Pichia pastoris Strains as Powerful Cell Factories

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Pichia pastoris is the most widely used microorganism for the production of secreted industrial proteins and therapeutic proteins. This yeast has been repurposed as a cell factory for the production of chemicals and natural products.

Pichia pastoris | natural product | cell factory

1. Introduction

Methylotrophic yeasts are microorganisms capable of utilizing methanol as the sole source of carbon and energy and are hence considered as potential hosts for green bio-manufacturing. Meanwhile, these microbes, when used as cell factories, have some other advantages such as high fermentation density, low accumulation of toxic metabolites, ability to grow in cheap basal salt media, and capability of complete protein modifications and processing.

Pichia pastoris, which has been renamed as *Komagataella phaffii* [1], is one of the typical methylotrophic yeasts and plays an important role in the production of recombinant proteins. Using the *P. pastoris* expression system, thousands of recombinant proteins have been synthesized so far, some of which have successfully entered the market, such as human insulin and human interferon-α [2].

P. pastoris is a unicellular microorganism with the advantages of high expression levels of recombinant proteins, ease of large-scale cultivation, and low cultivation costs [3]. Its cell density can reach 130 g/L during industrial production [4], and the yield of recombinant proteins can reach tens of grams per liter [5]. Recombinant proteins can be expressed both intracellularly and in a secreted form in *P. pastoris*. The efficiency of secretory expression in *P. pastoris* is generally higher than that in *Saccharomyces cerevisiae*, which is another commonly adopted workhorse for microbial bio-production [6]. *P. pastoris* has a clear genetic background and is excellent in protein folding and post-translational modifications, especially glycosylation [7][8]. In *P. pastoris*, oligosaccharide chains added to proteins after translation (8–14 mannose residues per side chain) are shorter than in *S. cerevisiae* (50–150 mannose residues), and *O*-linked glycosylation is very minimal, potentially avoiding the risk of excessive glycosylation (**Figure 1**) [9]. Moreover, *P. pastoris* contains a strong methanol utilization pathway and is able to provide some key cofactors [10]. Another major difference between these two yeasts is that the homologous recombination (HR) pathway in *P. pastoris* is significantly weaker than the non-homologous end joining (NHEJ) pathway [11].

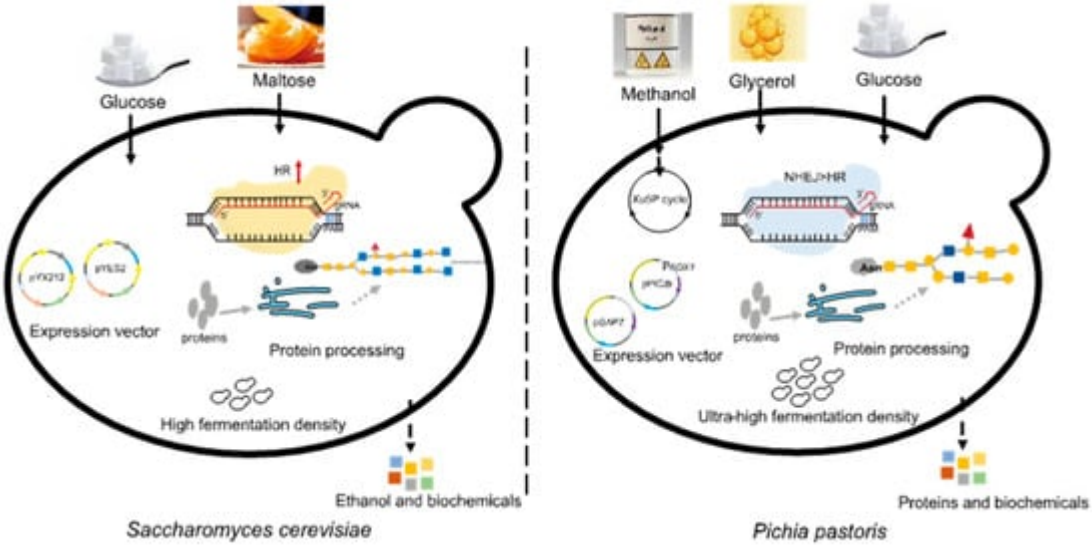


Figure 1. Differences between *Pichia pastoris* and *Saccharomyces cerevisiae*.

2. Physiological Characteristics of *Pichia pastoris*

P. pastoris belongs to the Ascomycetes class [12]. Its colony is generally milk-white with a smooth surface, showing a bulge (**Figure 2**). This yeast mainly exists in the haploid form in the asexual growth phase. Under nutrient limitation, two haploid cells can be induced with different physiological types to mate and fuse into diploids [13].

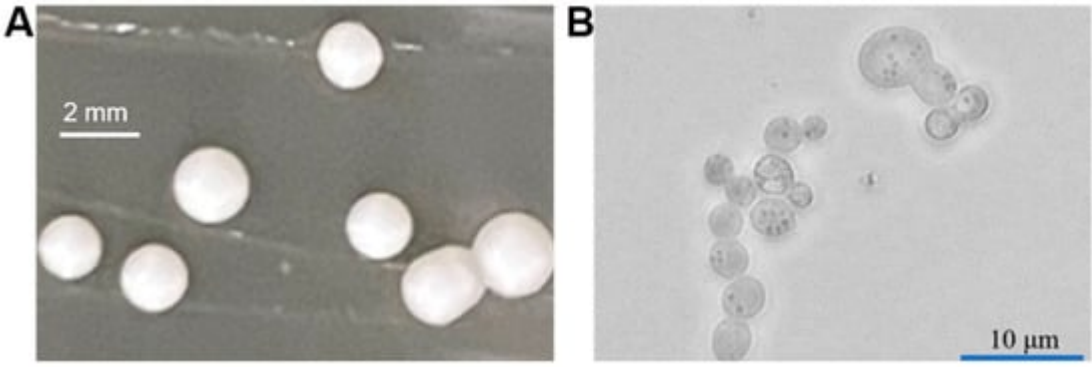


Figure 2. Colony morphology (**A**) and microscopic image (**B**) of *P. pastoris*.

P. pastoris grows optimally at 28–30 °C [14] with a tolerance of pH ranging from 3 to 7 [15]. The carbon source includes a variety of compounds such as glucose [16], glycerol [17], L-rhamnose [18], formate [19], and so on. This microbe can also use methanol as its sole source of carbon and energy owing to the presence of enzymes in the peroxisomes essential to methanol metabolism, such as alcohol oxidase, dihydroxyacetone synthase, and peroxidase [20]. Among these, the expression of alcohol oxidase is strictly induced and controlled by methanol, so the enzyme activity can only be detected in the presence of methanol [21]. *P. pastoris* can use ammonium sulfate, proline, and peptone as the nitrogen source. When ammonium sulfate is adopted, the expression levels of major genes related to methanol utilization (gene *MUT*) and peroxisome biogenesis/degradation (gene *PEX*) are the highest [22]. Meanwhile, an appropriate NH_4^+ concentration is beneficial for cell growth and heterologous protein

expression [22][23]. In some cases, the use of complex nitrogen sources such as casamino acids and peptone can effectively alleviate proteolytic degradation of heterologous proteins [24].

3. The *Pichia pastoris* Expression System

3.1. Strains

At present, the commonly used *P. pastoris* strains for heterologous protein expression include X-33, GS115, KM71, SMD1163, and MC100-3. The characteristics of these strains have been described in detail in other reports [25][26]. Based on their ability to utilize methanol, *P. pastoris* strains are mainly divided into three phenotypes, i.e., Mut^+, Mut^S, and Mut^-. Strain X-33 is a wild-type strain carrying genes *AOX1* and *AOX2* (both encoding alcohol oxidase) and the phenotype is Mut^+ (methanol utilization plus), which is often used to express recombinant plasmids containing zeocin resistance. Both GS115 and KM71 strains are histidine deficient. The GS115 strain contains *AOX1* and *AOX2*, and its own phenotype is Mut^+. In comparison, the KM71 strain is Mut^S (methanol utilization slow) because the gene *AOX1* is replaced by the *S. cerevisiae Arg4*, and the strain can only utilize methanol at a slower rate dependent on the weakly controlled gene *AOX2*. Strain MC100-3 is deficient in both *AOX* genes and hence cannot grow on methanol, thus performing the Mut^- (methanol utilization minus) phenotype. SMD1163 is a protease deficient strain in which the *pep4* gene encoding protease A and the *prb1* gene encoding a subtilisin-like protease are knocked out. This strain is favorable for the expression of heterologous proteins sensitive to proteases [27].

3.2. Expression Vectors

According to the location of the expressed proteins, *P. pastoris* vectors can be divided into intracellular expression vectors (such as pPIC3, pPICZ, pPHIL-D2, etc.) and secretory expression vectors (such as pPIC9, pPIC9K, pPICZα, etc.), in which the secretory expression vector usually contains a signal peptide sequence inserted behind the promoter. The characteristics of the expression vectors commonly used at present are summarized in detail in a recent report [28].

Integrative plasmids are frequently used for exogenous gene expression in *P. pastoris*. Such vectors are typically constructed as *E. coli*/*P. pastoris* shuttle vectors. On the one hand, these vectors contain elements for plasmid amplification in *E. coli*, including an origin of replication and a selection marker, which is usually antibiotic resistance. On the other hand, these vectors carry components required for heterologous gene expression in *P. pastoris*, including the promoter/terminator, the multiple cloning site, and a proper selection marker. The selection marker can be an auxotrophic marker (HIS4, ARG4, URA3, etc.) or an antibiotic resistance marker (zeocin, G418, etc.).

To introduce a foreign gene into *P. pastoris*, episomal vectors are generally accompanied with easier manipulation and higher efficiency compared with gene integration into the chromosome. However, natural autoreplicative vectors have limited applications in *P. pastoris* due to their instability and the uneven distribution among progeny

cells during cell division. To solve these issues, the genome of *P. pastoris* has been analyzed and the chromosome-2 centromeric DNA sequence has been identified to facilitate stable autoreplication and accurate distribution of the plasmid [29]. The stability of the episomal vectors can also be improved by introducing the autonomously replicating sequences (ARS) from other organisms, such as the panARS derived from *Kluyveromyces lactis* [30].

3.3. Promoters

3.3.1. *AOX1* Promoter

Upon entry into *P. pastoris*, methanol is decomposed into formaldehyde and hydrogen peroxide by alcohol oxidase encoded by *AOX1* and *AOX2*. However, the enzyme activity is mostly provided by *AOX1* due to the low expression level of *AOX2* [21]. The endogenous promoter controlling *AOX1* expression, i.e., P_{AOX1} upstream of *AOX1*, can be efficiently induced by methanol at low concentrations, with the optimal concentration ranging from 0.5% to 2.0% [6].

In recent years, with the continuous mechanistic research on the transcriptional regulation of P_{AOX1}, the cis-acting elements and transcription factors that affect the transcriptional activity of this promoter have been discovered. Inan et al. divided P_{AOX1} into five segments (A~E), of which fragments B and E play a promotional role for *AOX1* expression (**Figure 3**A) [31]. In *P. pastoris*, the response of P_{AOX1} to methanol is positively regulated by a cascade of transcription factors including Mit1, Mxr1, and Prm1, which bind to different sites of P_{AOX1} [32]. Among them, Mxr1 is closely related to the carbon-source-induced repression of P_{AOX1}. When methanol is used as the carbon source, Mxr1 is transferred from the cytoplasm to the nucleus, leading to the derepression of P_{AOX1}. At this point, Prm1 activates its own expression and the expression of Mit1, thus inducing strong activation of P_{AOX1}. The strength of P_{AOX1} can be improved via Mit1 overexpression [33]; however, Mit1 exerts feedback inhibition of Prm1 expression (**Figure 3**B) [34]. In consequence, the expression level of Mit1 needs to be delicately tuned to achieve the optimal expression of genes controlled by P_{AOX1}.

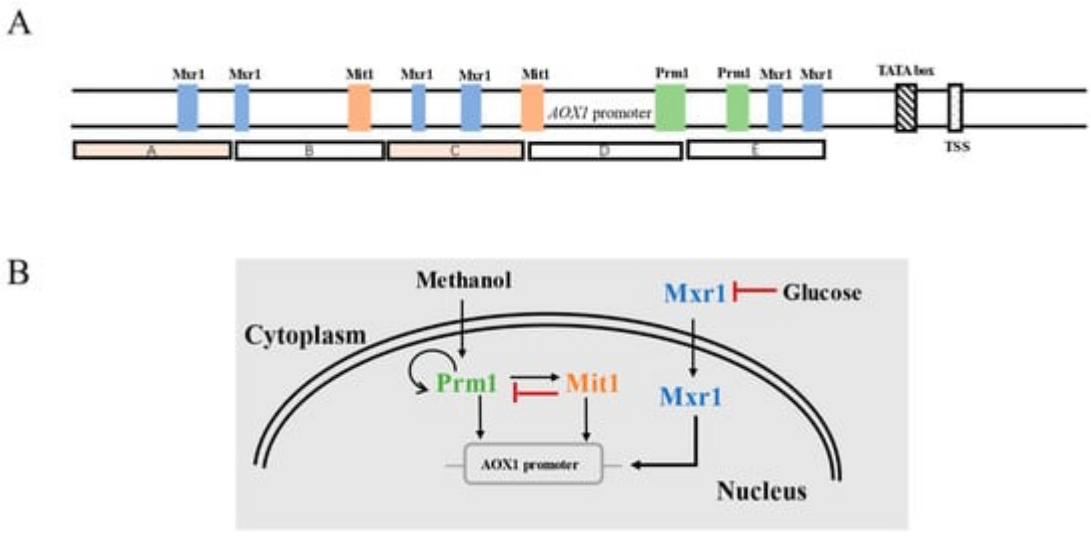


Figure 3. (**A**) The structure of P_{AOX1}. (**B**) Transcriptional regulation of P_{AOX1}.

3.3.2. GAP Promoter

Inducible promoters generally have some intrinsic disadvantages, such as costs and toxicity associated with the inducers and concerns of leaky expression of the target genes. A preferred alternative is the constitutive promoter. Genes regulated by constitutive promoters can be normally expressed during cell growth without the involvement of inducers. In *P. pastoris*, the most commonly used constitutive promoter is the glyceraldehyde-3-phosphate dehydrogenase promoter (P_{GAP}), which is considered as a standard promoter for methanol-free expression systems [35][36]. This promoter is regulated by the metabolism of carbon sources, and its transcription level is the highest in the presence of glucose and is the lowest when cells are fed with methanol [37].

P_{GAP} is generally weaker than P_{AOX1} and attempts have hence been made to improve the strength of P_{GAP}. Ata et al. [38] analyzed the transcription factor binding sites of P_{GAP}. Via targeted deletion or overexpression of these sites, a promoter library was constructed with different strength in initiating gene expression.

3.3.3. Other Promoters

Although the use of methanol as the sole source of carbon and energy is a major advantage of the *P. pastoris* cell factories, the toxicity and safety issues of methanol impose many limitations on practical industrial applications [39]. To solve this problem, methanol-free induction systems have been developed based on P_{AOX1}. Chang et al. synthesized a positive feedback circuit in which P_{AOX2}-driven Mxr1 promotes the transcription of P_{AOX1}, while P_{AOX1} is induced under glycerol starvation or in the absence of carbon sources [40]. Kinases have been proposed as potential targets for regulating the repression of P_{AOX1} in the presence of a common carbon source such as glycerol [41]. By targeting the genes encoding glycerol kinase (*gut1*) and dihydroxyacetone kinase (*dak*), non-methanol-inducible P_{AOX1} expression systems were constructed using glycerol and dihydroxyacetone as carbon sources to induce P_{AOX1} expression, respectively, although the induction was not as efficient as that induced by methanol [42].

3.3.4. Synthetic Core Promoter Engineering

The core promoter region plays a pivotal role in the regulation of gene expression, and its genetic modification is an important content of promoter engineering. The core promoter is the region necessary for RNA polymerase to recognize and initiate transcription, and it consists of the RNA polymerase binding site, the TATA box, and the transcription start site. In *P. pastoris*, fully synthetic core promoters and the 5′-untranslated region have been designed and applied to P_{AOX1}, resulting in a series of promoter libraries with different expression levels [43].

3.4. Signal Peptides

Secretory expression of proteins is generally dependent on cleavable signal peptides (usually 15–50 amino acids) that direct the transmembrane transfer of the newly synthesized peptides and proteins. These signal peptides, mostly located at the N-termini of the secreted proteins, usually contain three domains, i.e., the positively charged basic N-terminus (1–5 amino acids), the hydrophobic center that forms a helical structure (7–15 amino acids), and the highly polar C-terminus (3–7 amino acids) that serves as the cleavage site [44]. With a great impact on the

extent of protein folding and the rate of protein secretion, signal peptides play a crucial role in high-level expression and secretion of functional proteins [45].

The signal peptides commonly used for protein secretion in *P. pastoris* include the signal sequence of α-factor and invertase-2 (SUC2) of *S. cerevisiae*, and *P. pastoris* acid phosphatase signal peptide (PHO1). Among these, α-factor is used most frequently and is mainly suitable for the secretory expression of peptides and small proteins. To further improve the efficiency of secretion, this signal peptide has been engineered through codon optimization, modification of the hydrophobic region, addition of spacer sequences, and site-directed mutagenesis [46][47][48].

3.5. CRISPR/Cas9 Genome Editing in Pichia pastoris

Since *P. pastoris* is an important workhorse for the synthesis of various bio-products, it is crucial to establish efficient and concise gene editing technologies for the genetic modifications of this microbe. Traditionally, gene insertion/deletion/replacement of *P. pastoris* relies on homologous recombination, which is inefficient with a low rate of success even when long homologous arms (sometimes more than 1 kb) are used. This is due to the domination of NHEJ over homologous recombination in this yeast [11]. Deletion of *KU70* impairs NHEJ and significantly facilitates homologous recombination at the expense of a lower transformation efficiency and slower cell growth.

CRISPR/Cas9 introduces breaks in DNA sequences complementary to the sgRNA, which are then repaired by host cells. Thereby, genetic modifications can be introduced programmably at desired locations by using sgRNA with particularly designed sequences. Compared with traditional homologous recombination-guided genomic modification, CRISPR/Cas9 is highly flexible in the sense that only sgRNA needs to be re-designed for each independent genomic modification process. As one of the most potent and convenient gene editing technologies, CRISPR/Cas9 has been explored extensively in the engineering of *P. pastoris* [8][49][50].

CRISPR/Cas9-mediated genomic editing in *P. pastoris* relies on the correct expression of Cas9 and sgRNA in the nucleus, which can be affected by a series of factors. To improve the expression, researchers used RNA Pol II promoter for sgRNA expression, added ribozyme sequences both upstream and downstream of sgRNA, and optimized the codon of Cas9. After such optimization, near 100% efficiency could be achieved in *P. pastoris* for gene deletion, and multiplex gene deletion and targeted gene insertion was achieved efficiently with the aid of NHEJ [51]. This system was further introduced into a *KU70*-knockout strain, so that DNA breaks could be repaired through homologous recombination. Despite lower cell viability, near 100% efficiency of gene integration was achieved [52].

The biosynthetic pathways of value-added compounds (natural products and bulk chemicals) are often complex and involve multiple pathway genes. Therefore, a competent genetic tool that can manipulate multi-gene pathways has important implications for the application of *P. pastoris* as a cell factory. Based on the *KU70* knockout strain, a CRISPR/Cas9-mediated marker-less multi-site gene integration method has been developed, for which various sgRNA targets are designed within 100 bp upstream of the promoter or downstream of the terminator. Using this

method, the integration efficiency of double-locus could reach 57.7%–70% [53]. The same method was used to establish a standardized CRISPR-based synthetic biology toolkit, in which the integration efficiency of double-locus could reach ~93% [54]. This toolkit allowed for one-step assembly of the biosynthetic pathways of 2,3-butanediol, *β*-carotene, zeaxanthin, and astaxanthin [54].

4. Adaptive Laboratory Evolution of *Pichia pastoris*

Adaptive laboratory evolution (ALE) is a method to artificially simulate the mutation and selection process in natural evolution under laboratory conditions such that the directed evolution of microorganisms can be achieved within a short period of time and mutated microbes with desired traits can be screened [55]. Compared with metabolic engineering, ALE only focuses on the generation of appropriate interference factors without detailed information on the intricate and intersecting metabolic networks, thus demonstrating broad applicability and strong practicability. ALE is one of the most effective methods of strain construction toward high-level synthesis of bio-products [56][57]. Although widely used in *S. cerevisiae* and *E. coli*, ALE only has limited applications in *P. pastoris*, and there is still a large space for development.

Efficient use of carbon sources and substrates is key to the high-level microbial production of bio-products, and ALE has been adopted in promoting the metabolic performances of *P. pastoris* on various nutrients or substrates. Moser et al. investigated the effect of growth media on cell growth and recombinant protein production in *P. pastoris* X-33 using methanol as a carbon source for continuous subculture in eutrophic medium YPM and low-nutrient medium BMM. After approximately 250 generations, evolved strains showed higher growth rates. Whole genome sequencing identified mutations in the *AOX1* gene involving the methanol binding region and its vicinity, leading to, surprisingly, a decline in AOX activity, possibly due to less intracellular accumulation of the toxic compound formaldehyde. Such methanol adaptation led to significantly higher titers of recombinant human serum albumin and fused lobes hexosaminidases [58].

5. Practical Applications of *Pichia pastoris* as a Cell Factory

Since Philips Petroleum Company released the *P. pastoris* expression system to academic research laboratories in 1993, the expression system has developed rapidly [59]. *P. pastoris* has gradually replaced *S. cerevisiae* as the eukaryotic expression system because it secretes very few endogenous proteins and has glycosylation similar to that in mammalian cells. *P. pastoris* has thus been gradually developed into a common host for the expression of medical and industrial enzymes, and thousands of recombinant proteins have been successfully produced [60]. In addition, natural products with diverse structures have also been synthesized in this host (**Figure 4**), which overcomes the disadvantages associated with chemical synthesis or extraction from plants that are traditionally used for their production. These accomplishments have promoted the engineering and development of *P. pastoris* as a potent and potential microbial platform [8].

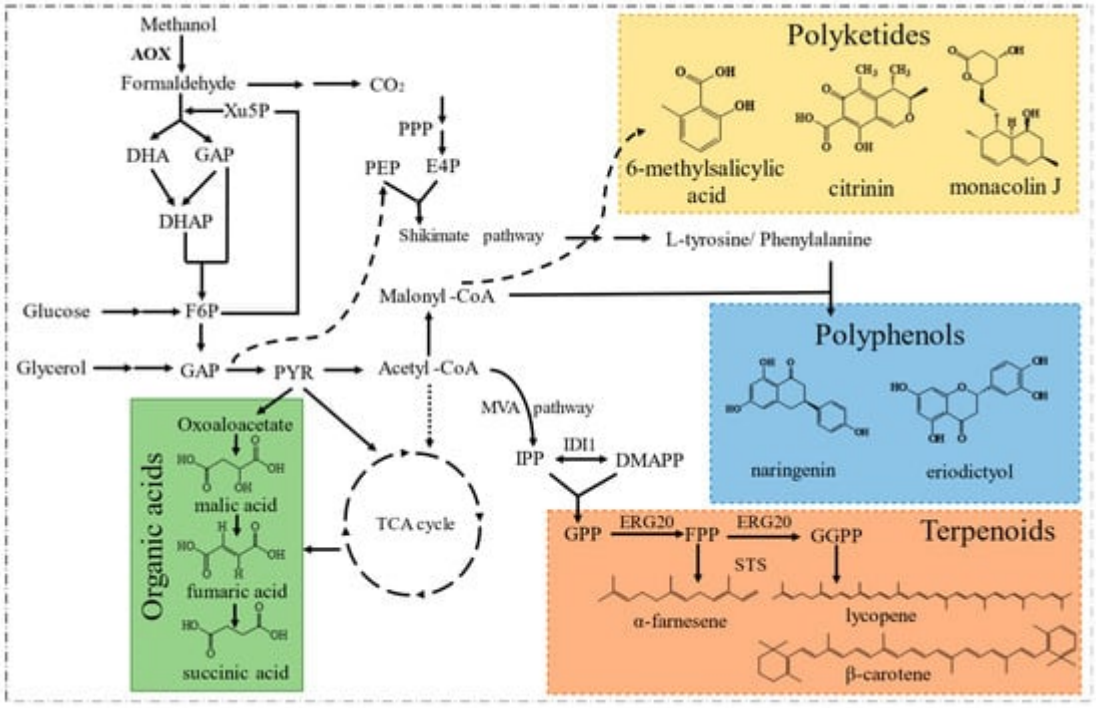


Figure 4. The portfolio of typical compounds produced by engineered *P. pastoris*.

5.1. Recombinant Proteins

5.1.1. Nanobodies

Nanobodies, the natural antibodies first found in the serum of camels and sharks, are the smallest units known to bind antigens [61][62]. Compared with traditional antibodies, nanobodies have unique properties such as strong antigen binding, low immunogenicity, high solubility and stability, and low molecular weight, which offer potential advantages in disease diagnosis and treatment [63]. For example, nanobody neutralization therapy has been employed in the treatment of the coronavirus COVID-19 [64]. Nanobodies can be stably expressed in *P. pastoris* besides prokaryotic hosts [65].

5.1.2. Human Proteins

Human serum albumin (HSA) is the major protein in human plasma and is widely used for drug delivery. Its recombinant expression in *P. pastoris* GS115 has been reported, and a high yield of 8.86 g/L was obtained after process optimization [66]. Human coagulation factor XII plays an important role in thrombosis, and its abundant supply is necessary for inhibitor screening in the development of antithrombotic drugs. The recombinant serine protease domain of human coagulation factor XII has been expressed in *P. pastoris* X-33 with a yield of 20 mg/L and a clotting activity similar to that of its natural counterpart [67]. Compared with these cellular proteins which can be expressed easily in the soluble form, high-level microbial expression of human membrane proteins can be a real challenge. Nonetheless, a human multichannel membrane protein named sterol Δ8-Δ7 isomerase has been successfully expressed in the form of a GFP fusion in *P. pastoris* as well as in *E. coli* and *S. cerevisiae*, with the best expression achieved in *P. pastoris* at 200 mg/L in shake flasks and 1000 mg/L in condensed culture [68].

5.2. Value-Added Compounds

5.2.1. Terpenoids

Terpenoids are secondary metabolites with isoprene as the basic structural unit, and they mainly include monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids, and polyterpenoids [69]. Most of these compounds have anti-tumor, anti-inflammatory, and immunomodulatory effects, and are widely used in food processing and pharmaceutical manufacturing industries [70]. Yeast cells generally produce more precursors than bacteria for terpenoid biosynthesis, including DMAPP (dimethylallyl diphosphate) and IPP (isopentenyl diphosphate) [71]. Compared with other yeast chasses, *P. pastoris* can reach a higher fermentation density, produce fewer metabolic byproducts, and present strong tolerance to complex environments. These traits make *P. pastoris* suitable for terpenoid biosynthesis.

5.2.2. Polysaccharides

Polysaccharides widely exist in nature and participate in important cellular processes as an energy source and organizational structure. Polysaccharides can be used as efficient drug carriers due to their biodegradability, biocompatibility, and generally low costs [72]. Polysaccharide biosynthesis using engineered microorganisms has been attracting increasing attention, with some of the focus laid on *P. pastoris* due to its abundant supply of sugar precursors.

5.2.3. Polyketides

Polyketides are a class of widely distributed natural products produced during the secondary metabolism of plants or microorganisms, with rich chemical properties and unique physiological activities. Polyketide biosynthesis in *P. pastoris* was first reported in 2013 for the fungal polyketide compound 6-methylsalicylic acid (6-MSA) [73]. The genes encoding *Aspergillus nidulans* phosphopantetheinyl transferase (*npgA*) and *Aspergillus terrus* 6-MSA synthase (*atX*) were integrated into the genome of *P. pastoris* via homologous recombination, thus producing 2.2 g/L of 6-MSA in a 5 L bioreactor upon 20 h of methanol induction. On this basis, a more structurally complex polyketide citrinin was also synthesized in *P. pastoris* by assembling *npgA*, *Monascus purpureus* citrinin polyketide synthase gene *pksCT*, and several genes in the citrinin gene cluster [74].

6. Conclusions

Pichia pastoris has been developed to produce proteins for four decades and has emerged as a new chassis to produce diverse chemicals and natural products in recent years. With the unique property of utilizing methanol as a sole carbon source, metabolic engineering of *P. pastoris* is attracting increasing attention amid the great concern on global energy security, given the potential of methanol as a supply of energy and carbon source for biomanufacturing. *P. pastoris* has shown great success in the biosynthesis of some natural products with the titers reaching >1 g/L, indicating very promising applications for industrialization and commercialization.

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