l^{-1}, α-MF	[244]
Pancreatic triglyceride lipase	S, 75 ml l^{-1}, PHO1	[245]
Papain nitrile hydratase	S, 5 mg l^{-1}, α-MF	[246]
Placental alkaline phosphatase (PLAP)	S, 2 mg l^{-1}, PHO1	[247]
Placental protein-14 (PP-14)	S, α-MF	[248]
Plasminogen kringles 1–4	S, 17 mg l^{-1}, PHO1	[50]
Plasminogen kringles 1–4, angiostatin protein	S, 10% total protein, PHO1	[249]
Procarboxypeptidase A2	S, 180 mg l^{-1}, α-MF	[250]
Procathepsin B	S, 20 mg l^{-1}, α-MF	[251]
Procolipase	S, 30 mg l^{-1}, native	[252]
Protein kinase C interacting protein 1 (PKCI-1)	I, 0.25 mg l^{-1}	[253]
Proteinase 3, Wegener's antigen	S, 670 mg l^{-1}, α-MF	[254]
Proteinase inhibitor 8	I, 15% total protein	[255]
scFv (against ovarian carcinoma)-biotin mimetic peptide	S	[256,257]
scFv (against squamous carcinoma)	S, 50 mg l^{-1}, α-MF	[239]
Serum albumin	S, 3 g l^{-1}, native	[58,258-260]
Serum transferrin N-lobe	S, 240 mg l^{-1}, α-MF	[261-263]
Sex steroid binding protein	S, 4 mg l^{-1}, α-MF	[264]
Single-chain urokinase-type plasminogen activator	S, 5 mg l^{-1}, pre *Mucor pusillus* rennin signal	[49]
Thrombomodulin	S	[33]
Tissue factor extracellular domain	S, 10 mg l^{-1}, PHO1	[265]
Tissue kallikrein	S, 30 mg l^{-1}, α-MF	[266,267]
Tissue-type plasminogen activator kringle 2 domain	S, 170 mg l^{-1}, α-MF	[9,26,53,268–270]
Transforming growth factor β receptor extracellular domain	S, 10 mg l^{-1}, α-MF	[271]
Tumor necrosis factor α (TNF)	I, 10 g l^{-1}	[272,273]
Type 1 plasminogen activator inhibitor (PAI-1)	S, 3 mg l^{-1}, α-MF	[274]
Type III collagen (with prolyl 4-hydroxylase)	I, 15 mg l^{-1}	[28]
Urokinase-type plasminogen activator-annexin V chimeras	S, 600 IU ml^{-1}, pre *Mucor pusillus* rennin signal	[275]
Vascular endothelial growth factor (VEGF165)	S, 40 mg l^{-1}, PHO1	[276]
Viruses		
A/VICTORIA/3/75 influenza virus neuraminidase head domain	S, 3 mg ml^{-1}, α-MF	[277,278]
Bovine herpes virus-1 glycoprotein D	S, 20 mg l^{-1}, α-MF	[279,280]
Dengue virus type 1 structural gene recombinant E protein	S, PHO1, prM virus signal sequence	[281]
Hepatitis B virus surface antigen	I, 400 mg l^{-1}	[31,282]
Hepatitis B virus surface antigen-HIV gp41 epitope chimera	I	[283]
Hepatitis E virus ORF3	I	[284]
Human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein, gp120 (ENV)	S, 20 mg l^{-1}, α-MF	[285]
Polyomavirus large T antigen	I, 0.5 mg l^{-1}	[286]
Reovirus lambda 1 core protein	I, 0.8 mg l^{-1}	[287]
Reovirus sigma 1 protein	I	[288]
Vaccinia virus complement control protein	S, 3 mg l^{-1}, α-MF	[289]

I = intracellular (with subcellular location), S = secreted, S* = secreted to plasma membrane. Amounts are highest reported for particular protein. Signal sequences: α-MF (*S. cerevisiae* α-mating factor); PHO1 (*P. pastoris* acid phosphatase); SUC2 (*S. cerevisiae* invertase).

as human pharmaceuticals in clinical trials. IGF-1 in a treatment for amyotrophic lateral sclerosis [57] and human serum albumin (HSA) in a serum replacement product [58] have passed through clinical trials and are awaiting final approval. The angiogenesis inhibitors endostatin and angiostatin are in or rapidly approaching clinical trials [59]. Another protein, hepatitis B surface antigen, is currently on the market as a subunit vaccine against the hepatitis B virus in South America. A complete list of heterologous proteins expressed successfully in *P. pastoris* is shown in Table 3.

Yet, despite the success of the *P. pastoris* system, opportunities exist to develop a larger range of proteins that can be expressed in the system. The new alternative promoters and marker/host strain combinations make possible the expression of heterooligomeric proteins and essential cofactors. Still little is known about *AOX1* promoter regulation at the molecular level. Such studies could lead to

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modified *AOX* promoters with increased transcriptional strength or to the identification and overexpression of factors that limit transcription of P_{AOX1}.

Studies are also needed to address problems associated with the secretion of mammalian proteins from *P. pastoris*. A better understanding of secretion signals, glycosylation, and endogenous *P. pastoris* proteases would be extremely helpful in developing and improving the *P. pastoris* heterologous expression system.

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