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Research review paper

Engineering strategies for enhanced production of protein and bio-products in *Pichia pastoris*: A review

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ABSTRACT

Pichia pastoris has been recognized as one of the most industrially important hosts for heterologous protein production. Despite its high protein productivity, the optimization of *P. pastoris* cultivation is still imperative due to strain- and product-specific challenges such as promoter strength, methanol utilization type and oxygen demand. To address the issues, strategies involving genetic and process engineering have been employed. Optimization of codon usage and gene dosage, as well as engineering of promoters, protein secretion pathways and methanol metabolic pathways have proved beneficial to innate protein expression levels. Large-scale production of proteins *via* high cell density fermentation additionally relies on the optimization of process parameters including methanol feed rate, induction temperature and specific growth rate. Recent progress related to the enhanced production of proteins in *P. pastoris via* various genetic engineering and cultivation strategies are reviewed. Insight into the regulation of the *P. pastoris* alcohol oxidase 1 (AOX1) promoter and the development of methanol-free systems are highlighted. Novel cultivation strategies such as mixed substrate feeding are discussed. Recent advances regarding substrate and product monitoring techniques are also summarized. Application of *P. pastoris* to the production of biodiesel and other value-added products *via* metabolic engineering are also reviewed. *P. pastoris* is becoming an indispensable platform through the use of these combined engineering strategies.

1. Introduction

The methylotrophic yeast *Pichia pastoris* has been established as a successful protein production platform, especially in the sector of industrial enzymes and the biopharmaceutical industry. As a "generally regarded as safe" (GRAS) microorganism, it has been used for the production of over 500 pharmaceutical proteins and more than 1000 recombinant proteins as of 2009 (Fickers, 2014). Driven by increasing demands in the food and feed industries, *P. pastoris* has also become an important host to produce enzymes such as xylanase and phytase, which are relevant to these sectors (Spohner et al., 2015). Recently, *P. pastoris* has also been favored in the expression of eukaryotic membrane proteins, facilitating advances in structural biology (Byrne, 2015; Goncalves, 2013). Using cell surface display techniques, *P. pastoris* has been used to synthesize biofuels and other chemicals (Tanaka et al., 2012). The success of *P. pastoris* as such a versatile system is mainly attributed to its ability to grow to a high biomass concentration on defined media, its capacity to perform complex post-translational modifications which include correct protein folding, disulfide bond formation as well as glycosylation, its high secretion efficiency and its repertoire of both inducible and constitutive promoters.

The successful development of high-yield yeast strains is imposed with strain- and product-specific challenges. To overcome these challenges, engineering strategies comprising genetic and process engineering approaches have been employed (Fig. 1). Extensive progress has been made for protein expression in *P. pastoris*. In this review, we will focus on recent progress related to the production of proteins and other by-products, aiming to update our previously published review (Potvin et al., 2012) from an engineering perspective. Strategies involving genetic and bioprocess engineering will be discussed. State-of-the-art monitoring techniques for substrates and products will also be briefly summarized.

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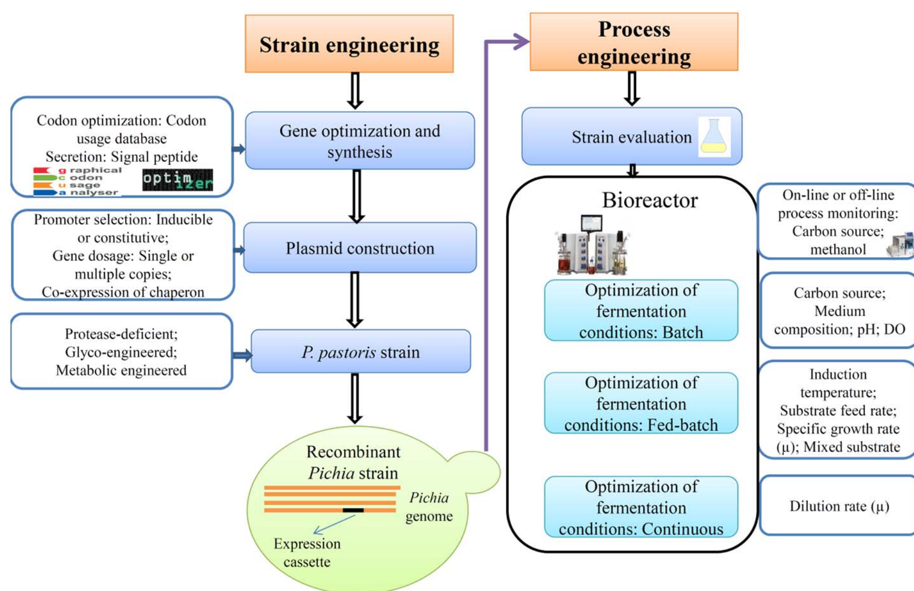

Fig. 1. General strategies for enhanced production of recombinant proteins in *P. pastoris*. Strategies are divided into strain engineering wise and process engineering wise. Considerations were indicated for each stage. Single or combined strategy could be used for product-specific purposes.

2. Genetic engineering strategies for improved protein production

2.1. Promoters

2.1.1. Advances of AOX1 promoter regulation

The alcohol oxidase I (AOX1) promoter regulates the metabolism of methanol and catalyzes the first step of methanol assimilation, converting methanol to formaldehyde. It is widely used to drive heterologous protein expression due to its tight regulation and strong inducibility when methanol is used as the sole carbon source. Although it is the most widely studied promoter in *P. pastoris*, the mechanisms for the regulation of P_{AOX1} are still gaining great attention. This is because insights into its regulation profile could facilitate the fine-tuning of P_{AOX1} and the development of methanol-free expression systems. The regulation of AOX1 expression mainly occurs at the transcriptional level. It has been recognized that the AOX1 promoter is strongly induced by methanol and repressed by glucose, glycerol and ethanol (Vogl and Glieder, 2013). Kim and coworkers investigated the regulation of AOX1 under methanol-limited and oxygen-limited conditions by visualizing the localization and expression of green fluorescent protein (GFP)-fused proteins (Kim et al., 2013). Their results confirmed that the rate of methanol consumption plays a crucial role in the final maximal protein yield. Methanol induction is regulated through the interaction between cis- or trans-acting elements. Through deletion and insertion analysis, it was determined that a region, D, which is located between positions −638 and −530 (relative to the transcription start site) of the AOX1 promoter is a cis-acting element (Xuan et al., 2009). The expression level was enhanced to 157% of that of the wild type when three copies of this region were inserted into a promoter lacking region D. The 5′-untranslated region (5′-UTR) of the AOX1 promoter consists of both positive and negative cis-acting elements which, according to a systematic analysis of this region, affect the translational efficiency of the AOX1 promoter (Staley et al., 2012). Mxr1 is a key transcription factor which regulates the activation of methanol utilization. Mxr1 contains a region which allows the binding of 14-3-3 proteins in response to various carbon sources (Parua et al., 2012). More recently, transcription factor 1 (Mit1) was identified and found to be critical to the activation of P_{AOX1} (Wang et al., 2016c). The study also provided a regulatory model for P_{AOX1} to elucidate the transduction of the methanol induction signal from the cytoplasm to the nucleus. The response to methanol induction is accomplished through a cascade of Mit1, Mxr1 and Prm1. Furthermore, the tight regulation of P_{AOX1} is attributed to the repression of methanol utilization genes.

2.1.2. Development of methanol-free P_{AOX1} systems

To address the issues associated with the use of methanol, expression systems based on non-methanol-induced P_{AOX1} may be established by activation of the methanol activation pathway or by inactivation of the catabolite repression pathway. A methanol-free, P_{AOX1}-based strain, MF1, was recently developed (Wang et al., 2017). This strain was constructed by deleting three transcription repressors associated with catabolite repression and by overexpressing the transcription activator Mit1, as previously described. The activity of P_{AOX1} in glycerol cell cultures reached 36% that of the wild type cultured in methanol. A fermentation strategy termed "Glucose-glycerol-shift" was designed based on the fact that MF1 is repressed by high concentrations of glucose, while repression is removed by glycerol or low concentrations of glucose (Wang et al., 2017). This strategy consists of batch and fed-batch phases to support biomass growth and P_{AOX1} repression, followed by a glycerol induction phase. An insulin precursor was shown to accumulate to a titer of 2.46 g/L, reaching 58.6% that of the wild type strain. Oxygen demand was also significantly reduced, making this strain a promising methanol-free system for protein production. Shen and coworkers screened 92 kinase mutants to identify two candidates: Δ*gut1* and Δ*dak* showed high AOX1 activities when cultured in non-methanol carbon sources (Shen et al., 2016). The Δ*gut1* mutant was introduced with a glycerol dehydrogenase from *Hansenula polymopha* to obtain Δ*gut1*-HpGCY1, a new strain that may be induced by glycerol. The Δ*dak* mutant on the other hand, can be induced by dihydroxyacetone (DHA). Both strains reached better expression levels than the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter and 50–60% that of P_{AOX1}, indicating the potential of those strains as novel methanol-free systems. Lately, a transcriptional repressor PpNrg1 was found to repress transcription of the AOX1 gene by binding directly to five sites on P_{AOX1}, two of which overlap the binding site of the key activator, Mxr1 (Wang et al., 2016b). The repression of P_{AOX1} was partially released through the deletion of a hexose transporter, HXT1. This suggests that this transporter may be involved in the catabolite repression of P_{AOX1}. The HXT1-deficient *P. pastoris* strain has the potential for further engineering as a methanol-free induction strain (Zhang et al., 2010).

2.1.3. GAP promoter

The P_{GAP} promoter offers an alternative to P_{AOX1} when the toxicity of methanol is of concern, such as in the food industry. It is featured by a high-level constitutive expression and is more suitable for continuous cultivation due to simpler process controls. The P_{GAP} system can

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