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Recombinant expression of glycerol-3-phosphate dehydrogenase using the *Pichia pastoris* system

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ABSTRACT

In the present study, the GPD2 gene from *Saccharomyces cerevisiae*, which codifies for the enzyme glycerol-3-phosphate dehydrogenase (GPDH), was cloned from the pPICZ-α expression vector and used with the purpose of inducing the extracellular expression of the glycerol-3-phosphate dehydrogenase under the control of the methanol-regulated AOX promoter. The presence of the GPD2 insert was confirmed by PCR analysis. *Pichia pastoris* X-33 (Mut$^+$) was transformed with linearized plasmids by electroporation and transformants were selected on YPDS plates containing 100 μg/mL of zeocin. Several clones were selected and the functionality of this enzyme obtained in a culture medium was assayed. Among the mutants tested, one exhibited 3.1×10^{-2} U/mg of maximal activity. Maximal enzyme activity was achieved at 6 days of growth. Medium composition and pre-induction osmotic stress influenced protein production. Pre-induction osmotic stress (culturing cells in medium with either 0.35 M sodium chloride or 1.0 M sorbitol for 4 h prior to induction) led to an increase in cell growth with sorbitol and resulted in a significant increase in GPDH productivity with sodium chloride in 24 h of induction approximately fivefold greater than under standard conditions (without pre-induction).

1. Introduction

Forty years ago, Kochi 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, the Phillips Petroleum Company developed media and protocols for growing *Pichia pastoris* on methanol. The following decade, Phillips Petroleum contracted the Salk Institute Biotechnology/Industrial Associates, Inc. (SIBIA, La Jolla, CA, USA) 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 SPC process and strong, regulated expression of the alcohol oxidase promoter led to surprisingly high levels of foreign protein expression. In 1993, Phillips Petroleum sold its *P. pastoris* expression system patent to Research Corporation Technologies, which is the current patent holder. Moreover, Phillips Petroleum licensed Invitrogen Corporation to sell components of the system; this arrangement continues under Research Corporation Technologies [2].

P. pastoris is methylotrophic yeast also known as *Endomyces pastori*, *Komagataella pastori*, *Petasospora pastori*, *Saccharomyces pastori*, *Zigosaccharomyces pastori*, *Zygowillia pastori* and *Zymopichia pastori*. In recent years, the methylotrophic yeast *P. pastoris* has developed into a highly successful system for the production of a variety of heterologous proteins. The increasing popularity of this expression system can be attributed to several factors: the simplicity of techniques needed for the molecular genetic manipulation of *P. pastoris* and their similarity to those for *Saccharomyces cerevisiae*, which is one of the most well-characterized experimental systems in modern biology [1]; the ability of *P. pastoris* to produce high levels of foreign proteins [3]; the capability of performing a large number of eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation and proteolic processing [4]; and the availability of the expression system as a commercially available kit [5].

Bibliographic reviews on the *P. pastoris* production system demonstrate that the best conditions (medium, pH, etc.) vary depending on the strain used and/or the foreign protein expressed

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[6,7]. According to Invitrogen, the most common medium for obtaining high cell density in fermentations with this yeast is the basal salt medium (BSM). BSM is supplemented with micronutrients such as Fe, Mn, Cu and biotin, normally using a solution with salt traits (PTM1), as proposed by Invitrogen.

Methanol concentration is one of the key parameters in the *P. pastoris* expression system. It is important to monitor and control this variable, as high levels of this inductor substrate can be toxic to cells and low levels of methanol may be insufficient to initiate AOX transcription [8]. Studies comparing different species of methylotrophic yeasts in relation to the carbon source report that glucose represses the protein expression, while the addition of glycerol or another carbon source is dependent on the promoter and yeast [9]. Maintaining a constant methanol concentration during the induction phase has a positive effect on the production of foreign protein [2]. Thorpe and coworkers [10] compared methanol/glycerol and methanol/sorbitol; this mixed substrate is one approach to reducing induction time and increasing the cell density and volumetric productivity of *P. pastoris* [7].

Cereghino and Cregg [2] describe some of the proteins expressed in the system *P. pastoris*, some originally found in bacteria (α-amylase originally from *Bacillus licheniformis*, D-alanine carboxypeptidase from *Bacillus stearothermophilus*), others found in fungi (glucoamylase from *Alternaria alt*, catalase L from *Aspergillus fumigatus*), in protists (hexose oxidase from the red alga *Chondrus crispus*, acid α-mannosidase from *Trypanosome cruzi*), in plants (phytochrome B from potato, acid phosphatase from soy grain nodules) and in invertebrates (bright green protein from the jelly fish *Aequorea vistoria*), in some non-human vertebrates [bovine β-casein, bovine pancreatic trypsin inhibitor (aprotitine), gelatinase B from rats, intestinal carrier of peptide (PEPt1 and 2 from rats)] and from humans (seric albumin, thrombomodulin).

In this paper, the expression system EasySelect® (Invitrogen) was used on *P. pastoris* with the purpose of inducing the extracellular expression of glycerol-3-phosphate dehydrogenase (GPDH, NAD^+: oxido-redutase EC.1.1.1.8), which catalyzes the reduction of the glycolytic intermediate dihydroxyacetone phosphate to glycerol-phosphate in a reversible way, which is then dephosphorylated through the enzyme glycerol kinase for the accumulation of glycerol. This enzyme is induced under hyperosmotic stress and the induction partially depends on the high-osmolarity glycerol pathway. Hence, the GPD2 gene defines physiological roles in the adaptation of *S. cerevisiae* to high-osmolarity and anoxic conditions [11]. Bruinemberg [12] found that *Debaryomyces hansenii* cells exhibited high levels of glycerol-3-phosphate dehydrogenase in a medium containing glucose and 8% NaCl. Studies report the considerable influence of medium composition and pre-induction osmotic stress over scFv production in *P. pastoris* [13].

The objective of the present study was to investigate the influence of induction medium pH, medium composition and pre-induction osmotic stress over recombinant GPDH production.

2. Experiment

2.1. Yeast strain, vector selection and cloning

The EasySelect® *Pichia* expression system was used for these genetic engineering studies. All primers, vectors, zeocin, pyrobest Taq DNA polymerase and host cells were obtained from the Invitrogen Corporation (San Diego, USA). The required restriction enzymes and T4-DNa ligase were obtained from New England Biolabs (USA). All other chemicals were of analytical grade and obtained from local commercial resources. The expression vector pPICZ-α and host cells of *P. pastoris* X-33 containing the *AOX 1* promoter, which allows for rapid growth using methanol as the sole carbon source, were used for the heterologous protein expression.

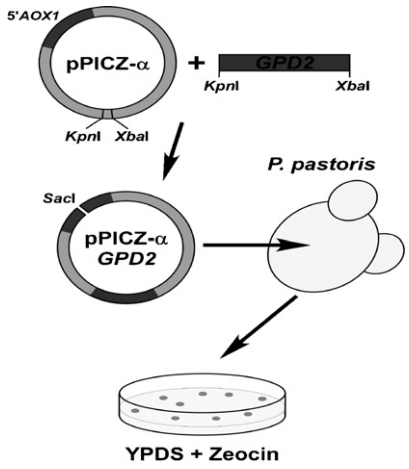

Fig. 1. General diagram for the construction of the *Pichia pastoris* recombinant strains.

Expression of any exogenous gene in *P. pastoris* requires three basic steps: insertion of the gene into an expression vector; integration of the expression vector into the yeast genome; and search for clones that produce the protein of interest. The coding sequence of *GPD2* gene was amplified from *S. cerevisiae* genomic DNA using the primers 5′-GG**GGTACC**ATGCTTGCTGTCAGAAG-3′ and 5′-GC**TCTAGA**CCTTCGTCATCG ATGTC-3′ and cloned in pPICZ-α KpnI/XbaI sites for extracellular glycerol-3-phosphate dehydrogenase expression. The construction obtained was linearized at the 5′ *AOX1* gene with SacI and used to transform competent X-33 cells by electroporation. Forty isolated transformed colonies were selected in YPDS containing 100 μg/mL zeocin (Fig. 1) for the extracellular production of recombinant GPDH.

2.2. Conditions for cell growth and induction of protein synthesis

Cells were cultivated in YPDA medium (1% yeast extract, 2% peptone, 2% glucose, 2% agar) at 30 °C for 24 h. Inocula for the shake-flask culture were grown for 24 h in baffled shake-flasks at 30 °C operating at 120 rpm in 25 mL of buffered methanol-complex BMGY medium [1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer at pH 6.0, 1.34% yeast nitrogen base without amino acid (YNB), 4×10^{-5}% biotin, 1% glycerol]. Shake-flask cultures were conducted in 50 mL of buffered methanol-complex BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol), with an initial cell concentration of 0.050 mg/mL incubated at 30 °C and 120 rpm. Volumes of methanol were added daily for the final concentration of 0.5% required for maintaining the induction. Samples of the culture media were taken every 24 h for analysis of biomass and protein production. The different peptone sources used in the BMMY medium were Difco, Merck and Acumedia (peptone A). The pH of the culture was maintained and controlled by the addition of the buffer used in the experiment and ranges from 4.0 to 6.0.

2.3. Pre-induction osmotic stress conditions

Cells were grown as described by Shi et al. [13], centrifuged, re-suspended and incubated in BMGY medium containing either 0.35 M NaCl or 1 M sorbitol with an initial cell concentration of 0.050 mg/mL for 4 h at 30 °C and 120 rpm prior to induction in 50 mL of buffered methanol-complex BMMY medium. Volumes of methanol were added daily for the final concentration of 0.5% required for maintaining the induction. Samples of the culture

media were taken every 12–24 h for analysis of biomass and protein production.

2.4. Ultrafiltration of recombinant GPDH

After growth, cells from the two culture flasks were harvested by centrifugation for 10 min at 4 °C and 15,000 × *g*. The supernatants were concentrated 120 times using a filter membrane (Millipore YM-10 cut-off: 10,000 MW, CENTRIPLUS® Centrifugal Filter Devices) for approximately 4 h at 17 °C and 2800 × *g* for subsequent determination of the enzyme and total protein.

2.5. Biomass analysis

Biomass was expressed as dry cell weight and was determined spectrophotometrically at a wavelength of 570 nm following appropriate dilution of the sample with deionised water. A conversion factor for dry cell weight of 0.88 was obtained from a standard curve relating absorbance to dry weight of the cell suspension.

2.6. Analytical methods and enzyme assay

2.6.1. Determination of glycerol-3-phosphate dehydrogenase activity

The activity secreted into the medium was adapted for an enzymatic procedure [14] based on the ultraviolet assay for glycerol proposed by Wieland [15]. The reaction mixture was prepared as follows: 42 mM NAD^+, 13.6 mM glycerol-phosphate (substrate) and 10 mM manganese sulfate in glycine/NaOH buffer at pH 9.8 (containing 1.09 M glycine and 2.25 M hydrazine added to deviate the equilibrium toward glycerol-phosphate consumption). The reaction was started by adding fresh diluted extracts to the assay mixture (final reaction volume of 3.3 mL). The NADH resulting from the GPDH activity was monitored spectrophotometrically at 340 nm every 15 s for the first 90 s of reaction time. Background reductions of NADH were eliminated from the assay using blank cuvettes containing all reagents except the substrate. One enzymatic unit was defined as the amount of enzyme required to form 1 μmol of NADH (molar absorption coefficient of 6200 M^{-1} cm^{-1}) per minute at 25 °C under the conditions specified.

2.6.2. Protein assay

Total protein was assayed using the method described by Lowry and modified by Layne [16], using bovine serum albumin as the standard protein. Variations (SD) in the protein content of the cultures were 15.19 ± 2.09 mg/mL.

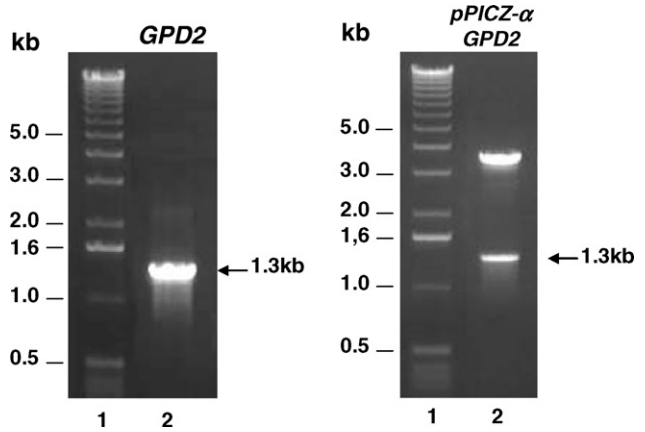

Fig. 2. (A) PCR amplification. Lane 2 shows amplification product of *GPD2* gene (1.3 kb). (B) Analysis of pPICZ-α-*GPD2* cloning. Lane 2 shows KpnI/XbaI double digestion of plasmid construction. Lane 1 in each panel contains 1 kb ladder (Invitrogen).

Table 1
Analysis of the clone selection regarding GPDH expression and protein.

Clones	Protein (mg/mL)	Activity	
		U/mL	U/mg × 10^{-2}
1	16.69 ± 0.17	0.240 ± 0.08	1.44 ± 0.48
2	15.06 ± 0.23	0.318 ± 0.01	2.11 ± 0.07
4	15.44 ± 0.23	0.477 ± 0.14	3.10 ± 0.91
5	16.33 ± 0.45	0.371 ± 0.05	2.27 ± 0.31
6	14.27 ± 0.17	0.143 ± 0.02	1.00 ± 0.14
8	13.10 ± 0.05	0.159 ± 0.02	1.21 ± 0.15
9	14.64 ± 0.20	0.143 ± 0.02	1.00 ± 0.10
10	14.32 ± 0.20	0.159 ± 0.02	1.11 ± 0.20
11	15.02 ± 0.30	0.159 ± 0.02	1.06 ± 0.20
12	15.46 ± 0.40	0.040 ± 0.00	0.30 ± 0.00

Table 2
Effect of induction medium pH on GPDH expression, biomass and protein.

pH	Biomass (mg/mL)	Protein (mg/mL)	Activity	
			(U/mL)	U/mg × 10^{-2}
4.0	14.27 ± 0.55	–	0.0	–
5.0	12.48 ± 0.88	14.68 ± 0.24	0.318 ± 0.0	2.17 ± 0.04
6.0	16.81 ± 0.08	17.27 ± 0.41	0.430 ± 0.0	2.49 ± 0.05

3. Results and discussion

3.1. Isolation of glycerol-3-phosphate dehydrogenase and introduction into P. pastoris

P. pastoris expression systems allow producing proteins in intracellular and extracellular forms. Extracellular production offers the advantage of releasing the protein of interest in the supernatant of the culture medium. Both expression models are widely studied and require enough cell mass to enable the proteins to be purified and used as products [2]. More than five hundred proteins have been cloned and expressed using the *P. pastoris* system [17].

P. pastoris generally produces higher amounts of heterologous proteins than other systems and is particularly advantageous for smaller proteins [2]. The coding sequence of the GPD2 gene was amplified through PCR analysis using primers that hybridize in the regions flanking the coding region of the gene. Fig. 2 illustrates the amplification of a fragment of 1.3 kb corresponding to the GPD2 gene. The fragments were cloned in the pPICZ-α expression vector. The plasmid constructs obtained release fragments corresponding to the cloned gene after digestion with cloning enzymes, confirming the insertion of the gene in the pPICZ-α vector.

3.2. Clone selection and time course

Ten clones were selected using southern blotting hybridization and revealed high levels of protein expression. The wild type *Pichia* strain was used to demonstrate the absence of extracellularly expressed recombinant protein, indicating that the procedures used for expression are appropriate for *P. pastoris* transformation systems (data not shown). Among the mutants tested, one was selected for exhibiting 3.1 × 10^{-2} U/mg of maximal activity

Table 3
Effect of nitrogen source on GPDH expression and biomass.

Peptone source	Biomass (mg/mL)	Activity	
		U/mL	U/mg × 10^{-2}
Difco	13.79 ± 0.07	0.48 ± 0.20	1.12 ± 0.04
Merck	11.81 ± 0.25	0.60 ± 0.08	3.15 ± 0.04
Acumedia (peptone A)	16.67 ± 0.72	0.58 ± 0.11	3.84 ± 0.07

Table 4
Effect of pre-induction osmotic stress on GPDH expression and biomass.

Time (h)	Control (without pre-induction)		0.35 M NaCl		1 M sorbitol	
	Biomass (mg/mL)	Activity (U/mL)	Biomass (mg/mL)	Activity (U/mL)	Biomass (mg/mL)	Activity (U/mL)
12	12.37 ± 0.03	0.143 ± 0.02	10.82 ± 0.18	0.0	ND[a]	ND[a]
24	12.36 ± 0.38	0.159 ± 0.00	14.05 ± 0.26	0.477 ± 0.01	18.78 ± 0.13	0.339 ± 0.09
48	16.67 ± 0.05	0.398 ± 0.08	17.79 ± 0.09	0.215 ± 0.09	22.73 ± 0.79	0.159 ± 0.02
72	18.83 ± 0.35	0.182 ± 0.08	19.98 ± 0.09	0.143 ± 0.02	27.85 ± 0.18	0.0

[a] ND = not determined.

(Table 1). Maximal enzyme activity of the system was achieved in cultures induced with 6 days of growth (Fig. 3).

3.3. Effect of pH on GPDH expression

While it is possible to increase heterologous protein expression in *P. pastoris* through the genetic manipulation of secretion signals, gene dosage and codon usage [18,19], the more practical approach of modifying cell growth conditions to optimize expression levels was chosen in the present study. The pH plays an important role in the maintenance and stability of heterologous protein expressed by *P. pastoris* and its control is necessary to minimize the proteolytic degradation of heterologous proteins [20,21]. The effect of pH on cell growth and GPDH expression was examined in a BMMY induction medium, which was buffered to limit protease activity. While the lowest level of protease activity was detected at pH 4.0, induction media with pH 6.0 were highly inductive to GPDH expression (Table 2). In *P. pastoris*, Saelens et al. [22] report that the pH of a culture medium has an important effect on the expression of A influenza virus and its best output was obtained at pH 8.0. According to Rosenfeld et al. [23], the recombinant hirudine, which is a powerful thrombin inhibitor, was expressed at pH 5.0. According to Choi and Park [24], when cultures were grown at pH 6.0, the activity of α-amylase remained stable and high levels of enzyme expression were obtained. Therefore, the pH of the growth medium of the yeast *P. pastoris* is dependent on the target protein.

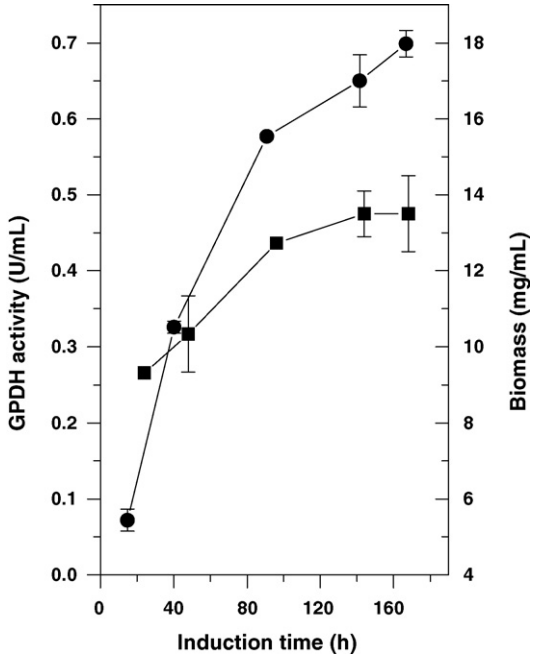

Fig. 3. Effect of induction time of the clone selected on GPDH activity (■) and biomass (●). Each point represents the average of two determinations.

Table 5
Analysis of productivity on GPDH expression and biomass.

Productivity	Control	0.35 M NaCl	Increase (fold)
Activity (U/mL/h)	0.0075	0.04	5.33
Biomass (mg/mL/h)	0.13	0.19	1.46

3.4. Effect of media composition on GPDH expression

The nitrogen source is another important factor to obtaining success in the expression of heterologous protein. According to Cos et al. [25], one of the most important factors in the BSM and FM22 media is the nitrogen source, which is added in the form of ammonium hydroxide in order to control the pH. Kobayashi et al. [26] found that, for the production of lipase in *Rizopus prize*, the lack of nitrogen was directly related to the increase in proteolytic activity and, consequently, the degradation of extracellular proteins. In the present case, cells grown in the BMMY medium containing peptone A (peptic digest of animal tissue) exhibited increased growth and GPDH production ($3.84\,U/mg \times 10^{-2}$) (Table 3).

3.5. Effect of pre-induction on GPDH expression

According to Cos et al. [25], the optimization of heterologous protein expression, which consists of maximizing output and productivity, depends on different phenotypes with promoter AOX1, other promoters, culture media and operational strategies. Thus, culturing *P. pastoris* in hypertonic media containing either 0.35 M sodium chloride or 1.0 M sorbitol prior to induction and then transferring it to induction medium led to an increase in cell growth with sorbitol and resulted into a significant increase of GPDH productivity with 0.35 M NaCl in 24 h of induction approximately fivefold greater than under standard conditions (Tables 4 and 5).

4. Conclusion

P. pastoris expression systems allow producing proteins in intracellular and extracellular forms. Extracellular production offers the advantage of releasing the protein of interest in the supernatant of the culture medium. Both expression models are widely studied and require enough cell mass to enable the proteins to be purified and used as products. The expression procedures employed in the present study are appropriate for *P. pastoris* transformation systems.

Acknowledgments

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