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Using *Pichia pastoris* to produce recombinant glycerol kinase

Werner Damião Morhy Terrazas[2]; Raquel Aizemberg[1]; Edwil Aparecida de Lucca Gattás[1*]

[1] Departamento de Alimentos e Nutrição, Faculdade de Ciências Farmacêuticas de Araraquara, UNESP – São Paulo, Brasil.
[2] Departamento de Tecnologia de Alimentos, Centro de Ciências Naturais e Tecnologica, Universidade do Estado do Pará.

ABSTRACT

The methylotrophic yeast *Pichia pastoris* has been developed into an efficient expression system for the production of recombinant protein under the tight control of the methanol-induced alcohol oxidase promoter (pAOX1). In this study, a 2.5-liter culture system was developed for the growth of a *P. pastoris* strain bearing the GUT1 gene from *Saccharomyces cerevisiae* for the expression of recombinant glycerol kinase (GK). The best culture conditions to produce high levels of secreted GK were investigated by growing the recombinant strain of *P. pastoris* in shake flasks and a fermenter. Cell growth and enzyme production were found to be optimal after two days of growth. Enzyme production was affected by the nitrogen source, Difco peptone being the most appropriate for this purpose. Three different rates of air flow (1 to 3 L/min) were tested to observe their effect on cell growth and the secretion of GK into a medium containing 1% methanol as the sole carbon source. Increasing the rate of air bubbling in the culture medium enhanced both cell growth and GK activity, reaching a dry biomass of 7.84 mg/mL, cell viability of 98.4% and a maximal GK activity of 1.57 U/mL, at a flow rate of 2.0 L/minute, at 30° C and pH 6.0. Moreover, the enzyme activity in the *P. pastoris* culture medium was 2.3 times higher under these conditions than in the shake-flask culture, demonstrating the significant influence of aeration on biomass production and GK activity secreted by *P. pastoris.*

Keywords: Pichia pastoris. Glycerol kinase. Oxygen. Biomass.

Autor correspondente: Edwil Aparecida de Lucca Gattás, Departamento de Alimentos e Nutrição, Faculdade de Ciências Farmacêuticas, UNESP-Campus de Araraquara, 14801-902 Araraquara, SP, Brasil;
Email: gattas@fcfar.unesp.br

INTRODUCTION

Glycerol kinase (GK) (ATP:glycerol phosphotransferase, EC 2.7.1.30) converts glycerol into glycerol-3-P by phosphorylation with adenosine triphosphate (ATP). The glycerol-3-P is then reduced to dihydroxyacetone phosphate, both phosphates being important intermediates in carbohydrate and fat metabolism. GK is thus a key enzyme in the regulation of glycerol metabolism, lipid synthesis and energy production (Janke et al., 2010). GKs have been characterized and purified from several microorganisms, including the bacteria *Escherichia coli* and *Pediococcus pentosaceus* (Pasteris & Saad, 1998; Thorner & Paulus, 1973) and the yeasts *Debaryomyces hansenii*, *Candida mycoderma*, *Pichia pastoris* and *Saccharomyces cerevisiae* (Aizemberg et al., 2011; Aragon et al., 2008; Janson & Cleland, 1974; Nilsson et al., 1989).

The methylotrophic yeast *P. pastoris* has been developed into an efficient expression system for the production of recombinant proteins (Macauley-Patrick et al., 2005; Cregg et al., 2000). A strongly inducible, tightly regulated alcohol oxidase 1 promoter (pAOX1) is the main component allowing control of this process. The production of GK by *P. pastoris* is induced by methanol in the growth medium, the concentration of which is one of the most important parameters in this expression system. The monitoring and control of methanol content are important elements of this process, as high levels of this inducing substrate can be toxic to cells, while low levels may not be sufficient to initiate AOX transcription (Cereguino & Cregg, 2000). Maintaining a constant methanol concentration during the induction phase thus enhances the production of foreign proteins (Chiruvolu et al., 1997).

Many strategies have been proposed and implemented to control the methanol feed, with the objective of maximizing protein production. The commonest of these strategies involves maintaining a constant dissolved oxygen (DO) content. Pichia cells utilize methanol through the oxidative pathway only when oxygen is non-limiting. The oxygen concentration in the culture and the optimal methanol feed rate are thus interrelated. In order to prevent methanol accumulation in the medium, an efficient methanol-induction strategy, coupled with proper

DO control, is essential for optimal protein production with Pichia expression systems (Potvin et al., 2012; Resina et al., 2004). However, the limitation in DO can be overcome by improving the oxygen mass transfer coefficient (KLa) by the addition of oxygen vectors to the medium (Zhang et al., 2008).

Boze et al. (2001) obtained appreciably higher levels of secretory protein production in a bioreactor than in flasks, when *P. pastoris* was grown at high cell density in a fed-batch bioreactor or continuous fermenter, by controlling pH and the supply of oxygen. According to Choi & Park (2006), three phases generally need to occur in the production of a heterologous protein with *P. pastoris* in a fermenter: first, the accumulation of biomass, with glycerol as the carbon source; next, a transition phase to adapt the cells, in which the addition of glycerol is controlled to limit growth rates, and finally induction, by the slow addition of methanol. According to Aizemberg et al. (2011), in BMGY medium, glycerol concentration is one of the most important factors for biomass production, while the rate of supplementation of methanol as a carbon source is the most important factor for the production of recombinant GK.

In the present study, *P. pastoris* containing the *S. cerevisiae* GUT1 gene, which codes for GK, was chosen as the model system. The first step was to understand the effects of pH and the nitrogen source in the medium on yeast growth and GK production in shake flasks. The effects of oxygen induction on *P. pastoris* cell growth and recombinant GK expression were tested in a 2.5-L fermenter.

MATERIALS AND METHODS

Microorganism and culture conditions

Pichia pastoris strain X-33, bearing the GUT1 gene expressing recombinant GK on the expression vector pPICZ A, was constructed and selected as described elsewhere (Aizemberg et al., 2011). The organism was maintained on slants of YPDA medium (1% yeast extract, 2% peptone, 2% D-glucose, 2% agar) at 30º C for 24 h. Inocula for the shake-flask culture were grown for 24 h in 25 mL of buffered methanol-glycerol-complex (Invitrogen, 2000) BMGY medium at 30º C, shaken at 120 rpm. The BMGY medium contained 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer at pH 6.0, 1.34% yeast nitrogen base without amino acids (YNB), 4 x 10-5% biotin, 1% glycerol and 1% methanol. The cells were pelleted by centrifuging, suspended in buffer and used to inoculate the shake-flask cultures.

Growth and induction in shake flask

Shake-flask cultures were grown in 25 mL of buffered methanol-complex (Invitrogen, 2000) BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% YNB, 4 x 10-5% biotin and 1% methanol) or BSM medium (40 g of glycerol, 26.7 mL/L of 85% phosphoric acid, 0.93 g/L of CaSO4.2H2O, 18.2 g/L of K2SO4, 14.9 g/L of MgSO4.7H2O and 4.13 g/L of KOH) plus trace element solution (Chiruvolu et al., 1999), with an initial cell concentration of 0.100 mg/mL, incubated at 30º C and 120 rpm. Volumes of methanol were added daily to a final concentration of 1%, required to maintain induction. Samples of culture media were removed every 24 hours to analyze the production of biomass and protein. Various peptone sources were used in the BMMY medium and the pH of the culture was maintained and controlled by the addition of the buffer used in each experiment (range: 5.0 to 7.0).

Growth and induction in 5-L fermenter

A seed culture was inoculated with a single colony of *P. pastoris* in a 250 mL flask containing 25 mL of BMGY medium and incubated in a rotary shaker at 30° C, 120 rpm for 24 h. Cells were then pelleted by centrifugation at 3000 rpm for 5 minutes, re-suspended in buffer and transferred to a fermenter. Fermentations were carried out in 2.5 L BMMY medium in an INFORS HT-Minifors 5-L vessel, incubated at 30° C and 120 rpm. Methanol was added daily to a final concentration of 1%. Samples of the culture media were taken every 24 hours for the analysis of biomass production and protein secretion. The reactor was oxygenated by a stream of air bubbles from a compressor, introduced at the base of the reactor. Air flow rate during the experiment ranged from 1 to 3 L/min. In all fermentations, the initial pH of 6.0 was maintained throughout by the addition of 100 mM phosphate buffer.

Biomass assay

Biomass was expressed as dry cell weight and determined spectrophotometrically by absorbance at 570 nm (Ultrospec 2100 spectrophotometer, Amersham Bioscience). The conversion factor was 0.88 mg dry wt cells/mL per absorbance unit, obtained from a standard curve relating absorbance to dry weight of the cell suspension (7.21 mg wet cells / mL per AU).

Supernatant concentration

After fermentation, the contents of the two shake flasks and 2.5-L fermenters were centrifuged at 15,000 g for 10 min in a Sorvall RC5C Plus centrifuge, and the cell pellets were discarded. The supernatants were concentrated by lyophilization or by means of a filter membrane (Millipore YM-10, cut-off 10 kDa, CENTRIPLUS® Centrifugal Filter Devices, USA), which afforded threefold concentration. During the concentration of recombinant GK, the centrifuge was operated at 17º C and 2800 g.

Measurement of glycerol kinase activity

The reaction of GK was assayed by a method described by Kennedy (1962). The following mixture of reagents was used: 0.07 mL of 20 mM NAD+, 0.07 mL of 50 mM ATP, 0.9 mL of 6 mM glycerol, 0.9 mL of 100 mM glycine-NaOH buffer, pH 9.8, containing 880 mM hydrazine hydrate, 0.210 mL of 200 mM magnesium sulfate, 0.330 mL of 100 mM calcium chloride, 0.220 mL of enzyme (concentrated supernatant) diluted three times in 100 mM Tris buffer, pH 7.2, containing 10 mM NaF, totaling 3.3 mL. The NADH generated in the reaction was read at 340 nm and recorded for the first 60 s of reaction. One enzyme unit was defined as the amount of enzyme required to form 1 mol of NADH (molar absorption coefficient 6200M-1 cm-1) per minute at 25º C under the conditions specified.

Protein assay

Total protein was assayed (Layne, 1975) with bovine serum albumin as the standard protein. The variation (SD) in the protein content of the cultures was 15.03 □ 1.93 mg/mL.

RESULTS

Influence of complex medium on glycerol kinase production

In the first phase of production, the strains are batch cultured in a defined medium containing glycerol, to generate biomass but repress heterologous gene expression. The second phase is the addition of methanol to the culture at a slow rate, to induce protein expression.

High levels of total protein expression and secretion into the shake-flask culture medium were achieved by incorporating the *S. cerevisiae* □-factor signal sequence into the pPICZ□-GUT1 construct (Aizemberg et al., 2011). In this study, the transformants had maximal GK activity (0.662 □ 0.44 U/mL) in the BMMY medium after two days of induction with methanol (Figure 1). The total protein was 18.43 ± 1.26 mg/mL and the amount of secreted GK was 1.1-fold greater in the lyophilized enzyme extract. (Table 1)

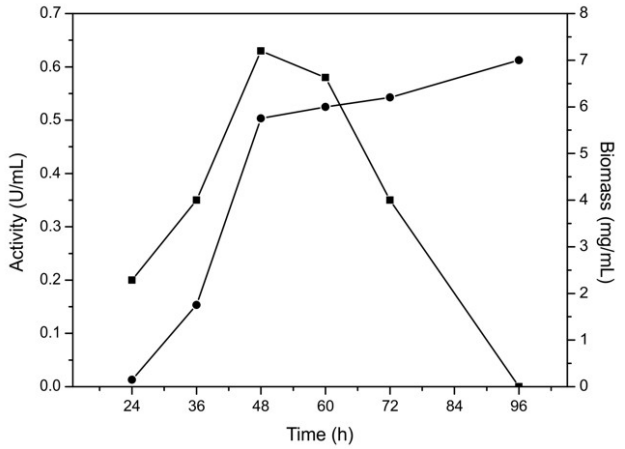

Figure 1. Effect of time of induction of *Pichia pastoris* growing in BMMY medium in shake flasks on recombinant GK activity (n) and biomass (l). Each point represents the average of two determinations.

Influence of pH of medium on glycerol kinase production

The pH also plays an important role in the maintenance and stability of heterologous proteins secreted by *P. pastoris* and it is necessary to control this variable to minimize the proteolytic degradation of the heterologous proteins. When the cultures were grown at pH 6.0, GK activity remained stable and high levels of enzyme expression were achieved. The expression of protein by *P. pastoris* and the extracellular GK activity depend on the pH of the growth medium and the nature of the buffer. The best GK production attained in BMMY medium was 0.67 U/mL (Table 2) and the total protein was 18.43 ± 1.26 mg/mL (Table 1). Optimization of the culture pH, buffer and medium composition for effective expression of

Table 1. Effect of complex methanol medium (BSM and BMMY) on GK activity secreted by *Pichia pastoris*.

Medium	Enzyme without / with lyophilization	GK Activity (U/mL)
BSM**	Without	0
BMMY	Without	0.662 □ 0.44
BMMY***	With	0.755 □ 0.00
BMMY	With*	0.318 □ 0.09

*Plus 2 mmol/L □-mercaptoethanol and 10 mmol/L MnSO4 ; ** total protein = 6.23 ± 0.04 mg/mL; *** total protein before lyophilization = 18.43 ± 1.26 mg/mL.

Table 2. Effect of pH of induction medium (BMMY) on biomass, viability and GK activity secreted by *Pichia pastoris* in shake-flask culture.

Value of pH	Biomass (mg/mL)	Viability (%)	GK Activity	
			(U/mL)	(U/mg protein secreted)
5.0*	5.30 □ 0.03	93.0	0	-
6.0*	5.91□ 0.01	94.2	0.26 □ 0.03	0.015
6.0**	5.02□ 0.01	65.6	0	-
6.0***	5.48□ 0.05	93.0	0.67 □ 0.06	0.036
7.0***	5.18□ 0.02	82.4	0.31 □ 0.04	0.022

Buffer 100 mmol/L: * potassium citrate-phosphate, ** potassium citrate, *** potassium phosphate.

Table 3. Effect of nitrogen source on biomass and GK activity secreted by *Pichia pastoris* growing in BMMY medium in shake flasks.

Nitrogen Source (2% w/v)	Biomass (mg/mL)	Total protein (mg/mL)	GK Activity	
			(U/mL)	(U/mg protein)
Without peptone	3.02 □ 0.00	14.1□ 0.18	0	-
Bacto Peptone	5.99 □ 0.07	13.6□ 3.31	0.509 □ 0.44	0.037
Acumedia (peptone A)	5.50 □ 0.04	19.2□ 3.07	0.700 □ 0.48	0.036
Difco Peptone	6.69 □ 0.07	19.5□ 4.15	0.764 □ 0.00	0.039
Merck Peptone	7.10 □ 0.02	15.4□ 2.95	0	-
(NH4)2SO4	6.51□ 0.00	24.0□ 2.71	0.682□ 0.05	0.028

Table 4. Specific growth rate of *Pichia pastoris* and GK specific activity at various air flow rates in BMMY medium in 5-L bioreactor.

Air flow (L/min)	1.0	2.0	3.0	none*
Final Biomass (mg/mL)	5.63	7.84	5.10	5.69
Viability (%)	99.1	98.4	97.9	99
Specific growth rate/h	0.12	0.16	0.11	0.12
GK activity (U/mL)	1.31 □ 0.05	1.57 □ 0.03	0.90□ 0.00	0.69□ 0.07
Specific activity (U/mg protein)	0.083	0.098	0.057	0.043

* Growth and induction in shake-flask.

heterologous enzyme was carried out. From the reported results, the optimal pH for GK specific activity was 6.0 and the best buffer phosphate (Table 2).

Influence of nitrogen source on glycerol kinase production

The culture medium composition exerts an effect on heterologous protein production in yeast through changes in cell growth and viability. In general, all *P. pastoris* strains grow on defined medium supplemented with a specific nitrogen source such as peptone or casamino acids, in order to reduce or prevent proteolytic degradation of the target protein (Gonçalves et al., 2013). The effects of various sources of nitrogen (Bacto peptone, Acumedia peptone A, Difco peptone, Merck peptone and ammonium sulfate) on the production of the GK from *Saccharomyces cerevisiae* in *P. pastoris* are shown in Table 3.

Influence of oxygen on glycerol kinase production

The effects of varying the nitrogen source and the pH were determined in shake-flasks. The influence of agitation and oxygen limitation in the shake-flask experiments was revealed by conducting assays in 5-L Minifors (INFORS HT) bioreactors equipped with pH, stirring and air-flow controls (Table 4).

DISCUSSION

Over the past 30 years, the methylotrophic yeast *P. pastoris* has been 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 the techniques needed for the molecular/genetic manipulation of *P. pastoris* and their similarity to those used for *S. cerevisiae*, one of the most well-known experimental systems in modern biology (Werner, 1990); the ability of *P. pastoris* to produce high concentrations of foreign proteins; the possibility of performing a large number of eukaryotic post-translational protein modifications, such as glycosylation, disulfide bond formation and proteolytic processing, and the availability of the expression system as a commercial kit (Cereguino & Cregg, 2000). The methanol-inducible pAOX1, to which the success of *P. pastoris* owes its success as a heterologous protein expression system, is used in the study. Several strategies are available for protein production in *P. pastoris* strains in growing under the control of pAOX1, as presented by Cereguino and Cregg (2000). GK activity varies in response to the conditions provided by the medium. Thus, the optimal pH, oxygen flow, nitrogen source and induction time for growth were determined.

The pH of growth and induction media plays an important part in heterologous protein production. In mouse □-amylase production by *P. pastoris*, the highest □-amylase activity was obtained when the pH was controlled at 6.0 (Choi & Park, 2006). Higher pH values decrease cell viability and may lower the stability or activity of the secreted product. Degradation due to pH-mediated destabilization, however, depends greatly on the stability of the recombinant protein itself, which is often enhanced by glycosylation and/or disulfide bond formation. Serine and aspartic acid proteases, both secreted by *P. pastoris*, are activated at low pH values, which may explain the pH-dependence of proteolytic activity (Cregg et al., 2000).

The nitrogen source is another important factor to the successful expression of heterologous proteins. Potvin et al., (2012), found that, for the production of lipase in Rhizopus oryzae, lack of nitrogen was directly related to an increase in proteolytic activity and, consequently, the degradation of extracellular proteins. Nutrient starvation leads to autophagic cell degradation and lysis, leading to the release of vacuolar proteases. Complex or enriched media not only prevent limitation, but may inhibit protease activity by proving competing enzyme substrates. In the present study, initial experiments were performed in shake flasks with four different nitrogen sources, leading to improvements in the growth of *P. pastoris* and various effects on protein expression. Cells grown in the BMMY medium with added Difco peptone exhibited increased growth (at least twice that in BMMY without peptone), total protein (18.4 ± 4.15mg/mL) and GK production (0.764 U/mL). The addition of ammonium sulfate prevented the degradation of the protein secreted by *P. pastoris* during growth. An increase in the concentration of ammonium in the culture medium extended the lag phase and thus inhibited cell growth initially, especially at concentrations equal to or greater than 0.6 mol/L (data not shown).

Other authors report several strategies for the production of heterologous proteins in *Pichia pastoris* strains in bioreactors under the control of AOX1 (Gonçalves, et al., 2013). First, the strains are batch cultured in a defined medium containing glycerol, to generate biomass but repress heterologous gene expression. The second stage is the addition of methanol to the culture at a slow rate, to induce the protein expression. However, it is not only the feeding strategy that controls the yield of GK production; factors such as pH, temperature, dissolved oxygen and medium formulation also affect enzyme expression.

Oxygen limitation is the commonest problem encountered during *Pichia pastoris* fermentation (Zhang et al., 2008). The oxygen is used by the methylotropic yeast to oxidize methanol to formaldehyde, as a side-reaction in the formaldehyde dissimilation pathway, which eventually lead to higher recombinant product formation.

The oxygen level was varied by conducting the assays in bioreactors equipped with agitation and air-flow control. The production of GK by *Pichia pastoris* was 1.74-times higher with air bubbling at 2.0 L/min than in the shake-flask culture. The results showed that the total protein was in the range 15.8 to 16.3 mg/mL, while the final biomass, specific growth rate and enzyme activity increased, up to 2.0 L/min of air flow, above which they gradually declined, though viability was maintained.

The final samples were lyophilized before the determination of GK activity and total protein. Lyophilization is one of the best-known preservation processes for pharmaceuticals and food. Improved levels of activity and increased stability can be obtained in lyophilized enzymes by a variety of methods, such as: addition of salts, sorbitol, trehalose, cyclodextrins, etc. This activation may occur by the most varied mechanisms: raising the solubility of stabilizer solutes around the protein, forming a protective layer, enabling the enzyme to keep an ionic balance and maintain its biocatalytic functions longer,

owing to the preservation of three-dimensional structure after lyophilization.

The following cell growth conditions were chosen as optimal: BMMY medium at pH 6.0 (phosphate buffer), 2% Difco peptone and air bubbling at 2.0 L/min.

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RESUMO

Produção de glycerol quinase recombinante utilizando o sistema Pichia pastoris

A levedura metilotrófica *Pichia pastoris* possui um sistema de expressão eficiente para a produção de proteínas recombinantes. A indução da produção da proteína de interesse é feita com metanol, que é capaz de ativar a transcrição do gene de interesse clonado sob controle do promotor do gene AOX1. Um meio de cultura de 2.5 litros foi elaborado para o crescimento da cepa *Pichia pastoris* construída com o gene GUT1 de *Saccharomyces cerevisiae* para expressar a enzima recombinante glicerol quinase (GK). As condições ideais de cultura, para alcançar altos níveis de expressão de GK foram investigados em crescimentos realizados em frascos e fermentador. Crescimento celular e produção de enzima atingiram valores ótimos em dois dias de cultura. A produção enzimática foi afetada pela fonte de nitrogênio no meio. Peptona da marca Difco foi a fonte de nitrogênio mais adequada para a expressão desta enzima. Três diferentes concentrações (1-3 L / min) de fluxo de ar foram analisados em ensaios de crescimento celular e secreção da GK, no meio contendo 1 % de metanol como única fonte de carbono. O aumento do fluxo do ar no meio de cultura produziu melhores resultados para o crescimento celular e atividade da GK, atingindo 7,84 mg / mL de biomassa seca e 98,4% de viabilidade. A máxima atividade de GK foi de 1,57 U / mL, com a concentração de fluxo de ar de 2,0 L / minuto a 30 ° C e pH 6.0. O aumento da atividade enzimática foi 2,3 vezes maior no meio de cultura da *Pichia pastoris* nestas condições, revelando a influência deste parâmetro na produção de biomassa e atividade da GK.

Palavras-chave: Pichia pastoris. Glicerol quinase. Oxigênio. Biomassa.

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