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# Characterization of glycerol kinase from baker's yeast: Response surface modeling of the enzymatic reaction

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**Abstract**

The present study describes a methodology of dosage of glycerol kinase (GK) from baker's yeast. The standardization of the activity of the glycerol kinase from baker's yeast was accomplished using the diluted enzymatic preparation containing glycerol phosphate oxidase (GPO) and glycerol kinase. The mixture was incubated at 60 °C by 15 min and the reaction was stopped by the SDS solution addition. A first set of experiments was carried out in order to investigate the individual effect of temperature (*T*), pH and substrate concentration (*S*), on GK activity and stability. The pH and temperature stability tests showed that the enzyme presented a high stability to pH 6.0–8.0 and the thermal stability were completely maintained up to 50 °C during 1 h. The $K_m$ of the enzyme for glycerol was calculated to be 2 mM and $V_{max}$ to be 1.15 U/mL. In addition, modeling and optimization of reaction conditions was attempted by response surface methodology (RSM). Higher activity values will be attained at temperatures between 52 and 56 °C, pH around 10.2–10.5 and substrate concentrations from 150 to 170 mM.

This low cost method for glycerol kinase dosage in a sequence of reactions is of great importance for many industries, like food, sugar and alcohol. RSM showed to be an adequate approach for modeling the reaction and optimization of reaction conditions to maximize glycerol kinase activity.
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*Keywords:* Glycerol kinase; Baker's yeast; Partial purification; Response surface methodology; Stability

## 1. Introduction

Glycerol kinase (GK, EC 2.7.1.30; ATP: glycerol 3-phosphotransferase) catalyzes the transfer of the terminal phosphate of ATP to glycerol, to form *sn*-glycerol-3-phosphate and ADP. This enzyme plays a physiologically important role for the formation for glycerol-3-phosphate in the biosynthesis of phospholipids. In addition, the enzyme is industrially important and useful for the clinical determination of the blood triglyceride level in combination with lipase, glycerol-3-phosphate oxidase and peroxidase (PO) [1]. Up to now, GKs have been purified from several microorganisms [2–7] and a vertebrate [8], and subsequently characterized. The genes of GK from *Escherichia coli* [9], *Bacillus subtilis* [10], *Saccharomyces cerevisiae* [11], *Thermus flavus* [12], *Flavobacterium meningosepticum* [13] and humans [14] have been cloned into *E. coli*, and the primary structures of their enzymes were determined. Comparison of these sequences suggests that the amino acid sequence of GK is relatively well conserved among various GKs. For example, the identity in the amino acid sequence between *E. coli* GK and human is 51%. Comparisons of kinetic constants for enzymes from four microorganisms (*C. mycoderma*, *S. cerevisiae*, *E. coli*, and *B. stearothermophilus*) also indicated little variation among them [15], the $K_m$ found was 5 mM for glycerol for the salt-tolerant yeast *Debaryomyces hansenii* [16]. This is probably because GK is a key enzyme in glycerol metabolism and therefore the enzyme cannot be structurally and functionally divergent during evolution.

Beyond the colorimetric assay [17], glycerol kinase activity has been determined by several methods, including photomet-

*Abbreviations:* GK, glycerol kinase; GPO, glycerol phosphate oxidase; PO, peroxidase; RSM, response surface methodology; $R^2$, determination coefficient (quadratic correlation coefficient); $R^2_{adj}$, adjusted $R^2$.

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ric [18], spectrophotometric [3,8,17,19–24], radioisotopic [21], radiochemical [25–29], and chemiluminescent [30].

In this study, a GK was semipurified from dry baker's yeast and characterized. In a first set of experiments, the effect of temperature (*T*), pH and substrate concentration (*S*), on GK activity and stability was investigated, in order to determine the most adequate variation ranges for each variable for GK activity. Afterwards, response surface methodology (RSM) was used for modeling and optimization of reaction conditions for glycerol kinase, as a function of reaction temperature (46–64 °C), substrate concentration (48–162 mM of glycerol) and pH of the reaction medium (8.6–10.4).

RSM consists on a set of mathematical and statistical methods developed for modeling phenomena and finding combinations of a number of experimental factors (variables) that will lead to optimum responses [31,32]. With RSM, several variables are tested simultaneously with a minimum number of trials, according to special experimental designs, which enables to find interactions between variables [31,32]. This is not an option with classical approaches. In addition, RSM has the advantage of being less expensive and time-consuming than the classical methods.

The RSM has been used on modeling and optimization of several bioprocesses such as fermentation and enzymatic reactions [33–42], as well as on adsorption processes [43,44].

## 2. Experimental

### 2.1. Microorganism

Dry baker's yeast from Mauri Brazil Ind. Com. e Imp. Ltda.

### 2.2. Cell disruption and crude cell extract preparation for enzymatic assay

#### 2.2.1. Glycerol kinase and glycerol phosphate oxidase (GPO) of the dry baker's yeast

The cells (10 g, dry weight) of baker's yeast were suspended in 100 mL of 2 mmol/L sodium citrate buffer, at pH 6.2 containing 2 mmol/L of β-mercaptoethanol, and 100 g of glass beads (425–600 μm) was added to the cells suspension and the disruption was carried out for 7 min in a Bead Beater (Biospec Products Inc., USA), cooled by ice and operated under full speed. The cells debris was separated by centrifugation for 10 min at 8,130 × *g*.

Cell extract was treated with 1% of streptomycin sulfate, and the clarified supernatant was obtained by centrifugation at 16,260 × *g* for 20 min [45]. The first ammonium sulfate precipitation, a result from treatment with streptomycin sulfate, was added to the supernatant (35% saturation), and the precipitate obtained by centrifugation (16,260 × *g*/20 min) after 30 min at 4 °C was discarded. In the supernatant fraction of the present enzyme was added ammonium sulfate in the range 60% of saturation, and after one night at 4 °C the suspension was centrifuged (16,260 × *g*/20 min). The precipitate was dissolved in 1 mL 10 mmol/L Tris/HCl buffer at pH 7.2, and the fraction of enzyme was dialyzed for 24 h at 4 °C against a solution of 2 mmol/L sodium citrate at pH 5.5 containing 2 mmol/L β-mercaptoethanol, and 10 mmol/L $MnSO_4$, and this fraction, after centrifugation (11,292 × *g*/10 min) was used as the source of GK and GPO.

#### 2.2.2. Horseradish peroxidase

The crude cell extract was obtained by disruption of the 500 g of peeled horseradish in an electric liquidizer with 20 mL of 0.1 M acetate buffer at pH 5.0. The homogenate was filtered in gauze and it was clarified by centrifugation (1,807 × *g*, 10 min) in refrigerated centrifuge, and used as peroxidase source.

### 2.3. Glycerol phosphate oxidase assay

GPO activity was determined by the peroxidase chromogen method as described by Šůcková et al. [46] with modifications. The absorbance at 500 nm (molar absorption coefficient of $6.65 \times 10^3\ M^{-1}\ cm^{-1}$) was measured using a mixture of 750 μL of glycerol-phosphate 0.5 M in 0.1 M Tris/HCl buffer pH 8.0 containing 0.1% Triton X-100; 150 μL of 4-aminoantipyrine 0.1%, 300 μL of phenol 0.1%; 15 μL (1.5 U) of horseradish peroxidase; 100 μL water. The reaction was started by adding 15 μL of the preparation of enzyme diluted 10-fold, and stopped with 300 μL of SDS 10%, after incubation at 60 °C for 120 min. The assay measures the production of the oxidated derivatives (quinone-imine) of 4-aminoantipyrine. One unit (U) of enzyme was defined as the amount of the enzyme producing 1 μmol of $H_2O_2$ per minute.

### 2.4. Peroxidase assay

PO activity was determined by a spectrophotometric method at 460 nm using a mixture of 0.2 mL of *o*-dianisidine 15 mM; 0.2 mL of hydrogen peroxide 30 mM; 5 μL of horseradish extract and 2.595 mL of 0.1M citrate-phosphate buffer at pH 5.5. The assay measures the production of the oxidated derivatives of *o*-dianisidine during the first 15–90 s of the reaction rate (molar absorption coefficient of $11.3 \times 10^3\ M^{-1}\ cm^{-1}$). One unit (U) of enzyme was defined as the unit of absorbance generated per minute in the assay conditions.

### 2.5. Glycerol kinase assay

Extracts and fractions were assayed as described by Huang et al. [17]. The reaction mixture contained 70 μL of 50 mM ATP, 50 μL of 0.2 M magnesium sulfate, 500 μL of 0.05 M glycerol in 0.4 M glycine/NaOH buffer pH 10.0, 160 μL of the preparation of enzyme diluted 60-fold containing glycerol-3-phosphate oxidase, 150 μL of 4-aminoantipyrine 0.1%, 300 μL of phenol 0.1%. The reaction was started by adding 100 μL (10 U) of horseradish peroxidase, and stopped with 300 μL of SDS 10%, after incubation at 60 °C for 15 min. The absorbance at 500 nm (molar absorption coefficient of $6.65 \times 10^3\ M^{-1}\ cm^{-1}$) was measured. One unit (U) of enzyme was defined as the amount of the enzyme catalyzing the formation of 1 μmol of glycerol-3-phosphate/min at 60 °C.

### 2.6. Kinetic parameters

The kinetic parameters, $K_m$ and $V_{max}$, for glycerol were estimated from the Lineweaver–Burks plots.

### 2.7. First set of experiments

The following experiments were carried out in order to determine the experimental domain for modeling and optimization of GK reaction conditions by RSM.

#### 2.7.1. Effect of pH on glycerol kinase activity and stability

The effect of pH on glycerol kinase activity was determined by assaying the enzyme activity at 60 °C at various pH levels, between 7.0 and 11.0 (Tris/HCl buffer, pH 7.0–9.0; glycine/NaOH buffer, pH 9.5–11.0). The pH stability of the enzyme was determined by measuring the enzymatic activity under standard enzyme assay conditions after incubating the enzyme at 30 °C during 25 days, the following 0.01 M buffer systems of varying pH were used: for pH values of 5.0 and 6.0 (sodium citrate buffer), 7.0, 8.0 and 9.0 (Tris/HCl buffer), and 10.0 (glycine/NaOH buffer).

#### 2.7.2. Effect of temperature on glycerol kinase activity and thermal stability

The optimum temperature of glycerol kinase activity was determined by assaying enzyme activity at pH 10.0 in 0.4 M of glycine/NaOH buffer, at temperatures of 40, 50, 60, 70 and 80 °C. The thermal stability of the enzyme was determined by measuring the enzymatic activity under standard enzyme assay conditions after incubating the enzyme solution for 1 h, at various temperatures of 30, 40, 45, 50, 55 and 60 °C.

### 2.8. Protein assay

Total protein was assayed according to the method of Lowry, modified by Layne [47], using bovine serum albumin as the standard protein. The levels of total protein were 2.53–3.30 mg/mL for horseradish extract, and around of 12.5–13.2 mg/mL for yeast extract.

### 2.9. Response surface experiments

#### 2.9.1. Experimental design

The best conditions for the enzymatic reaction were established via RSM. In this study, the experiments were carried out following a full factorial design with the addition of centre points, as a function of reaction temperature, substrate concentration and pH of the reaction medium. This matrix was replicated one time. Thus, a total of 22 experiments were carried out (Table 1): 16 factorial points (coded levels as (+1) and (−1)); and 6 centre points (coded as 0). Temperature varied from 46 to 64 °C, substrate concentration (glycerol) varied from 48 to 162 mM and pH from 8.6 to 10.4. The establishment of these variation ranges was based on the previous results obtained in preliminary experiments. These ranges contain the apparent optimum value for each factor. Concerning glycerol concentration, the range chosen corresponds to the values suggested by the preliminary experiments where no substrate limitation was observed, i.e. where zero-order kinetics was attained.

Table 1
Coded and decoded experimental design followed

| Experiment no. | Replicate | Coded matrix | | | Decoded matrix | | |
|---|---|---|---|---|---|---|---|
| | | Temperature | [Substrate] | pH | Temperature (°C) | [Substrate] (mM) | pH |
| 1 | 1 | −1 | −1 | −1 | 46 | 48 | 8.6 |
| 2 | 1 | 1 | −1 | −1 | 64 | 48 | 8.6 |
| 3 | 1 | −1 | 1 | −1 | 46 | 162 | 8.6 |
| 4 | 1 | 1 | 1 | −1 | 64 | 162 | 8.6 |
| 5 | 1 | −1 | −1 | 1 | 46 | 48 | 10.4 |
| 6 | 1 | 1 | −1 | 1 | 64 | 48 | 10.4 |
| 7 | 1 | −1 | 1 | 1 | 46 | 162 | 10.4 |
| 8 | 1 | 1 | 1 | 1 | 64 | 162 | 10.4 |
| 9 (C) | 1 | 0 | 0 | 0 | 55 | 105 | 9.5 |
| 10 (C) | 1 | 0 | 0 | 0 | 55 | 105 | 9.5 |
| 11 (C) | 1 | 0 | 0 | 0 | 55 | 105 | 9.5 |
| 12 | 2 | −1 | −1 | −1 | 46 | 48 | 8.6 |
| 13 | 2 | 1 | −1 | −1 | 64 | 48 | 8.6 |
| 14 | 2 | −1 | 1 | −1 | 46 | 162 | 8.6 |
| 15 | 2 | 1 | 1 | −1 | 64 | 162 | 8.6 |
| 16 | 2 | −1 | −1 | 1 | 46 | 48 | 10.4 |
| 17 | 2 | 1 | −1 | 1 | 64 | 48 | 10.4 |
| 18 | 2 | −1 | 1 | 1 | 46 | 162 | 10.4 |
| 19 | 2 | 1 | 1 | 1 | 64 | 162 | 10.4 |
| 20 (C) | 2 | 0 | 0 | 0 | 55 | 105 | 9.5 |
| 21 (C) | 2 | 0 | 0 | 0 | 55 | 105 | 9.5 |
| 22 (C) | 2 | 0 | 0 | 0 | 55 | 105 | 9.5 |

The addition of six centre points (55 °C, 105 mM of substrate and pH 9.5) allows checking the curvature of the response surface fitted to the experimental points. With this experimental design, three levels for each factor were used which enables to fit second-order polynomials to the experimental data points. Therefore, curved surfaces can be fitted to the experimental data. Partial differentiation of these polynomial equations may be used to find the optimum points, i.e. the stationary points [31]. However, the identification, for each variable, of the regions corresponding to optimal responses, may be directly achieved by visual examination of the response surfaces and/or contour plots.

### 2.9.2. Statistical analysis

All the considered data presented standard deviation of up to 5%. The results of the 22 experiments were analyzed using the software "Statistica$^{TM}$", Version 6, from Statsoft, Tulsa, USA. Both linear and quadratic effects of each of the independent variables (factors) under study, as well as their interactions, on glycerol kinase activity were calculated. Their significance was evaluated by analysis of variance. A surface, described by a first- or a second-order polynomial equation was fitted to the set of experimental data points. 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 model was evaluated by the determination coefficients ($R^2$) and adjusted $R^2$ ($R^2_{adj}$) [31,48]. In practice, $R^2$ should be at least 0.75 or greater; values above 0.90 are considered to be very good [48].

## 3. Results and discussion

### 3.1. Optimization of the glycerol kinase assay

The linear range of the assay was adjusted by shorting the reaction time and determining the dilution of enzyme (Fig. 1). The glycerol kinase kinetics is well described by the Michaëlis–Menten model (Fig. 2). For glycerol concentrations higher than about 50 mM, a zero-order reaction is observed. A 15-min reaction time, a dilution of enzyme up to 60 times, and a concentration of peroxidase of 100 μL and substrate of 50 mM, seems to be the best conditions for the determination of the GK activity.

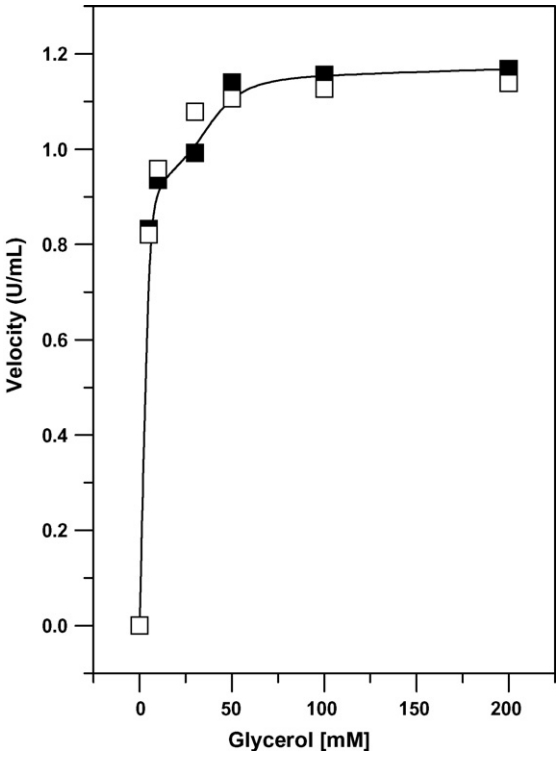


Fig. 2. GK activity obtained for different standard solution of glycerol. The data in closed squares represent the experimental data and in the open symbols are the theoretical values estimated by the equation of Michaëlis–Menten.

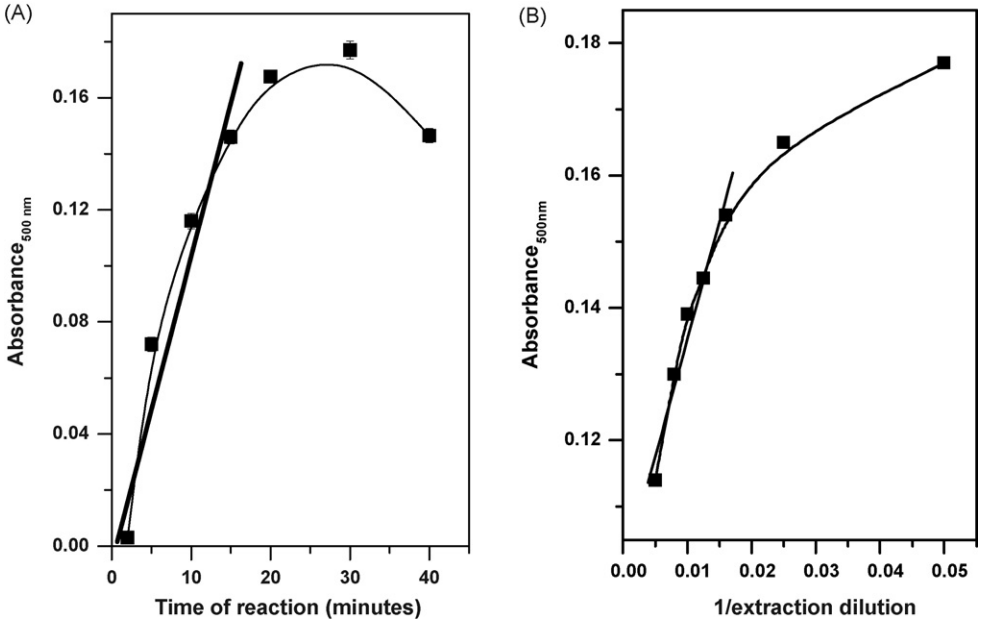


Fig. 1. Absorbance values obtained for standard solution of glycerol at different reaction time (A) and dilutions of the cellular extract (B).

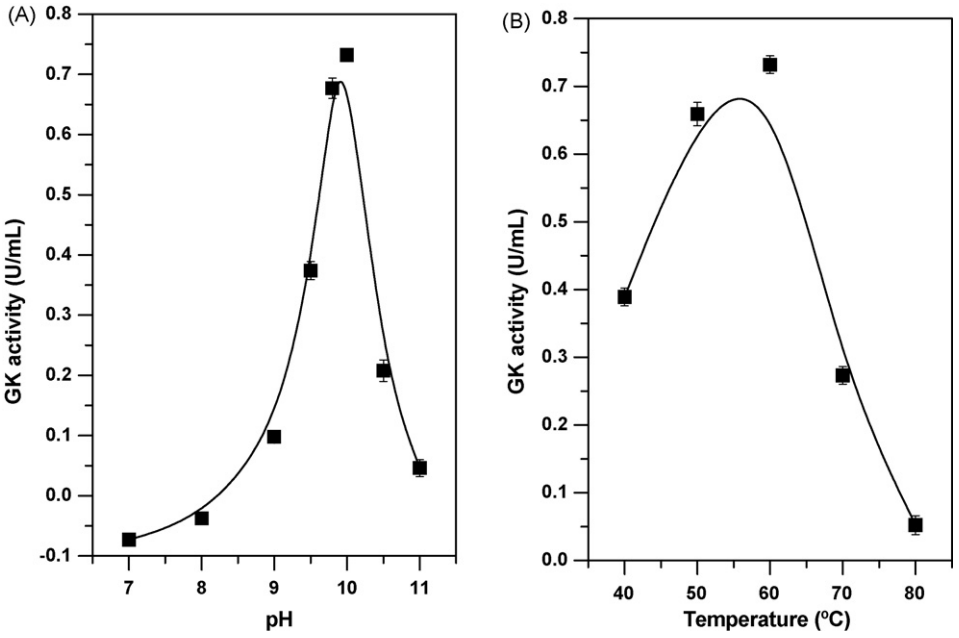


Fig. 3. Influence of pH (A) and temperature (B) on glycerol kinase activity. The GK was assayed in the pH range 7.0 and 11.0, using various buffers (Tris–HCl buffer, pH 7.0–9.0; glycine/NaOH buffer, pH 9.5–11.0). The optimum temperature of glycerol kinase activity was determined by assaying enzyme activity at pH 10.0 in 0.4 M of glycine/NaOH buffer, at temperatures of 40, 50, 60, 70 and 80 °C.

### 3.2. Effect of pH and temperature on glycerol kinase activity

Crude cellular extracts from dry baker's yeast were obtained as described above, and the pH and temperature curves for the assay of the activity of the glycerol kinase can be observed in Fig. 3. The highest activity was found at pH 10.0 and at a temperature of 60 °C. The optimum pH [17,49,50] and temperature [17,50] for the assay system have been established by others. Our values of temperature and pH are similar to those reported in the literature for glycerol kinase of *T. flavus* [17] and for *E. coli* [3], respectively. However, these values may be only pseudo-optimum pH and temperature values, since the effect of interactions among factors was not considered. In the second set of experiments, the RSM will be used as an attempt to find out the real optimal conditions for GK activity.

### 3.3. Stability of glycerol kinase activity at different pHs and temperatures

The thermal stability was obtained by measuring the activity, after incubation of the enzyme for 1 h at temperatures of 30, 40, 45, 50, 55 and 60 °C, as shown in Fig. 4; in relation to the pH (5–10) stability, the activity was determined during 25 days at 30 °C, as shown in Fig. 5. The results obtained showed that the enzyme presented a wide range stability to pH (6.0–8.0), and the thermal stability was completely maintained up to 50 °C, after which its activity gradually decreased. This glycerol kinase in *S. cerevisiae* was stable in a wide range of pH and coincides with other microorganisms [13]. The enzyme was unexpectedly thermostable, and its thermostability is similar to that of GK from *B. stearothermophilus*, a moderate thermopile [13].

### 3.4. Catalytic properties

In the experiments by varying the concentrations of substrate, the kinetic data were obtained by double reciprocal plot of initial reaction rates and the concentration of glycerol. The $K_m$

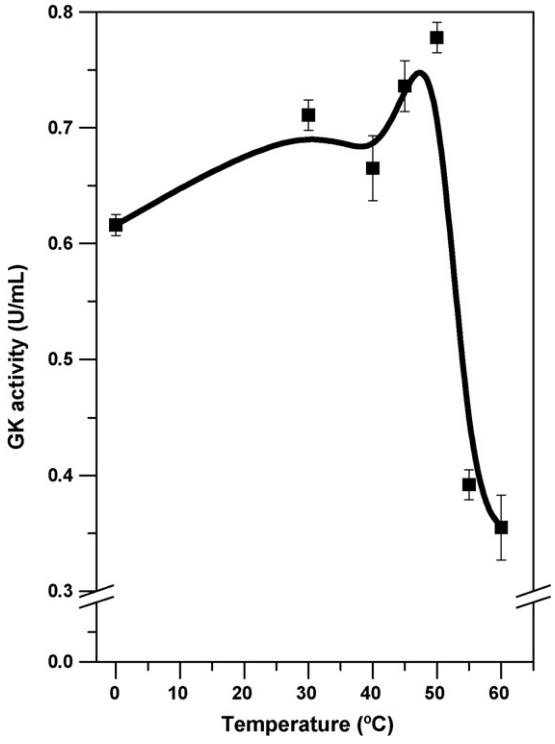


Fig. 4. Effect of temperature on GK stability. In the experiments, the enzyme solution was incubated for 1 h, at various temperatures of 30, 40, 45, 50, 55 and 60 °C.

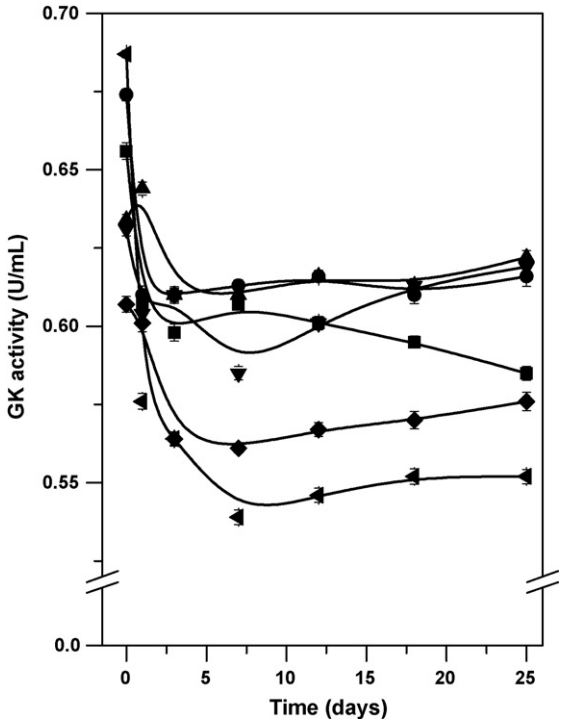


Fig. 5. Effect of pH on GK stability. In the experiments, the buffers used were as follows: 0.01 M sodium citrate buffer (pH 5.0 (■) and pH 6.0 (●)), Tris/HCl buffer (pH 7.0 (▲), pH 8.0 (▼) and pH 9.0 (♦)), and glycine/NaOH buffer (pH 10.0 (◄)).

of the enzyme for glycerol was calculated to be 2 mM and $V_{max}$ to be 1.15 U/mL, being values similar to the literature [16].

### 3.5. Response surface experiments

The significant effects of temperature ($T$), initial substrate concentration ($S$), pH value and interactions ($T \times$ pH and $S \times$ pH) on glycerol kinase activity are shown in Table 2. Temperature showed to have significant effects, both at linear and quadratic levels, on enzyme activity. The negative quadratic effect indicates that the effect of temperature on glycerol kinase activity may be described by a convex surface. Also, the increase in substrate concentration and in pH of the reaction medium will be accompanied by a linear increase in enzyme activity (positive linear effects). No significant effects of $S$ or pH at quadratic levels were observed.

Table 2
Effect estimates of the factors temperature ($T$), substrate concentration ($S$) and pH, at linear (L) and quadratic (Q) levels, and of the respective interactions on the activity of glycerol kinase (only significant effects of $p \leq 0.5$ or those having a confidence range smaller than the value of the effect, or smaller than the standard deviation (data not shown), were considered)

| Factor/interaction | Effect | $p$ value |
|---|---|---|
| Temperature (L) | −0.1734 | 0.0000 |
| Temperature (Q) | −0.7773 | 0.0000 |
| Substrate (L) | 0.0646 | 0.0006 |
| pH (L) | 0.4386 | 0.0000 |
| $T \times$ pH | −0.2516 | 0.0000 |
| $S \times$ pH | 0.0279 | 0.0802 |

A significant negative interaction between $T$ and pH ($T \times$ pH) and a positive significant interaction between $S$ and pH ($S \times$ pH) were observed. Thus, a simultaneous increase in $T$ and pH will conduct to a decrease in enzyme activity. Conversely, a simultaneous increase in $S$ and pH will be accompanied by an increase in enzyme activity. However, no significant interaction between $T$ and $S$ ($T \times S$) was detected.

The experimental data points can be described by the following second-order polynomial model:

$$\text{Activity} = -23.6088 + 0.6665T - 0.0048T^2 - 0.0020S + 1.0754\text{pH} - 0.0156\,(T \times \text{pH}) + 0.0003\,(S \times \text{pH})$$

where the activity is expressed in U/mol, $T$ is the temperature (°C) and $S$ is the substrate concentration (mM). In this model equation, only the factors and the interactions showing significant effects of $p \leq 0.5$, or having a confidence range smaller than the value of the effect, or smaller than the standard deviation (data not shown), were considered. In fact, these later effects have a lower probability, but their values are not small enough to be neglected [48].

This model has high values of both $R^2$ (0.9928) and $R^2_{adj}$ (0.9899), indicating that it explains more than 99% of the variability of the experimental data. Therefore, a close agreement between the experimental results and the theoretical values predicted by this model is attained [48,51]. This can be illustrated by Fig. 6, showing a close correlation between predicted values versus observed values. Also, this model fitted to the experimental points of enzyme activity is represented by a saddle-like surface. Since 3 variables (factors) were considered, the response is in a 4th axe. Thus, the 4-dimensional saddle-like response surface is represented by two 3-dimensional surfaces, as a function of 2 of the 3 variables, keeping the 3rd variable constant and equal to its centre point value (Fig. 7). Saddle-like surfaces do not have a defined stationary point (maximum) but they are characterized by having infinity of data points that maximize the response. Thus, in this case, the analysis of contour plots is useful to find out the best regions in the experimental domain (Fig. 8). Higher

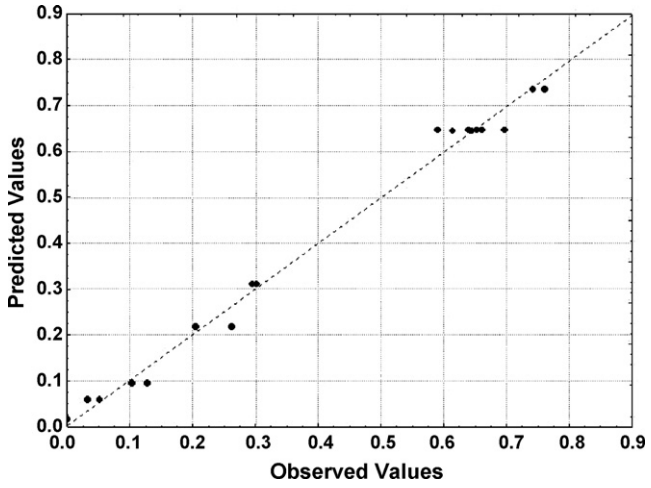


Fig. 6. Linear relationship between the experimental values and the corresponding values predicted by the second-order polynomial model.

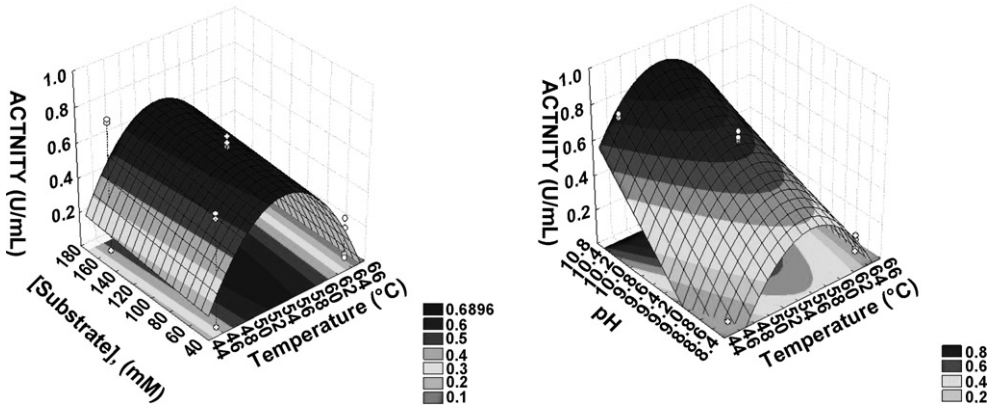


Fig. 7. Response surface fitted to the experimental data points corresponding to the activity of glycerol kinase, as a function of temperature and initial substrate concentration, at pH of 9.5, and as a function of temperature and pH, at initial substrate concentration of 105 mM.

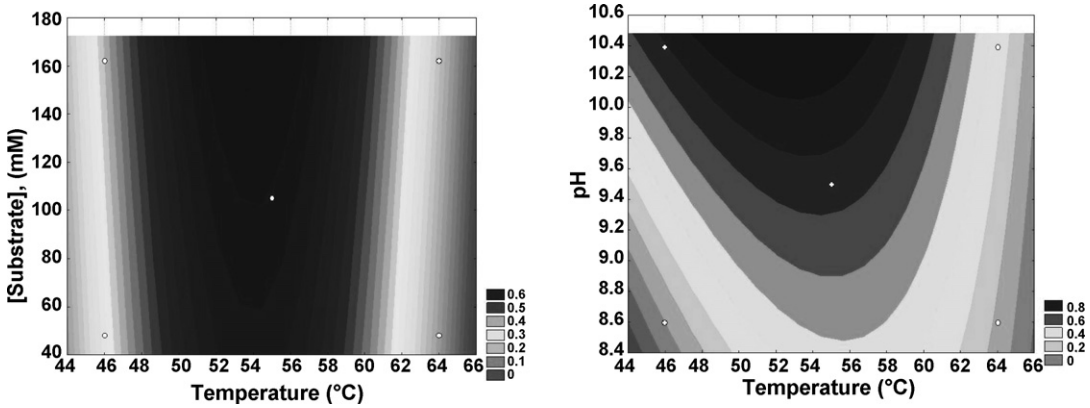


Fig. 8. Contour plots of the response surface presented in Fig. 7.

activity values will be attained at temperatures between 52 and 56 °C, pH around 10.2–10.5 and substrate concentrations from 150 to 170 mM.

The response surface and respective contour plot show that the effect of temperature in GK activity is more pronounced than that of pH and much more than that of substrate concentration, inside the experimental range tested. An increase in temperature up to values around 50 °C is accompanied by an increase in enzyme activity; for temperatures higher than 55–60 °C, a thermal inactivation is observed. The interaction between pH and temperature is well observed both in response surfaces and in contour plots, showing that it cannot be ignored. The effect of substrate concentration is not very pronounced because the values tested were rather high. However, the $T$ and pH ranges suggested by RSM are not very far from the apparent optima suggested by the preliminary experiments ($T = 60$ °C; pH 10). The differences may be explained by the existence of significant interactions ($T \times$ pH) and ($S \times$ pH) that were ignored in the classical approach followed in the preliminary experiments.

## 4. Conclusion

The results obtained showed that the colorimetric method using enzymatic preparations, obtained from dry baker's yeast, turned into a simple methodology with low cost-effectiveness, with wide application, and will be used in the glycerol determination. This method compared with a commercial kit for this purpose, and containing purified enzymes (Sigma®), presented a reduction of costs of approximately 88.4%. RSM showed to be an adequate approach for modeling the reaction and optimization of reaction conditions to maximize glycerol kinase activity.

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