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Naturally occurring novel promoters around pyruvate branch-point for recombinant protein production in *Pichia pastoris* (*Komagataella phaffii*): Pyruvate decarboxylase- and pyruvate kinase- promoters

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ABSTRACT

Novel strong promoter discovery is crucial for the design of novel strains of the industrial yeast *Pichia pastoris* for recombinant protein (r-protein) production. In order to remedy the deficiency, transcriptome and proteome data of *P. pastoris* were analysed. Genes having higher expression levels than glyceraldehyde-3-phosphate-dehydrogenase (GAP) gene were identified as promoter sources. Pyruvate kinase- (P_{PYK}) and pyruvate decarboxylase- (P_{PDC}) promoters around pyruvate-node were determined as promising candidates, and *in silico* analysis of the putative promoter regions was performed. The putative promoters were introduced into the plasmid pGAPZαA::*hGH* harboring human growth hormone (hGH) gene, instead of P_{GAP}. Single-copy *hGH* gene carrying strains under P_{PYK}, P_{PDC}, and for comparison under P_{GAP} were tested in high-cell-density rhGH fermentations, respectively, abbreviated as BR_{PYK}, BR_{PDC}, and BR_{GAP}. Comparative promoter strength assessment was made by the mRNA-transcription-copy-number (mTCN) and r-protein concentration measurements, and by constrained flux analysis. P_{PDC} and P_{PYK} exhibited higher activity compared to P_{GAP}. Highest rhGH titer was obtained in BR_{PDC} as 122 mg dm^{-3} at t = 15 h, in BR_{PYK} as 101 mg dm^{-3} at t = 12 h, and in BR_{GAP} as 58 mg dm^{-3} at t = 9 h; while, with P_{PDC} and P_{GAP} the mTCNs were high and close. The flux distributions demonstrated regulatory effects of the novel promoters in the engineered strains and validated cross-pathway regulatory interactions.

1. Introduction

Pichia pastoris (syn. *Komagataella phaffii*) has become a highlighted yeast platform for extracellular production of heterologous recombinant proteins (r-proteins) due to its hybrid characteristics such as the ease of genetic manipulations and fast growth like bacteria, and post-translational modification performance like higher eukaryotes [1,2]. *P. pastoris* has been reviewed in many aspects such as protein folding and secretion [3,4], metabolic engineering approaches as well as synthetic biology tools [4–12], and fermentation conditions and strategies [9,13–16]. In high-cell-density (HCD) aerobic fed-batch fermentations, major issues encountered to enhance production and productivity are, ***i)*** control of the substrate uptake rates [17], and ***ii)*** tuning the oxygen- transfer and uptake rates [18]. In HCD *P. pastoris* fermentations, in order to drive the intracellular reaction network towards the r-protein production, keeping the specific oxygen uptake rate at pre-determined value(s) is imperative. Thus, strong promoter discovery is crucial for design and construction of successful novel strains of the industrial yeast *Pichia pastoris* which can operate under low- to moderate- oxygen transfer conditions. Several inducible and constitutive *P. pastoris* promoters are available with distinct properties and strengths [9,15]. The inducible promoter alcohol oxidase I (P_{AOX1}) [19] and the constitutive promoter glyceraldehyde-3-phosphate dehydrogenase (P_{GAP}) [20] are widely used strong promoters with distinct growth- and production- phases. For biopharmaceutical protein production, P_{GAP}-based fermentation processes with glucose based defined media are more advantageous compared to P_{AOX1} based fermentations [7], since glucose based defined medium also may simplfy downstream purification and regulatory documentation. Recently, Ata et al. [21] analyzed P_{GAP} in terms of putative transcription factor (TF) binding sites and constructed a synthetic library with distinct regulatory properties through deletion and duplication of the identified putative TF binding sites to understand their

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roles on the heterologous protein production. A threefold higher expression was obtained with a P_{GAP} variant compared to the naturally occurring P_{GAP} [21]. Besides, to increase heterologous protein production, using double promoter expression system is a novel conspicuous design strategy [22] where two potential strong promoters are needed.

In this context, the intriguing operation of the metabolism stimulated us to focus on the discovery of naturally occurring novel promoters (NONPs) in *P. pastoris* having different expression patterns than the existing promoters, empowering design of novel strains for the r-protein production. Transcriptome and proteome data of *P. pastoris* obtained under different oxygen transfer conditions [23] were analysed to predict the NONPs. Thereafter, r-*P. pastoris* strains carrying the human growth hormone (hGH) gene under the control of the predicted NONPs were constructed; and, as promising substitutes for the constitutive P_{GAP}, their potentials and strengths in the r-protein production were investigated in biphasic HCD fermentations under well defined oxygen transfer conditions. Further, the flux distributions were calculated by constrained flux balance analysis for the fed-batch fermentations by the two r-*P. pastoris* strains each constructed with a NONP and, for comparison by the strain constructed with P_{GAP}. The flux distributions were analysed around glyceraldehyde-3-phosphate and pyruvate nodes to determine influences of the promoters governing the r-protein synthesis gene on the flow of carbon molecules through the central pathways.

2. Materials and methods

2.1. Strains and plasmids

pGAPZαA*::hGH* [24] was used as the parent plasmid. *E. coli* DH5α (Invitrogen, CA, USA) and *P. pastoris* X-33 (Invitrogen, CA, USA) were used in the construction of the expression cassettes with the novel promoters.

2.2. Engineering P. pastoris: construction of expression cassettes with novel promoters

New expression systems were designed and constructed with the NONPs using the determined putative promoter regions to drive the gene expression with *Saccharomyces cerevisiae* α-mating factor *pre-pro* sequences (α-MF) as secretion signal leader. The promoters were placed instead of P_{GAP} in the parent plasmid pGAPZαA*::hGH*, having *Nsi*I and *Eco*RI restriction sites in 5′ and 3′ ends (Fig. 1). The complete sequences of the putative promoter regions were synthesized by GenScript (Piscataway, NJ, USA). The synthesized DNA sequences and parent plasmid were double-digested with *Nsi*I and *Eco*RI, and then ligated. The constructed plasmids were transformed into *E. coli*. The recombinant plasmids carrying novel promoters were isolated from the transformants and sequences were analysed (ABI Prism 310 Genetic Analyzer, Applied Biosystems, USA). The sequence authenticity was checked with NCBI (NIH, USA) nucleotide BLAST tool.

2.2.1. E. coli *and* P. pastoris *transformation*

E. coli DH5α was transformed using calcium chloride method. Isolated plasmids from recombinant *E. coli* strains were linearized with *Nsi*I restriction enzyme prior to transformation. Lithium chloride method was used to perform the transformation into *P. pastoris*. pPDC*::hGH* and pPYK*::hGH* were integrated into *pdc1* and *pyk* locus respectively in chromosome 3 and 2. Transformed *P. pastoris* cells were recovered by incubating at 30 °C for 4 h in YPD medium, contained (in g dm^{-3}): peptone, 20; yeast extract, 10; glucose, 20; and then plated on YPD plates, contained (in g dm^{-3}): peptone, 20; yeast extract, 10; glucose, 20; agar, 20; Zeocin 0.1. After 48–72 h at 30 °C, randomly selected transformants were streaked on selective YPD plates and incubated for another 24 h at 30 °C, prior to genomic DNA isolation. Isolation of the genomic DNA from the putative *Pichia* transformants was performed as reported by Massahi and Çalık [24].

2.2.2. Pre-screening and hGH gene copy number determination

For the determination of the *hGH* gene copy number, quantitative polymerase chain reaction (qPCR) experiments were conducted (Corbett Rotor-Gene 6000, QiaGen, Germany) in triplicate where each colony was duplicated in each run, and all the strains were analyzed in the same run (Table S1; Fig. S1). The primers were synthesized by Sentromer (Istanbul, Turkey). *P. pastoris* strains carrying single-copy *hGH* gene under P_{PYK}, P_{PDC}, and for comparison under P_{GAP} were selected.

2.3. Laboratory-scale bioreactor experiments

All *P. pastoris* strains were stored in microbanks at -80 °C. Extracellular rhGH producing *P. pastoris* was streaked onto YPD agar containing Zeocin 0.1 g dm^{-3}, and incubated for 48 h at 30 °C. After incubation, the culture was inoculated into buffered BMGY medium, containing (in g dm^{-3}): yeast extract, 10; peptone, 20; yeast nitrogen base without amino acids and ammonium sulfate, 3.4; ammonium sulfate, 10 g; biotin, 4×10^{-5}; glycerol, 10; chloramphenicol, 34 mg; and 0.1 M potassium phosphate buffer (pH = 6.0), and grown in 250 cm^3 shake-bioreactors (Sartorious Certomat, Germany) at 30 °C ± 0.1 °C and 200 rpm. The cells were harvested at a final OD_{600} of 18, by centrifugation at 2000 g for 10 min at 20 °C.

The biphasic HCD fermentations were designed in 3 dm^3 controlled bioreactors (BBraun CT2-2, Germany) with a working volume of 0.8–2.2 dm^3, started with the glycerol-batch phase for the cell growth, before shifting without interruption to a tailored glucose fed-batch (GFB) bioreactor operation which designed specifically for the cells to increase production without decreasing productivity. The harvested cells were transferred into bioreactor to an initial OD_{600} of 2 for the glycerol-batch phase fermentations. In the glycerol-batch phase, glycerol was used as sole carbon source in BSM medium, containing (in g dm^{-3}): glycerol, 25; $CaSO_4$, 0.93; $MgSO_4.7H_2O$, 14.9; K_2SO_4, 18.2; KOH, 4.13; and, H_3PO_4 (85%), 26.7 cm^3; 4.35 cm^3 of filter sterilized PTM1 solution containing (in g dm^{-3}) $CuSO_4{\cdot}5H_2O$, 6.0; NaI, 0.08; $MnSO_4{\cdot}H_2O$, 3.0; $Na_2MoO_4{\cdot}2H_2O$, 0.2; H_3BO_3, 0.02; $CoCl_2$, 0.5; $ZnCl_2$, 20.0; $FeSO_4{\cdot}7H_2O$, 65.0; biotin, 0.2; H_2SO_4 (95%–98%), 5.0 cm^3. Glycerol-batch phase was started at T = 30.0 ± 0.1 °C, pH = 5.0 ± 0.1 adjusted by 26% NH_4OH, N = 900 rpm and with oxygen transfer by keeping the dissolved oxygen (DO) concentration constant at 20% of air saturation. DO was measured on-line with an optical sensor. When the cell concentration reached OD_{600} ~50 ($C_X \sim 10$ g dm^{-3}), glycerol-batch phase was ended and the GFB bioreactor operation was started without interruption by continuous feeding of $C_S{}^\circ$ = 500 g dm^{-3} glucose stock solution, containing 12 cm^3 dm^{-3} PTM and 1 cm^3 dm^{-3}chloramphenicol with the feed-flow rate Q(t) designed and calculated with the pre-determined parameter $\mu = 0.15\ h^{-1}$ [24] using Eq. (1) [25]:

$$Q(t) = \frac{\mu\ V_o\, C_{X,o}}{Y_{X/S}(C_S^o - C_S)}\ \exp(\mu\, t) \quad (1)$$

where, Q(t) is volumetric flow rate of the continuous feed stream (CFS, $dm^3\ h^{-1}$), μ is the targeted pre-determined specific growth rate (h^{-1}), V_o is the initial volume of the bioreaction medium (dm^3), $C_{X,o}$ is the initial cell concentration (g dm^{-3}), $Y_{X/S}$ is cell yield on substrate (g g^{-1}), $C_S{}^o$ is feed substrate stock solution concentration (g dm^{-3}), and C_S is substrate concentration in bioreactor during the fermentation. The parameters of Eq. (1) are V_0 = 0.85 dm^3, $C_{X,o}$ =