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Review

Recombinant protein production in *Pichia pastoris* under glyceraldehyde-3-phosphate dehydrogenase promoter: From carbon source metabolism to bioreactor operation parameters

Pınar Çalık[a,b,*], Özge Ata[b], Hande Güneş[a], Aslan Massahi[b], Erdem Boy[a], Abdullah Keskin[a], Sibel Öztürk[b], Gül H. Zerze[a], Tunçer H. Özdamar[c]

[a] *Industrial Biotechnology and Metabolic Engineering Laboratory, Department of Chemical Engineering, Middle East Technical University, 06800 Ankara, Turkey*
[b] *Graduate School of Natural and Applied Sciences, Department of Biotechnology, Middle East Technical University, 06800 Ankara, Turkey*
[c] *Biochemical Reaction Engineering Laboratory, Chemical Engineering Department, Ankara University, 06100 Tandoğan, Ankara, Turkey*

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ABSTRACT

The yeast *Pichia* (*Komagataella*) *pastoris* has become a potential host system for recombinant protein (r-protein) production. Several strong inducible and constitutive promoters have been identified in *P. pastoris* which make the host ideal in r-protein expressions. The constitutive promoter of the glyceraldehyde-3-phosphate dehydrogenase gene, P_{GAP}, is promising as its use offers the advantage of associating product quantity to growth which can be optimized by a well-designed feeding strategy so long as the product itself is not toxic to the host. In this article, carbon source metabolism and the central carbon pathways of *P. pastoris* are reviewed. After comparing P_{GAP} with the available promoters of *P. pastoris*, the bioreactor operation parameters and bioreactor operation strategies are analyzed for r-protein production under P_{GAP} by focusing on response of the cells, final product concentration, yield, and productivity. The overview of the subject reveals the requirement for in-depth information on the overall regulation of *P. pastoris* metabolism that can enable fine-tuning bioreactor operation parameters in relation with the physiology of the microorganism in order to develop new strategies for the enhanced r-protein production.

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* Corresponding author at: Industrial Biotechnology and Metabolic Engineering Laboratory, Department of Chemical Engineering, Middle East Technical University, 06800 Ankara, Turkey. Tel.: +90 312 210 43 85; fax: +90 312 210 26 00.
E-mail address: pcalik@metu.edu.tr (P. Çalık).

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1. Introduction

There are 165 recombinant proteins (r-proteins) that have been approved by the United States Food and Drug Administration (FDA) and by European Medicines Agency (EMEA) for pharmaceutical use and this number is expected to reach over 200 in the next few years [1]. Forty-five percent of the r-proteins are produced in mammalian cell hosts, 39% in bacterium *Escherichia coli* and 15% in the yeast *Saccharomyces cerevisiae* [2]. Due to its rapid growth on inexpensive minimal medium and its ability to secrete r-proteins to the fermentation medium which simplifies purification [3], the yeast *Pichia pastoris* has recently become one of the most successful and popular host systems for heterologous protein production. Further, *P. pastoris* can produce structurally and functionally correct r-proteins, especially when the r-protein is derived from eukaryotic sources: *P. pastoris* has ability to perform post-translational modifications; it has the chaperones for proper protein folding and disulfide bond formation; and it has proteases for essential proteolytic processing [4]. Moreover, *P. pastoris* is a promising host for the production of human-like glycoproteins via engineering its glycosylation pathway using GlycoSwitch vector technology [5–8].

Several strong inducible and constitutive promoters of *P. pastoris* have been identified, providing excellent tools for producing r-proteins [9,10]. The constitutive promoter of the glyceraldehyde-3-phosphate dehydrogenase gene, P_{GAP}, is one of the benchmark promoters that has been widely used for constitutive expression of r-proteins [11]. P_{GAP} is generally used for the production of r-proteins which do not inhibit or is not toxic to *P. pastoris* growth: the foremost advantage is that r-protein yield is proportional to the cell mass, while it avoids problems of methanol use, and simplifies the production process by lowering costs [12].

Similar to other yeast cells, *P. pastoris* sense the amount and quality of external nutrients through multiple interconnected signalling networks, which allow them to adjust their metabolism, transcriptional profile, and developmental program to adapt readily and appropriately to changing nutritional states [13]. The complete genome sequence for *P. pastoris* [14–16] enabled the annotation of intracellular reactions based on gene–protein data and consequently allowed scientists to determine intracellular flux distributions which are the functions of gene and protein (enzyme) expression and the intracellular metabolite concentrations. However, metabolic flux distributions could be analyzed better by encountering the metabolic regulations being either at enzyme or gene level. As P_{GAP} is a carbon source dependent promoter for protein expression in *P. pastoris*, in this review, first the intracellular reaction network of *P. pastoris* is presented and the carbon sources that are utilized by *P. pastoris* are discussed. Thereafter, among the crucial steps for r-protein production, P_{GAP} and other promoters used in *P. pastoris* are reviewed. Afterwards, the bioreactor operation parameters, the composition of the culture medium, oxygen transfer (OT) conditions, pH, and temperature; moreover, the bioreactor operation modes, including batch, fed-batch, and continuous cultures, the r-protein production strategies applied under P_{GAP} are analyzed in terms of response of the cells, final product concentration, yield, and productivity. Finally, the perspectives are discussed with respect to the future of using P_{GAP} to control r-protein expression in *P. pastoris*.

2. Carbon source metabolism within the intracellular reaction network of *P. pastoris*

The production of r-proteins is frequently enhanced at the levels of transcription, codon usage, protein folding, and secretion; however, overproduction of heterologous proteins, also directly affects the primary metabolism of the cells [17,18]. Therefore, in this section, based on the complete genome sequence of *P. pastoris* [14–16], the carbon sources that can be utilized by *P. pastoris* were determined and the central carbon metabolism of *P. pastoris* is presented in Fig. 1. The genes and the encoded enzymes of the central carbon metabolism of *P. pastoris* are given in Table 1. Similar to other yeast cells, *P. pastoris* can produce energy through fermentation of sugars, i.e., glucose, fructose, galactose, maltose, etc., and sugar alcohols, i.e., sorbitol and mannitol, or through oxidation of a variety of fermentation products, such as glycerol, ethanol, and lactate. As widely used, the methylotrophic yeast *P. pastoris* can also use methanol as sole carbon and energy source.

The rate of the transport processes determines the growth kinetics of the microorganism [16]. The carbohydrates are taken by the cells in a certain order with intermittent lag phases due to the set of mechanisms controlled by glucose since the transcription of the genes encoding transporters is finely regulated by the glucose concentration in the medium. Thus, the presence or uptake of glucose has a negative impact upon the metabolism of other sugars [19]. In *S. cerevisiae* hexose uptake is mediated by HXT proteins [20] together with *GAL2* gene products. High abundance of hexose transporters encoded by more than 10 isogenes in the fermentative yeast *S. cerevisiae* leads to high glucose uptake rates. Nevertheless, respiratory yeasts like *P. pastoris* have a limited glucose uptake rate, as they contain less hexose transporter genes which encode energy dependent symporters. Additionally, K_S values are usually in the μM range for glucose in Crabtree-negative yeasts. In *P. pastoris* chemostat cultivations, the specific glucose uptake rate was found to be limited to $q_{\mathrm{Glc\,max}} = 0.35\,\mathrm{g\,g^{-1}\,h^{-1}}$ which was 8-fold lower than that obtained in fully aerobic *S. cerevisiae* ($q_{\mathrm{Glc\,max}} = 2.88\,\mathrm{g\,g^{-1}\,h^{-1}}$) at the maximum specific growth rate $\mu_{\max} = 0.19\,\mathrm{h^{-1}}$ [21,22].

Limited glucose uptake rates keep *P. pastoris* from overflow metabolism, which causes reduced cell yield and desired product formation. Among the putative sugar transporters of *P. pastoris*, two sequences have similarities to hexose transporters of *S. cerevisiae* with corresponding PIPA IDs of PIPA00236 and PIPA08653 [16]. PIPA00236, the ortholog of *HXT6* gene in *S. cerevisiae*, encodes the major low affinity glucose transporter of *P. pastoris* [23]. Disruption of this gene led to impaired growth on high concentrations of glucose while disruption of PIPA08653 did not show a distinct growth phenotype. Two high affinity glucose transporters of *P. pastoris* were reported to be PIPA02561 and PIPA00372 with high similarity to high affinity glucose transporters such as *Kluyveromyces lactis* Hgt1 [16]. *GTH1* gene of *P. pastoris* [24] corresponds to one of these genes reported by Mattanovich et al. [16]. Besides, PIPA01691 was found to be the potential transporter-like hexose sensor (*SNF2*). In another work conducted by Zhang et al. [25], two hexose transporters were identified experimentally, i.e., hexose transporter 1 and hexose transporter 2, encoded by the genes Pp*HXT1* and Pp*HXT2*, respectively; however, neither chromosomal location, nor