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# Optimal Conditions for Biomass and Recombinant Glycerol Kinase Production Using the Yeast *Pichia pastoris*

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**Summary**

The extracellular glycerol kinase gene from *Saccharomyces cerevisiae* (*GUT*1) was cloned into the expression vector pPICZα A and integrated into the genome of the methylotrophic yeast *Pichia pastoris* X-33. The presence of the *GUT*1 insert was confirmed by PCR analysis. Four clones were selected and the functionality of the recombinant enzyme was assayed. Among the tested clones, one exhibited glycerol kinase activity of 0.32 U/mL, with specific activity of 0.025 U/mg of protein. A medium optimized for maximum biomass production by recombinant *Pichia pastoris* in shaker cultures was initially explored, using 2.31 % (by volume) glycerol as the carbon source. Optimization was carried out by response surface methodology (RSM). In preliminary experiments, following a Plackett-Burman design, glycerol volume fraction ($\varphi$(Gly)) and growth time ($t$) were selected as the most important factors in biomass production. Therefore, subsequent experiments, carried out to optimize biomass production, followed a central composite rotatable design as a function of $\varphi$(Gly) and time. Glycerol volume fraction proved to have a significant positive linear effect on biomass production. Also, time was a significant factor (at linear positive and quadratic levels) in biomass production. Experimental data were well fitted by a convex surface representing a second order polynomial model, in which biomass is a function of both factors ($R^2$=0.946). Yield and specific activity of glycerol kinase were mainly affected by the additions of glycerol and methanol to the medium. The optimized medium composition for enzyme production was: 1 % yeast extract, 1 % peptone, 100 mM potassium phosphate buffer, pH=6.0, 1.34 % yeast nitrogen base (YNB), $4\cdot10^{-5}$ % biotin, 1 % methanol and 1 % glycerol, reaching 0.89 U/mL of glycerol kinase activity and 14.55 g/L of total protein in the medium after 48 h of growth.

*Key words*: *Pichia pastoris*, recombinant glycerol kinase, carbon source, biomass, response surface methodology

## Introduction

*Pichia pastoris* is a methylotrophic species of yeast. Taxonomically, it is placed in the kingdom Fungi, phylum Ascomycota, subphylum Saccharomycotina, class Saccharomycetes, order Saccharomycetales, family Saccharomycetaceae and genus *Pichia* (*1–3*). This yeast has been genetically engineered to express in high levels heterologous proteins that are prized for scientific research and industrial development, with a potential for high levels of expression (*4,5*). Efficient product secretion and growth to very high cell densities in a cheap medium have led the

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Phillips Petroleum Company (Bartlesville, OK, USA) to propose the use of this yeast as a food source (single cell protein: SCP) (*6*). Yeast SCP is a product with many applications in the food, feed, pharmaceutical and biotechnological industries. Productivity, yield and selling price are the major factors affecting the economics of SCP production. Given to the potential food and feed applications of SCP, the Generally Recognized as Safe (GRAS) or Qualified Presumption of Safety (QPS) status are additional requirements for such a yeast strain (*7*).

In a typical fermentation process in which *P. pastoris* is used to produce heterologous proteins, glycerol is initially added to a defined medium as the sole carbon source. During this phase, biomass is accumulated, but the gene expression of heterologous proteins is completely repressed. After the initial depletion of glycerol, there is a transition period in which glycerol is added to the culture at a limiting rate (nutritional stress). Finally, either methanol alone or a mixture of glycerol and methanol is added to the culture, to induce the expression of heterologous proteins (*8*).

In much of the work with *P. pastoris*, a complex medium is required for growth and induction of protein expression (*9*). Formulations of such media can be found in the manuals of the Invitrogen Corporation (Carlsbad, CA, USA) and they depend on the kind of strain used (*10*). The concentration of the substrate (glucose or glycerol) is a common limitation in large-scale production, since maintenance of the initial substrate at a low (non-inhibitory) concentration is essential for the optimization of biomass production and, consequently, the desired protein (*11*).

Basal salts medium (BSM) has been used for the production of angiostatin in a bioreactor containing methanol and other carbon sources along with it in the growth phase, such as glycerol, sorbitol, ammonium acetate and lactic acid (*12*). Other media have been used by a number of authors, such as buffered glycerol-complex medium (BMGY) for accumulation of biomass and buffered methanol-complex medium (BMMY) for protein induction (*13*,*14*). The Invitrogen formulations are useful in the studies on the induction of the expression of extracellular glycerol kinase, which is an enzyme used to assay glycerol, an important intermediate metabolite of lipid biosynthesis and glycolysis (*10*). This enzyme is industrially important and useful for the clinical determination of serum triglyceride levels, in combination with lipase, glycerol-3-phosphate oxidase and peroxidase (*15*), as well as of glycerol, and a number of important bioanalytical applications (patent number PI0205552-0) (*16*).

In this study, response surface methodology (RSM) was used to select the most important factors for biomass production and productivity, with a Plackett-Burman screening design. In a second set of experiments, modelling and optimization of medium composition and growth conditions, in terms of the previously selected factors, were attempted, following a central composite rotatable design.

In addition, the expression system EasySelect® (Invitrogen) was used on *P. pastoris*, with the purpose of inducing the extracellular expression of glycerol kinase (GK, ATP:glycerol-3-phosphotransferase, EC 2.7.1.30). The relation between the accumulated biomass and glycerol kinase activity was identified.

## Materials and Methods

### *Yeast strain, vector selection and cloning*

The EasySelect® *Pichia* expression system was used for genetic engineering experiments. All primers, vectors, zeocin, Taq DNA polymerase and host cells were obtained from the Invitrogen Corporation. The required enzymes and T4-DNA ligase were obtained from New England Biolabs (Ipswich, MA, USA). All other chemicals were of analytical grade and obtained from local commercial suppliers. The expression vector pPICZα and host cells of *P. pastoris* X-33 containing the *AOX*1 promoter, which allows rapid growth on methanol as the carbon source, were used for heterologous protein expression. The 2.1-kb coding region for the *GUT*1 gene from *Saccharomyces cerevisiae* genomic DNA was amplified, using the primers 5'-CG**GAATTC**ATGTTTCCCTCTCTC-3' and 5'-CC**CTCGAG**TCAGTGGTGGTGGTGGTGGTGT-3' cloned into the pPICZα A vector at the *Eco*RI and *Xho*I sites for extracellular GK expression. The constructs were linearized at the *AOX*1 promoter with *Sac*I and used to transform competent X-33 cells by electroporation. Fifty-five transformants were selected on yeast extract-peptone-dextrose-sorbitol (YPDS) medium containing 100 μg/mL of zeocin for the extracellular production of recombinant GK.

### *Conditions for cell growth and induction of GK production*

Cells from *Pichia pastoris* were cultived on YPDA (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 BMGY medium (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate buffer at pH=6.0, 1.34 % yeast nitrogen base (YNB) without amino acids, $4\cdot10^{-5}$ % biotin, 1 % glycerol). Shake flask cultures were then conducted in 25 mL of buffered methanol complex (BMMY) medium (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate buffer, pH=6.0, 1.34 % YNB, $4\cdot10^{-5}$ % biotin, 0.25 to 3.0 % methanol), with an initial cell concentration of 0.1 mg/mL, incubated at 30 °C and 120 rpm. Volumes of methanol were added daily to maintain the final volume fraction of 0.5 % required for induction. Samples of culture medium were taken every 48 h for the analysis of biomass, cell viability and glycerol kinase production. Various carbon sources (glycerol, sorbitol, lactic acid and ammonium acetate) were added to the BMMY medium at 1 % of the final volume fraction.

After fermentation, the content of two culture flasks was centrifuged at 15 000×*g* for 10 min in a Sorvall® Legend RT (Kendro Laboratory Products, Langenselbold, Germany) and the cells were discarded. The supernatant was concentrated through a Millipore filter, following the manufacturer's instructions, with an Ultracel® membrane,

Millipore User Guide cutoff of $M_r$=10 000. All operations during the concentration of recombinant GK were carried out at 17 °C and 2800×*g* in a centrifuge.

### *Biomass assay*

Absorbance (*A*) was measured at 570 nm with an Ultrospec 2100 spectrophotometer (Amersham Biosciences, Cambridge, UK). The $A_{570\ nm}$ was converted to mg of dry cells per mL by means of a standard curve relating the absorbance to the dry mass of *P. pastoris* cell suspension.

### *Measurement of glycerol kinase activity*

Glycerol kinase activity was determined using the method described by Wieland (*17*). The following reaction mixture 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 buffer (100 mM glycine-NaOH, pH=9.8, containing 880 mM hydrazine hydrate), 0.21 mL of magnesium sulphate (200 mM), 0.330 mL of 100 mM calcium chloride, 0.22 mL of enzyme extract (diluted in most cases) and 0.40 mL of GPDH (G6751, Sigma-Aldrich, St. Louis, MO, USA). The concentration of the NADH generated in the reaction was monitored by $A_{340\ nm}$ in the spectrophotometer.

### *Protein assay*

Lowry's method was used with serum bovine albumin as protein standard (*18*). Variations (S.D.) in the protein content of the cultures were (15.19±2.09) mg/mL.

### *Screening of factors affecting biomass production*

A first set of experiments, based on a Plackett-Burman design (*19*), was carried out at 30 °C, in order to screen the most important factors to include in subsequent experiments for the modelling and optimization of biomass production. In this design, the effect of the following factors was tested at three different levels (coded as –1, 0 and +1, respectively): yeast extract 0.5, 1 and 1.5 %, peptone 1, 2 and 3 %, glycerol 0.5, 1 and 1.5 %, YNB 0.67, 1.34 and 2.01 % and biotin 0.15, 0.20 and 0.25 % volume fractions, phosphate buffer 50, 100 and 150 mM, pH=5.5, 6.0 and 6.5 and growth time 24, 48 and 72 h. Three central points were added to the original Plackett-Burman design (total of 15 experiments). In each experiment, the amount of produced biomass (mg/mL) and biomass productivity (mg/(mL·h)) were assayed.

### *Modelling and optimization of biomass production*

In order to model and optimize the biomass production, a second set of experiments was carried out in a central composite rotatable design (CCRD), in which the two most important factors for biomass production, glycerol content and growth time, previously selected from the Plackett-Burman design results, were varied. In this design, five different levels were tested for each factor (coded as $-\alpha$, –1, 0, +1 and $+\alpha$): glycerol content varied from 0.19 to 2.31 % (by volume) and fermentation time from 14 to 82 h. All growth media were prepared in 50 mM phosphate buffer containing 1 % peptone and 0.15 % biotin. Fermentation was carried out at 30 °C. These conditions had been shown to promote the highest biomass production and biomass productivity.

### *Statistical analysis*

The results from each Plackett-Burman and CCRD experiment for biomass production and biomass yield were analysed with the software package STATISTICA™, v. 5, (StatSoft, Tulsa, OK, USA).

For CCRD, the linear and quadratic effects of the independent variables and their linear interactions on biomass production were calculated. Their significance was evaluated by analysis of variance. Three-dimensional surfaces were fitted to each set of the estimated biomass production and yield. These surfaces were described by the second-order polynomial equation. First- and second-order coefficients of these equations are usually unknown and, therefore, were estimated from the experimental data by using the statistical principle of least squares. The fit of the models was evaluated by the determination coefficients ($R^2$) and adjusted $R^2$ ($R_{adj}^2$) (*19,20*). In practice, $R^2$ should be at least 0.75 or greater; values above 0.90 are considered to be very good (*20*).

## Results and Discussion

### *Introduction of GK gene into Pichia pastoris and clone selection*

The EasySelect® (Invitrogen) expression system in *P. pastoris* (*10*) is used to provide the extracellular expression of proteins of interest from artificially integrated genes in the yeast genome. Chromosomal integration is performed by the transformation of the yeast by a plasmid containing the gene of interest, linearized with *Sac*I at the 5'-UTR of the *AOX*1 promoter from *P. pastoris* in the vector, leading to homologous recombination with the endogenous *AOX*1 copy. Extracellular production is conferred by the expression of the protein fused with the α-factor signal peptide from *S. cerevisiae*, so that the protein is secreted into the culture medium. The coding sequence of the *GUT*1 (glycerol kinase) gene was amplified by PCR, using a pair of primers that hybridize with flanking regions of the coding region of the *GUT*1 gene. Fig. 1a illustrates the amplification of a 2.1-kb fragment corresponding to the *GUT*1 gene. This fragment was cloned into the pPICZα A expression vector. The plasmid construct released the same fragment after digestion with the *Xho*I and *Eco*RI cloning enzymes, confirming the insertion of the gene in the pPICZα vector (Fig. 1b).

The integration of the GK gene into the *Pichia pastoris* genome was confirmed by Southern blotting hybridization (data not shown). The obtained transformants (4 clones) were subjected to induction of the expression of the enzyme of interest as suggested in the EasySelect® system instructions (Invitrogen). This induction is performed with methanol, which activates the transcription of the cloned gene of interest under the control of the *AOX*1 promoter. Among the four transformants tested

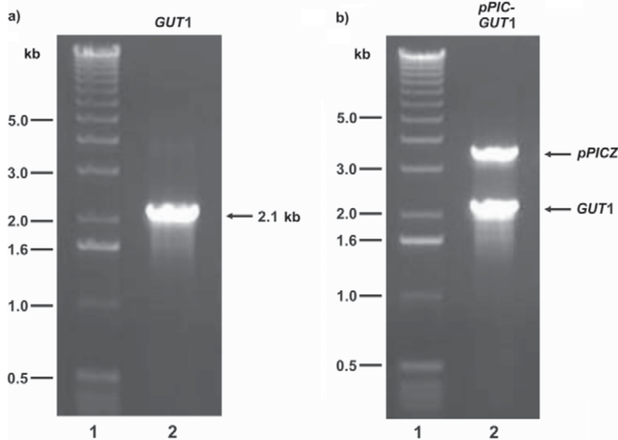


**Fig. 1.** PCR amplification of the *GUT*1 gene from *S. cerevisiae:* a) lane 2 shows *GUT*1 amplification product of 2.1 kb; cloning confirmation by restriction analysis, b) lane 2 shows the fragments corresponding to the *GUT*1 gene (2.1 kb) and the vector pPICZα A (3.6 kb). Lane 1 in both bands corresponds to the molecular mass standard (1 Kb Plus DNA Ladder™ by Invitrogen)

for GK production, clone 4 achieved the best enzyme activity and protein production (Table 1).

Table 1. Analysis of glycerol kinase (GK) activity and total protein in selected clones

| Clone | γ(total protein) | GK activity | |
|---|---|---|---|
| | mg/mL | U/mL | U/mg of protein |
| 1 | 12.11±0.2 | 0.21±0.05 | 0.017±0.002 |
| 2 | 12.38±0.1 | 0.13±0.02 | 0.011±0.001 |
| 3 | 12.47±0.2 | 0.0 | 0.0 |
| 4 | 12.56±0.3 | 0.32±0.0 | 0.025±0.002 |

## *Factors affecting biomass production*

In the screening step, the linear effects on biomass production and biomass yield were calculated for 8 potential factors (data not shown). A positive or a negative effect of a factor on a response (biomass production or productivity) indicates that an increase in the values of that factor is accompanied by an increase or a decrease in the response, respectively. Biomass production (in mg/mL) increased significantly with fermentation time (effect of 3.75; p=0.0015). In addition, although with a p-value higher than 0.05, the influence of a positive effect of glycerol and a negative effect of buffer molarity on the biomass production cannot be neglected. Thus, in order to promote biomass production, long growth time, high glycerol content and phosphate buffer solutions of low molarity must be used.

On the other hand, with respect to biomass productivity (mg/(mL·h)), a significant negative effect (effect of –0.0317; p=0.0176) of growth time was observed. This means that biomass yield decreases with long growth time. The negative effects of phosphate buffer molarity, biotin and peptone content also have to be considered. Therefore, higher biomass productivities are observed at short growth time when media prepared with low molarity phosphate buffer solutions and containing low amounts of biotin and peptone are used (data not shown).

## *Optimized biomass production*

The amount of biomass produced in each of the 12 experiments of the CCRD is presented in Table 2. From these results, linear and quadratic effects of each factor and of their linear interaction (φ(Gly)·time) were calculated (Table 3). Both glycerol volume fraction and growth time exhibited significant (p≤0.05) linear and positive effects on biomass production. The significant negative effect of time at the quadratic level (–1.56) indicates that biomass production is described by a convex response surface as a function of time. Although it has a p-value higher than 0.05, the interaction effect on biomass production should not be ignored. In fact, biomass production B (mg/mL) data are fitted well by a convex surface (Fig. 2) described by the following second-order polynomial model equation, as a function of glycerol volume fraction and fermentation time (h):

$$B = -0.989 - 0.365\varphi(\text{Gly}) + 0.167t - 0.001t^2 + 0.032(\varphi(\text{Gly})\cdot t) \quad /1/$$

The high values of both, the determination coefficient $R^2$ (0.946), and the adjusted $R_{adj}^2$ (0.916), together

Table 2. Central composite rotatable design (CCRD) used as a function of glycerol content and time of growth of *Pichia pastoris,* and respective experimental results of biomass production

| Experiment | φ(glycerol) | *t* | γ(biomass) |
|---|---|---|---|
| | % | h | mg/mL |
| 1 | 0.50 | 24 | 2.88 |
| 2 | 0.50 | 72 | 5.29 |
| 3 | 2.00 | 24 | 2.65 |
| 4 | 2.00 | 72 | 7.38 |
| 5 | 0.19 | 48 | 3.57 |
| 6 | 2.31 | 48 | 7.27 |
| 7 | 1.25 | 14 | 1.14 |
| 8 | 1.25 | 82 | 6.99 |
| 9 | 1.25 | 48 | 5.11 |
| 10 | 1.25 | 48 | 5.18 |
| 11 | 1.25 | 48 | 5.89 |
| 12 | 1.25 | 48 | 5.94 |

Table 3. Effects of glycerol volume fraction (φ(Gly)) and growth time on biomass production and respective p-levels analyzed in CCRD

| Factor | Effect | p-level |
|---|---|---|
| φ(Gly) linear | 1.77 | 0.0065 |
| φ(Gly) quadratic | –0.21 | 0.6870 |
| Time linear | 3.86 | 0.0001 |
| Time quadratic | –1.56 | 0.0183 |
| φ(Gly)·time | 1.16 | 0.1083 |

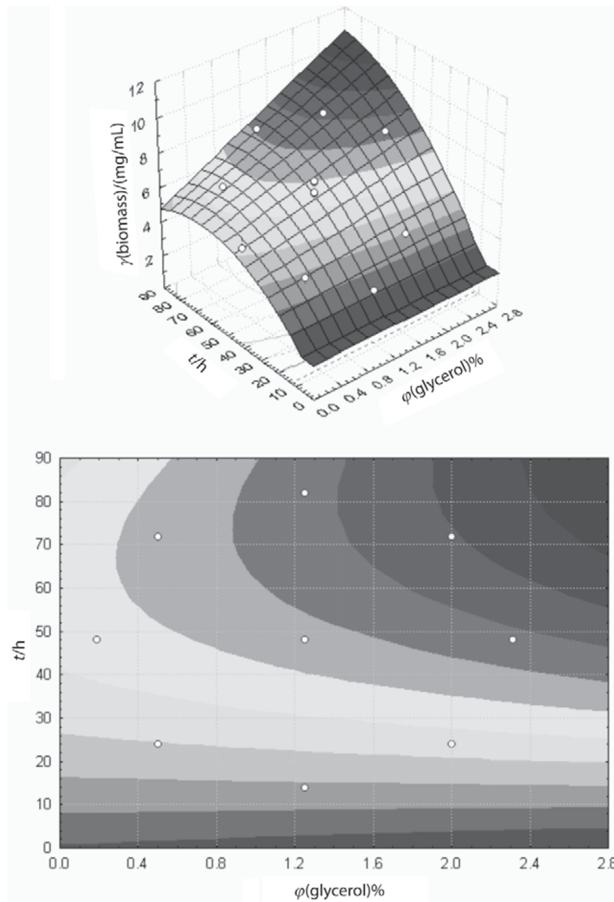


**Fig. 2.** Response surface of biomass production at 30 °C as a function of glycerol volume fraction (%) and growth time (h), and the respective contour plot

with the linear relationship between the observed and predicted values (Fig. 3) demonstrate the very good fit of this model to the experimental data. Although the maximum is outside the experimental domain, Fig. 2 shows that the highest biomass production values are obtained at higher $\varphi$(Gly) and longer growing times.

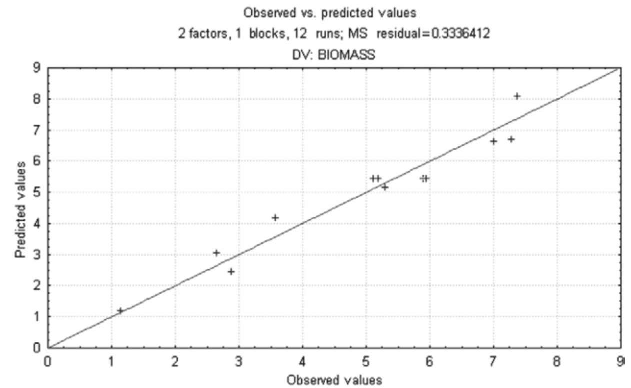


**Fig. 3.** Linear relationship between the observed values of biomass production and those predicted by the model

### *Effect of medium composition on GK expression*

The carbon source is another important factor for the successful expression of heterologous proteins. According to Xie *et al.* (*12*), one of the most important factors in the BMMY medium is the supplementation of the carbon source. Due to the high specificity of the control system for expression of protein on methanol, *Pichia pastoris* proved more effective when the methanol concentration was within its optimal range (Table 4). In the present case, cells grown in the BMMY medium containing both 1 % methanol and 1 % glycerol exhibited the highest GK activity and viability (Table 4).

The methylotrophic yeast *Pichia pastoris* is currently widely used as a host for the production of recombinant proteins, as it can be grown in a methanol-complex medium (*21*). Proteins can also be secreted into the culture medium with the aid of a suitable secretion signal. Glycerol is commonly used as the growth substrate for biomass accumulation in the first phase, whereas only methanol is fed in the second phase to induce the foreign protein expression (*22,23*). An alternative mixed-feed and fed-batch strategy has been developed, which has considerably increased the productivity of recombinant *Pichia* systems (*24,25*).

Table 4. Effect of methanol concentration and various carbon sources on biomass, viability, glycerol kinase (GK) activity and total protein production by *Pichia pastoris* grown in BMMY medium

| BMMY medium | $\gamma$(biomass) | Viability | $\gamma$(protein) | GK activity | |
|---|---|---|---|---|---|
| | mg/mL | % | mg/mL | U/mL | U/mg |
| Methanol (0.25) | 3.28±0.12 | 79.80±0.25 | 15.80±0.90 | 0.00 | 0.00 |
| Methanol (0.50) | 3.20±0.05 | 86.10±0.95 | 17.68±0.48 | 0.50±0.00 | 0.028 |
| Methanol (1) | 3.53±0.04 | 94.14±0.48 | 14.14±0.18 | 0.76±0.52 | 0.054 |
| Methanol (2) | 2.90±0.04 | 83.40±2.55 | 14.55±0.48 | 0.032±0.00 | 0.0022 |
| Methanol (3) | 2.78±0.01 | 78.80±0.55 | 14.34±1.36 | 0.00 | 0.00 |
| Methanol (1) and lactic acid (1) | 3.33±0.08 | 90.88±1.65 | 15.59±0.31 | 0.13±0.00 | 0.0082 |
| Methanol (1) and glycerol (1) | 6.02±0.11 | 98.31±0.33 | 14.55±0.95 | 0.89±0.00 | 0.118 |
| Methanol (1) and ammonium acetate (1) | 3.62±0.05 | 93.79±1.10 | 13.93±0.18 | 0.51±0.00 | 0.037 |
| Methanol (1) and sorbitol (1) | 5.67±0.15 | 94.14±0.52 | 14.94±0.54 | 0.00 | 0.00 |

Numbers in parentheses denote the fraction of the carbon source (%)

Table 5 displays the final activity of GK and biomass obtained for *Pichia pastoris* when 1 % methanol (control) plus 1 % glycerol (optimized medium) were added initially to the BMMY medium for enzyme synthesis by the yeast strain. The highest value of final biomass (6.02 mg/mL) was observed for recombinant *Pichia pastoris* after 48 h of growth in the optimized medium, with a 1.17-fold increase in GK activity. The specific yield for GK activity with respect to cell growth (U/mL)/mg fell to 0.68 of the control, but the maximum GK productivity rate (GK activity/growth time) was the highest (2.45-fold) for *Pichia pastoris* grown in the optimized medium.

Table 5. Analysis of glycerol kinase activity determined before and after the optimization of BMMY medium

| | Control* | After medium optimization** | Increase fold |
|---|---|---|---|
| Activity/(U/mL) | 0.76 | 0.89 | 1.17 |
| $\gamma$(biomass)/(mg/mL) | 3.53 | 6.02 | 1.71 |
| Specific yield per cell/(U/(mL·mg)) | 0.22 | 0.15 | 0.68 |
| Specific activity per protein/(U/mg) | 0.054 | 0.13 | 2.41 |
| Productivity rate per protein/(U/(mg·h)) | 0.0011 | 0.0027 | 2.45 |

Initial carbon source (% by volume): *methanol 1, ** methanol 1 and glycerol 1

## Conclusion

The regulation of heterologous gene expression by means of a mixed carbon source proved to be simple and cost-effective for industrial enzyme production. *Pichia pastoris* production system offers the advantage of secreting the protein of interest into the culture medium. The present study reports biomass production and accumulation of GK activity by the recombinant *Pichia pastoris* grown in an optimized medium. The optimization of biomass production (increasing with time) contrasted with that of biomass productivity (decreasing with time). However, since glycerol content favours biomass production and does not affect biomass productivity, media with higher glycerol contents should be used to promote biomass production. The highest activity (0.89 U/mL) and specific activity per mass of protein (0.13 U/mg) of GK in the medium were obtained in a mixture containing 1 % glycerol and 1 % methanol as carbon sources.

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