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# Comparative biochemical characterization of soluble and chitosan immobilized β-galactosidase from *Kluyveromyces lactis* NRRL Y1564

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ABSTRACT

An investigation was conducted on the production of β-galactosidase (β-gal) by different strains of *Kluyveromyces*, using lactose as a carbon source. The maximum enzymatic activity of 3.8 ± 0.2 U/mL was achieved by using *Kluyveromyces lactis* strain NRRL Y1564 after 28 h of fermentation at 180 rpm and 30 °C. β-gal was then immobilized onto chitosan and characterized based on its optimal operation pH and temperature, its thermal stability and its kinetic parameters ($K_m$ and $V_{max}$) using o-nitrophenyl β-D-galactopyranoside as substrate. The optimal pH for soluble β-gal activity was found to be 6.5 while the optimal pH for immobilized β-gal activity was found to be 7.0, while the optimal operating temperatures were 50 °C and 37 °C, respectively. At 50 °C, the immobilized enzyme showed an increased thermal stability, being 8 times more stable than the soluble enzyme. The immobilized enzyme was reused for 10 cycles, showing stability since it retained more than 70% of its initial activity. The immobilized enzyme retained 100% of its initial activity when it was stored at 4 °C and pH 7.0 for 93 days. The soluble β-gal lost 9.4% of its initial activity when it was stored at the same conditions.



## 1. Introduction

The enzymatic hydrolysis of lactose by β-galactosidase (β-gal) plays an important role in the processing of dairy products, such as the production of milk containing low concentrations of lactose, the prevention of crystallization in dairy products, and the use of galactosyltransferase for synthesizing galacto-oligosaccharides [1,2].

*Kluyveromyces* strains have been extensively studied for their production of β-gal, especially *Kluyveromyces lactis* and *Kluyveromyces marxianus*, which possess an ability to assimilate lactose when lactose is used as a carbon source [3]. The yeast *K. lactis* is a source of β-galactosidases (β-D-galactohydrolase, EC 3.2.1.23) [4] and is of great commercial importance, with the complex mixture of enzymes in Lactozym 3000 L HP G being the enzymatic preparation that is most widely used [5].

As it is relatively costly to produce the pure enzyme, an alternative to reduce the production cost would be to use more affordable culture media such as whey, which is the liquid portion that is formed upon the coagulation of casein in milk when cheese is produced. Milk consists of approximately 85 to 95% whey by volume, and whey contains 55% of the nutrients found in milk [6]. Cheese whey is treated and transformed into various food products. Among those products, whey powder accounts for a large fraction of the whey that is processed, therefore maintaining the quality of fresh whey for a longer period of time, facilitating manipulation and transport [7].

The main disadvantage of using soluble enzymes as industrial catalysts is their poor stability in aqueous solutions against extreme pH, temperature, high ionic strengths, among others [8]. Various approaches have been proposed to solve this problem, including enzyme immobilization, the use of glycols and other stabilizing additives, reticulation with bifunctional agents, the use of enzymes from thermophilic organisms, or a chemical modification of the enzyme [9,10]. The immobilization of enzymes is usually advantageous in numerous ways: the biocatalyst can be reused, the reaction volume can be reduced because the immobilized enzyme can be used at higher concentrations, there is an ease of operational control, it is easier to control the process parameters when the reactor is operated continuously, and the product can be more easily separated from the immobilized enzyme because the immobilized enzyme is not soluble in the reaction medium [11]. Pessela et al. [12] studied the immobilization of β-gal from *Escherichia coli* on different supports and observed that the recovery activity was dependent on the immobilization support, which may be associated with the

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different levels of enzyme distortion caused by immobilization. It has also been reported [13] that the cross-linking of the support with glutaraldehyde may enhance its hydrophobicity, which may affect the enzyme catalytic activity.

This work aims to study how *Kluyveromyces* strains can be used to produce β-gal from an agro-industrial by-product such as whey powder. It also aims to investigate the immobilization of the enzyme onto chitosan and determine its properties such as the optimal operating pH and temperature, the thermal stability of the enzyme, the thermal denaturation constant, the half-life (at 40 °C, 50 °C and 60 °C) and the kinetic parameters $K_m$ and $V_{max}$ of the enzyme β-gal from *K. lactis* strain NRRL Y1564.

Chitosan is the deacetylated form of chitin, the second most abundant polymer in nature after cellulose. It is a low cost, renewable, biodegradable natural product, which has very good biocompatibility, low toxicity, chemically inert and high hydrophilicity [14,15]. This biopolymer has a similar molecular structure to cellulose, differing only in its functional groups. Hydroxyl groups (OH) are present on the general structure of these biopolymers, but the main difference between them is the presence of amino groups ($NH_2$) in the structure of chitosan. Different configurations of chitosan can be obtained in the process of deacetylation of chitin and these configurations may be used in the process of enzyme immobilization. However, hydrogels and membranes are the most used forms due to the obtained physical changes, such as increasing the pore diameter of the support, suitable for the process of immobilization of enzymes. Different protocols may be employed for the immobilization of enzymes in chitosan, such as adsorption, encapsulation and covalent bounding. Among the different methods, the covalent immobilization onto glutaraldehyde activated chitosan is the method of choice due to the presence of amino groups in the structure of chitosan that react with this agent in mild conditions close to neutrality [14].

## 2. Materials and methods

### 2.1. Microorganisms

Three *K. marxianus* strains (LAMI CE 025; CCA 510; ATCC 36907 – CCT André Tosello) and two *K. lactis* strains (NRRL Y1564 and NRRL 4087) were tested. Stock cultures were maintained at 4 °C in YPD (yeast extract, peptone and dextrose) agar plates containing 10 g/L of yeast extract, 20 g/L of peptone and 20 g/L of dextrose.

### 2.2. Materials

Powdered chitosan was purchased from Polymar Ind. Ltda (Ceará, Brazil). Bovine serum albumin (BSA), glutaraldehyde solution Grade II 25% and the synthetic substrates *o*-nitrofenil-β-D-galactopyranoside (99%, ONPG) and o-nitrophenol (99%, ONP) were purchased from Sigma–Aldrich (St. Louis, USA). Dinitrosalicylic acid (DNS) was obtained from Vetec (São Paulo, Brazil). Glicose PAP Liquiform, the enzymatic kit for glucose determination, was obtained from Labtest Diagnóstica (Minas Gerais, Brazil). Whey powder was kindly provided by Alibra Ingredientes Ltda (Campinas, Brazil). All other analytical grade reagents were purchased from Synth (São Paulo, Brazil) and Vetec (São Paulo, Brazil).

### 2.3. Pre-treatment of the whey

The whey powder was diluted in distilled water to obtain a lactose concentration of 50 g/L and heated to 90 °C. Commercial lactic acid was then added to the whey solution under mild agitation to reach pH 4.8. Agitation was then interrupted and the solution was left to sit for several minutes to settle its contents. Whey was then filtered using qualitative filter paper [16].

### 2.4. Culture medium preparation

A synthetic medium was prepared according to a previous study [17], comprising 50 g/L of lactose, 5 g/L of $KH_2PO_4$, 1.2 g/L of $(NH_4)_2SO_4$, 0.4 g/L of $MgSO_4{\cdot}7H_2O$ and 1 g/L of yeast extract in 1 L of potassium phosphate buffer (0.2 M) at pH 5.5. A complex medium, described as "deproteinized whey" in this study, was formulated by adding 1 g/L of yeast extract to the pre-treated whey, where the pH was adjusted to 6.0 using a 4 mol/L solution of KOH. Both types of culture medium were placed in 250 mL Erlenmeyer flasks and sterilized at 115 °C for 15 min.

### 2.5. Inoculum preparation

The *Kluyveromyces* strains were initially grown in YPD agar plates (containing 10 g/L yeast extract, 20 g/L of peptone, 20 g/L of dextrose, 20 g/L of agar) and incubated at 30 °C for 24 h. Three colonies were then transferred into separate 250-mL Erlenmeyer flasks containing 50 mL of synthetic medium each [17]. The flasks were incubated at 30 °C and shaken at 180 rpm for 24 h. The optical density (OD) was then determined at a wavelength of 620 nm using a spectrophotometer and OD was adjusted to 1.0.

### 2.6. Batch cultivation conditions

All assays were conducted in 250-mL Erlenmeyer flasks containing 50 mL of culture medium that were shaken on a TE240 rotary shaker (Tecnal, São Paulo, Brazil) at 180 rpm and 30 °C [17]. Experiments were initiated by inoculating the culture medium with inocula to a concentration of 10% (v/v) and then maintaining the culture at 30 °C with shaking at 180 rpm. Batch cultivations lasted 24 h, when to the selection of lactose-consuming strains, and 30 h, when studying the effect of incubation time on the batch production of β-gal by the *K. lactis* NRRL Y1564 strain cultivated in deproteinized whey. Approximately 1 mL of sample was collected from each flask to determine the cell growth, the pH and the lactose concentration. The assays were performed in duplicate for each different microorganism.

The volumetric enzyme activity in terms of U/mL is defined as the enzyme activity per unit volume of culture medium, and it was used to investigate the production kinetics of β-gal [18]. The specific enzyme activity in terms of U/g was calculated by dividing the enzymatic activity by the mass of cells harvested prior to the extraction of the enzyme [18,19].

### 2.7. Selection of β-gal-producing yeasts

A screening of lactose-utilizing among six non-pathogenic *Kluyveromyces* strains was conducted in a synthetic culture medium. The criteria used for screening and microorganism selection were cell growth and lactose uptake. The strains that demonstrated both growth and lactose uptake were then cultivated in the complex medium to select yeasts that could produce β-gal using the lactose present in whey.

### 2.8. Extraction of the enzyme β-galactosidase from the culture medium

β-Galactosidase is an intracellular enzyme and has to be released from the cells; hence, cell autolysis was performed according to a previous literature report [20] with some modifications. Approximately 20 mL of the sample was collected and centrifuged at 10,000 × *g* for 15 min at 4 °C. The cell pellets obtained from centrifugation were washed once with 20 mL of cold distilled water and centrifuged again under the same conditions. After washing, the cell pellets were re-suspended in a potassium phosphate buffer at pH 6.6 containing 0.1 mM of $MnCl_2{\cdot}4H_2O$ to obtain a cell density of 2.9 mg/mL. Ethanol and toluene were added to the buffer at concentrations of 10% (v/v) and 2% (v/v) respectively to permeabilize the cells in 125 mL Erlenmeyer flasks, which were shaken at 150 rpm for 20 min at 37 °C. The suspension of the permeabilized cells was then centrifuged at 10,000 × *g*, for 15 min at 4 °C. The cell-free supernatant containing the crude enzyme extract was then collected and refrigerated.

### 2.9. Preparation and modification of supports used in enzyme immobilization

In this work, chitosan was used as a support for enzyme immobilization [21]. Powdered chitosan was solubilized in 2% (v/v) acetic acid to a concentration of 2.0 wt.% and homogenized for 30 min before 20 mL of this solution was placed in a 100 mL-glass beaker at 50 °C and left to stabilize for several minutes under mechanical agitation. After the stabilization of the chitosan solution, 30 mL of a KOH 0.5 M solution was added to the reactor, and the reactor was then further agitated for 30 min. Support activation was conducted by adding glutaraldehyde to the solution of chitosan coagulated with KOH to a concentration of 0.8% (v/v). After 30 min of activation at 50 °C, the particles were immediately washed with distilled water at room temperature to remove excess glutaraldehyde and then dried under a vacuum.

### 2.10. The immobilization of β-gal onto glutaraldehyde-activated chitosan

The immobilization of β-gal that was produced by *K. lactis* NRRL Y1564 was carried out in a batch reactor under mild agitation at 25 °C by mixing the enzyme in a 50 mM potassium phosphate buffer at pH 7.0, containing 0.1 mM manganese chloride and the previously activated support, for different contact times between the enzyme and the support (5, 10 and 24 h). In all experiments, the initial quantity of enzyme added to the buffer was 10 mg of protein/g support. To determine the immobilization yield, samples of supernatant were withdrawn and their hydrolytic activities were measured. A blank assay was also conducted to evaluate the possibility of enzyme deactivation in the buffer while it was undergoing immobilization. Therefore, a solution of β-gal in phosphate buffer with 0.1 mM of added manganese chloride was prepared in a separate reactor as a control experiment, but there were no chitosan supports placed in that reactor.

#### 2.10.1. Immobilization yield

After measuring the initial ($At_i$) and final ($At_f$) enzyme activities in the supernatant of the immobilization suspension and in the reference suspension, the remaining enzyme activity in the supernatant ($At_i - At_f$) and the immobilization yield (IY) could be calculated according to Eq. (1) [22]. The reference suspension is a control assay that was performed in the absence of support to discard any possibility of loss of enzyme activity during immobilization.

$$IY_{imob}(\%) = \frac{At_i - At_f}{At_i} \times 100 \quad (1)$$

#### 2.10.2. Recovery activity ($At_r$)

The theoretical activity ($At_t$) of immobilized β-gal onto the chitosan support could be calculated (Eq. (2)) as a product of the enzymatic activity offered per unit mass of support ($At_{off}$) and the immobilization yield, where the immobilization yield can be obtained from Eq. (1). After measuring the activity of the immobilized enzyme per unit mass of support ($At_d$), the recovery activity ($At_r$) could be calculated according to Eq. (3).

$$At_t = IY \times At_{off} \quad (2)$$

$$At_r = \frac{At_d}{At_t} \times 100 \quad (3)$$

### 2.11. The optimal operating pH and temperature of β-gal produced by the K. lactis NRRL Y1564 strain

The effects of pH and temperature on the activities of soluble and immobilized β-gal produced by the *K. lactis* NRRL Y1564 strain were evaluated. Samples were incubated at a pH ranging between 5.5 and 8.0 and a constant temperature of 37 °C to evaluate the effect of pH on enzymatic activity. The effect of temperature on enzymatic activity was determined at a constant pH of 6.6 [23] in a temperature range of 25 and 60 °C. In this work, the optimal operating pH and temperature refers to the maximum enzymatic activity.

### 2.12. An evaluation of the thermal stabilities of soluble and immobilized β-gal produced by the K. lactis NRRL Y1564 strain

Soluble and immobilized β-gal was placed in separate 50 mM sodium phosphate buffer solutions at pH 8.6 and kept at 3 different temperatures of 40, 50 and 60 °C for more than 4 h. Samples were periodically withdrawn and their residual activities were evaluated by analyzing their ability to hydrolyze ONPG. A first order deactivation model [24] was fitted to the experimental data using Origin 8.1 software (OriginLab Corporation, Northampton, USA). The knowledge of the exponential decay model and its parameters allows for the determination of the deactivation constants ($K_d$) and the half-lives ($t_{1/2}$) of the biocatalysts. Stabilization factors (SF) were obtained as the ratio between the half-lives of the immobilized β-gal and the soluble β-gal, see Eq. (4). The deactivation energy was estimated based on the Arrhenius equation [25].

$$SF = \frac{t_{1/2\,immobilized}}{t_{1/2\,soluble}} \quad (4)$$

### 2.13. Calculation of cultivation parameters

The specific cell growth rates ($\mu_X$), lactose uptake rate ($\mu_S$), and the specific product formation rates ($\mu_P$) were estimated according to the methodology described by Schmidell et al. [26] according to Eqs. (5) and (6). The relative magnitudes of these parameters provide an important classification of the fermentation process, where product formation may be associated, partly linked, or not linked at all to cell growth.

$$\mu_X = \frac{1}{x}\frac{dX}{dt} \quad (5)$$

$$\mu_P = \frac{1}{x}\frac{dP}{dt} \quad (6)$$

The volumetric productivity was calculated as the ratio of the component concentration to the fermentation time ($t$), see Eqs. (7) and (8):

$$P_X = \frac{X}{t} \quad (7)$$

$$P_1 = \frac{C_1}{t} \quad (8)$$

where, $P_X$ is the volumetric productivity of biomass, $P_1$ is the volumetric productivity of β-gal, $X$ is biomass concentration and $C_1$ is volumetric enzyme activity.

The substrate conversion, which in the case of this study was lactose, was calculated by Fogler [27] according to Eq. (9):

$$\Delta S(\%) = \frac{S_0 - S}{S_0} \times 100 \quad (9)$$

where $S_0$ refers to the initial substrate concentration and $S$ refers to the substrate concentration in the samples taken for analysis at each time interval.

### 2.14. Determination of the kinetic parameters in the hydrolysis of o-nitrofenyl-β-D-galactopyranoside

The kinetic parameters of ONPG hydrolysis were determined in the range of 0.1–7.5 mM in 50 mM potassium phosphate buffer containing 0.1 mM $MnCl_2{\cdot}4H_2O$ at 37 °C. The Michaelis–Menten constant ($K_m$) and the maximum reaction velocity ($V_{max}$) were calculated by fitting a non-linear Michaelis–Menten model to the experimental data using Origin 8.1 software. The catalytic efficiency of the immobilized enzyme was calculated based on a previous report [28], but with slight modifications:

$$\text{catalytic efficiency}(\%) = \frac{(V_{max}/K_m)_{Immob}}{(V_{max}/K_m)_{soluble}} \times \frac{[E_0]_{Immob}}{[E_0]_{soluble}} \times 100 \quad (10)$$

where $E_0$ refers to the initial enzyme activity.

### 2.15. The estimation of thermodynamic parameters

To determine the thermodynamic parameters of the immobilized enzyme, the enthalpy of inactivation ($\Delta H^*$), the Gibbs free energy of inactivation and the entropy of inactivation ($\Delta S^*$) were calculated according to Ustok et al. [25].

### 2.16. Reuse of the immobilized β-gal

The reuse of the immobilized enzyme was assayed by using 1 g of the biocatalyst in successive batches of ONPG hydrolysis. The assay conditions were the same as those described in Section 2.19.2. At the end of each batch, the support was removed from the reaction medium, washed with phosphate buffer to remove any remaining substrate or product, dried under a vacuum and subjected to a fresh batch of ONPG for hydrolysis. The residual activity was calculated as the activity of the immobilized enzyme measured after each cycle relative to the activity of the immobilized enzyme during the first cycle.

### 2.17. Storage stability of the immobilized β-gal

Biocatalyst samples were also stored at 4 °C in a 50 mM potassium phosphate buffer at pH 7.0, and their residual hydrolytic activities were determined after 93 days of storage to evaluate the storage stability of the soluble and immobilized enzyme.

### 2.18. Lactose hydrolysis by the soluble and immobilized β-gal

The hydrolysis of lactose by the soluble and immobilized β-gal was performed based on previous reports [12,28], but with slight modifications. Lactose hydrolysis was performed using pure lactose at 2% in 50 mM potassium phosphate buffer solution, pH 7.0, containing 0.1 mM $MnCl_2{\cdot}4H_2O$. The reaction was conducted in a 100 mL-glass beaker at 40 °C for 360 min under orbital agitation (150 rpm). Experiments were initiated by contacting 50 mL of lactose solution with 1 g or 1.3 mL of immobilized (3.8 U/g) or soluble enzyme (2.9 U/mL), respectively. Samples of supernatant were withdrawn and glucose release was measured using an enzymatic kit (Glicose PAP Liquiform) for glucose determination. The assays were performed in triplicate for each different biocatalyst.

### 2.19. Analytical methods

#### 2.19.1. Biomass concentration

Biomass concentration was determined by measuring the optical density of the samples (O.D.) at 620 nm using a Spectronic spectrophotometer (Genesys, Series 20, USA) and converted into concentration (g/L) according to the standard curve that was developed for the specific microorganism.

#### 2.19.2. Enzyme activity

The hydrolytic activity of soluble and immobilized β-gal was determined using *o*-nitrophenyl-β-D-galactopyranoside as a substrate at pH 6.6 and 37 °C, according to the methodology described in literature previous report [20] but with some modifications. A 50 μL enzyme sample was added to a test tube containing a 2 mL solution of 1.25 mM of ONPG, mixed into a 50 mM potassium phosphate buffer solution containing 0.1 mM $MnCl_2{\cdot}4H_2O$, and maintained at 37 °C for 5 min. The immobilized enzyme activity was measured by adding 0.05 g of immobilized enzyme to the test tube. The reaction was stopped and 0.5 mL of 1 M sodium carbonate was added. The *o*-nitrophenol product concentration was measured at a UV wavelength of 420 nm. One unit (U) of β-gal is defined as the quantity of enzyme that liberates 1 μmol of *o*-nitrophenol/min under the test conditions.

#### 2.19.3. Lactose and ethanol concentrations

Lactose and ethanol concentrations were measured using high performance liquid chromatography (Waters, Milford, MA, USA), with a Waters refractive index detector (Model 2414) and an Aminex HPX-87H column at 65 °C. The eluent was 5 mM $H_2SO_4$, and it was pumped through the column at a flow rate of 0.5 mL/min. The injection volume of the samples was 20 μL and the analysis time for each sample was 30 min.

**Table 1**
The cell growth and the lactose uptake of the different strains of *Kluyveromyces* cultivated in the synthetic medium containing 10 g/L of lactose at 30 °C and an agitation of 180 rpm after 24 h.

| Microorganism | Cell growth | Lactose consumption |
|---|---|---|
| *K. marxianus* CE 025 | + | – |
| *K. marxianus* CCA 510 | + | – |
| *K. marxianus* ATCC 36907 | + | + |
| *K. lactis* NRRL Y1564 | + | + |
| *K. lactis* NRRL Y4087 | + | + |

Response: (+) positive (cell growth or lactose uptake); (–), negative (no measurable cell growth or lactose uptake).

*2.19.4. Reducing sugars concentration*

The concentration of the total reducing sugars was determined colorimetrically by the dinitrosalicylic acid (DNS) method [29]. An appropriate calibration curve was used to convert absorbance to concentration.

*2.19.5. Protein concentration*

The protein concentration was determined according to the Bradford assay [30] at a wavelength of 595 nm using bovine serum albumin (BSA) as the standard.

## 3. Results and discussion

### 3.1. Selection of lactose-consuming strains

Literature reports [17,31] have shown that yeasts such as *K. marxianus* and *K. lactis* produce β-gal when lactose is used as the carbon source for cultivation. Therefore, *Kluyveromyces* strains were screened for their ability to uptake lactose, where five strains were grown in a synthetic medium containing 10 g/L of lactose as shown in Table 1.

All strains were able to grow in the synthetic medium as shown in Table 1. Nevertheless, only three of the five strains evaluated exhibited lactose uptake, namely the *K. marxianus* ATCC 36907 strain, the *K. lactis* NRRL Y1564 strain, and the *K. lactis* NRRL Y4087 strain. These three strains were selected to assess their β-gal production capabilities when grown on whey. The *K. marxianus* CE 025 and CCA 510 strains showed no lactose uptake and were therefore excluded from this study.

### 3.2. The selection of β-gal-producing yeasts from deproteinized whey

Table 2 shows the cell growth, the lactose uptake and the enzyme activity of β-gal after 24 h of cultivating the *K. lactis* NRRL Y1564 strain, the *K. lactis* NRRL Y4087 strain, and the *K. marxianus* ATCC 36907 strain in a deproteinized whey medium. The *K. lactis* NRRL Y1564 strain showed the highest cell growth, consuming approximately 2.2 times more lactose than the *K. lactis* NRRL Y4087 strain. However, the *K. marxianus* ATCC 36907 strain showed practically no lactose uptake when cultivated in the whey medium.

Of the yeasts used in this study, the *K. lactis* NRRL Y1564 strain was selected because it had a volumetric enzyme activity that was 72.6% greater than the enzyme produced by the *K. lactis* NRRL Y4087 strain and 67.1% more activity than the enzyme produced by the *K. marxianus* ATCC 36907 strain.

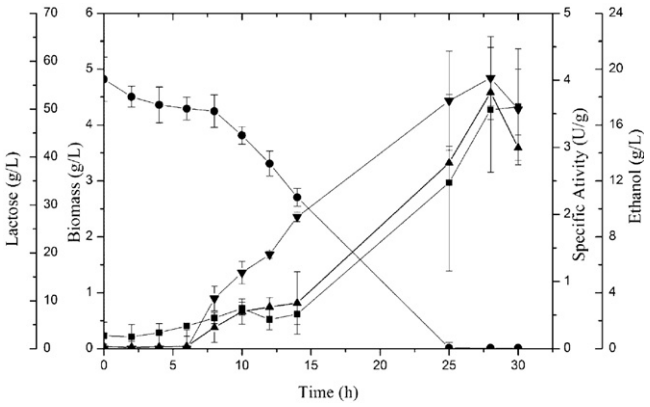


**Fig. 1.** Cultivation of a *K. lactis* NRRL Y1564 batch culture in deproteinized whey at 30 °C and 180 rpm as a function of time: (■) biomass (g/L); (●) lactose (g/L); (▲) enzyme activity (U/mL) and (▼) ethanol concentration (g/L).

Other authors [20] cultivated various strains of *K. lactis* and *K. marxianus* in a medium containing 10 g/L lactose as a carbon source. The values that they obtained for enzyme activity ranged between 0.7 and 1.3 U/mL for *K. lactis* and ranged between 0.7 and 1.0 U/mL for *K. marxianus*. Braga et al. [31] studied seven strains of *Kluyveromyces* and found that the *K. marxianus* ATCC 16045 strain was the best producer of β-gal in culture medium containing agro-industrial waste materials such as whey and water from parboiled rice (left over). The highest enzymatic activity achieved was 10.4 U/mL when whey was used as a culture medium, with concentrations of 120 g/L lactose, 5 g/L yeast extract, 15 g/L peptone, 15 g/L $(NH_4)_2SO_4$ and 30 g/L water that was left over from parboiled rice at pH 4.0.

### 3.3. The effect of incubation time on the batch production of β-gal by the K. lactis NRRL Y1564 strain cultivated in deproteinized whey

Fig. 1 shows the cell growth profile, the lactose uptake, the volumetric enzyme activity, and the ethanol concentration when the *K. lactis* NRRL Y1564 strain was cultivated in deproteinized whey that was supplemented with yeast extract. A maximum enzyme activity of 3.8 ± 0.2 U/mL and a maximum volumetric productivity of 0.14 U/mL/h was obtained after 28 h of cultivation. Other authors [18] also observed a peak in the enzyme activity and productivity when β-gal was produced from the *K. marxianus* CCT 7082 strain. The highest concentration of biomass was 4.3 g/L, which was achieved after 30 h of cultivation when the lactose was completely consumed. On the other hand, the maximum biomass productivity of approximately 0.035 g/L/h was achieved after 12–14 h of cultivation. During the same period, approximately 100% of the substrate was consumed, indicating that the final lactose concentration in the medium was less than 1 g/L. Ethanol was also produced as a result of cell cultivation, reaching a concentration of 16.9 ± 3.5 g/L after

**Table 2**
The cell growth, the lactose uptake and the production of β-gal by *K. lactis* NRRL Y1564, *K. lactis* NRRL Y4087 and *K. marxianus* ATCC 36907 strains cultivated in deproteinized whey at 30 °C and an agitation of 180 rpm for 24 h. Volumetric enzyme activity was defined as the hydrolytic β-gal activity/mL of culture medium while specific enzyme activity was calculated by dividing the hydrolytic activity by the amount of cell harvested before enzyme extraction. The initial lactose concentration ($S_0$) was 50 g/L.

| Microorganism | Initial biomass (g/L) | Final biomass (g/L) | Final lactose concentration (g/L) | Volumetric enzyme activity[a] (U/mL) | Specific enzyme activity[a] (U/g) |
|---|---|---|---|---|---|
| *K. lactis* NRRL Y1564 | 0.27 | 2.97 | 11.50 | 0.75 | 251.72 |
| *K. lactis* NRRL Y4087 | 0.27 | 1.35 | 20.05 | 0.09 | 68.96 |
| *K. marxianus* ATCC 36907 | 0.16 | 1.97 | 50.00 | 0.33 | 168.96 |

[a] Enzyme activity was determined at room temperature (27 °C).

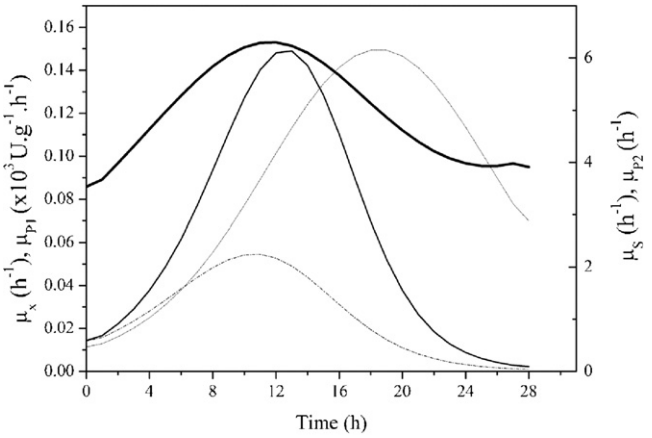


**Fig. 2.** Specific growth ($\mu_X$), lactose uptake rate ($\mu_S$), β-gal production ($\mu_{P1}$) and ethanol production ($\mu_{P2}$) rates by a *K. lactis* NRRL Y1564 strain cultivated in deproteinized whey at 30 °C and 180 rpm: (—) $\mu_X$ ($h^{-1}$); (▬▬) $\mu_S$ ($h^{-1}$); (-·-·-) $\mu_{P1}$ (U/g/h) and (----) $\mu_{P2}$ ($h^{-1}$).

25 h of cultivation. Other authors [32] have studied the production of β-gal by *K. lactis* and observed that the volumetric enzymatic activity increased during the exponential growth phase. They also observed the production of ethanol and mentioned that it is always a fermentation product when lactose is metabolized by *K. lactis*.

It can be observed through a comparison of cell growth and enzyme production as shown in Fig. 1 that β-gal production started early in the exponential phase and ran parallel to cell growth kinetics during that phase for up to 28 h, indicating that it was associated with cell growth. Furthermore, the similarity between the $\mu_X$ and the $\mu_P$ curves as shown in Fig. 2 suggests that ethanol is a primary metabolite produced by this strain when lactose is fermented [33]. According to Gaden [34], processes that show only one maximum for the specific rate curves, where the curves are virtually coincident, are usually known as growth-associated processes. It is therefore possible to say that the synthesis of β-gal by the *K. lactis* NRRL Y1564 strain in this experiment is associated with populational growth when cultivated in a medium containing lactose as the sole carbon source. Various studies [20,35,36] performed on enzyme production have made a common observation that the highest enzyme activity per unit of cell concentration is achieved at the beginning of the stationary phase and declines thereafter, even though lactose is still present in the medium.

Other authors [17,20] working with strains of *K. lactis* (NRRL Y 1564 and NRRL Y 8279) grown on a synthetic medium containing lactose, observed that the peaks in enzymatic activity were obtained after 24 h of fermentation with values ranging between 0.5 and 1.5 U/mL, which are lower than the 3.5 U/mL that has been obtained in this present work after 28 h of fermentation. Matheus and Rivas [37] optimized the β-gal production by *K. lactis* using deproteinized whey as a fermentation medium, which contained 4.85% lactose supplemented with 0.15% yeast extract and 0.1% $(NH_4)_2SO_4$. The enzyme activity varied between 1.29 and 7.43 U/mL, depending on the operational conditions.

### 3.4. Immobilization of β-gal onto chitosan activated with glutaraldehyde

The immobilization of β-gal onto chitosan activated with glutaraldehyde was investigated in this work, with the aim of obtaining a biocatalyst possessing operational and thermal stabilities. Table 3 shows the results of the effect of contact time between the enzyme and the support on the derivative activity ($At_d$), the recovery activity ($At_r$), and the immobilization yield (IY).

**Table 3**
Immobilization parameters of β-gal on chitosan activated with glutaraldehyde: derivative ($At_d$) and recovery activity ($At_r$), immobilization yield (IY), enzymatic activities of the soluble enzyme in the supernatant before ($At_0$) and after ($At_f$) immobilization. Immobilization conditions: 9 mL of enzyme solution mixed with 50 mM potassium phosphate buffer containing 0.1 mM manganese chloride at pH 7.0 and mixed with 1 g of support at 25 °C. Enzyme load: 10 mg protein/g support.

| Contact time (h) | $At_0$ (U/g) | $At_f$ (U/g) | $At_d$ (U/g) | $At_r$ (%) | IY (%) |
|---|---|---|---|---|---|
| 5 | 6.40 | 2.26 | 0.32 | 7.7 | 64.7 |
| 10 | 6.15 | 1.13 | 0.62 | 12.4 | 81.6 |
| 24 | 5.39 | 0.03 | 0.53 | 9.9 | 99.5 |

No expressive loss of enzyme activity was observed in the reference suspension during immobilization.

Table 3 shows that higher immobilization yields were achieved with an increased contact time. These results indicate that the first bonds between enzyme molecules and activated support are built up quickly. Nevertheless, the next bonds take more time to be formed since the protein itself becomes a barrier to enzyme molecules diffusion [38]. Thus, longer contact times are needed to overcome the intra-particle diffusion effects, caused by the presence of the enzyme inside the pores, and promote more enzyme molecules to link to the support.

The $At_d$ and the $At_r$ were observed to increase for 10 h (see Table 3). After 10 h, there was no further increase in enzyme and recovery activity despite the increased enzyme load on the support. Therefore, a contact time of 10 h was selected as the optimal contact time for further immobilization assays.

Afterwards, soluble and immobilized β-gal samples were stored in potassium phosphate buffer at a pH 7.0 and a temperature 4 °C for more than 3 months. The immobilized enzyme retained 100% of its initial activity after being stored for 93 days. In contrast, the soluble β-gal experienced a 9.4% reduction in its activity when stored under the same conditions for the same period of time. This is in accordance with the results that have been published in the literature [8,15,39,40]. Dwevedi and Kayastha [15] immobilized β-gal obtained from peas onto chitosan and observed no activity loss of the immobilized enzyme for up to 40 days. Ansari and Husain [39] immobilized β-gal obtained from *Aspergillus oryzae* onto a support constructed out of concanavalin A-Celite 545 and observed that the enzyme retained 78% of its initial activity after 2 months of storage while its soluble enzyme counterpart retained only 40% of its initial activity. Bayramoglu et al. [40] reported that β-gal immobilized on magnetic poly-(glycidyl methacrylate ethylene dimethyl methacrylate) beads retained 59% activity after 2 months of storage, while the free enzyme experienced a complete loss of activity after 5 weeks of storage. Other authors [8] stored the soluble and the immobilized β-gal from *K. lactis* at 5 °C in a Tris–HCl buffer. They observed that the soluble enzyme retained 46% of its activity after seven days, while the immobilized enzyme retained 60% after the same period of storage time. After 14 days, soluble enzyme lost more than 80% of its initial activity, while the immobilized enzyme only lost 59% of its initial activity.

### 3.5. The effects of temperature and pH on the activity of soluble and immobilized β-gal

The hydrolysis of ONPG was used as a model reaction to study the effects of temperature and pH on the properties of soluble and immobilized β-gal. The effect of temperature was investigated at temperatures ranging from 25 to 60 °C as shown in Fig. 3, at a constant pH of 6.6. The immobilized enzyme possessed a higher activity at a temperature range of 30–37 °C in comparison with the soluble enzyme. However, the immobilized enzyme exhibited a lower activity at temperatures above 45 °C. Furthermore, while the optimal operating temperature for the soluble enzyme was 50 °C, the

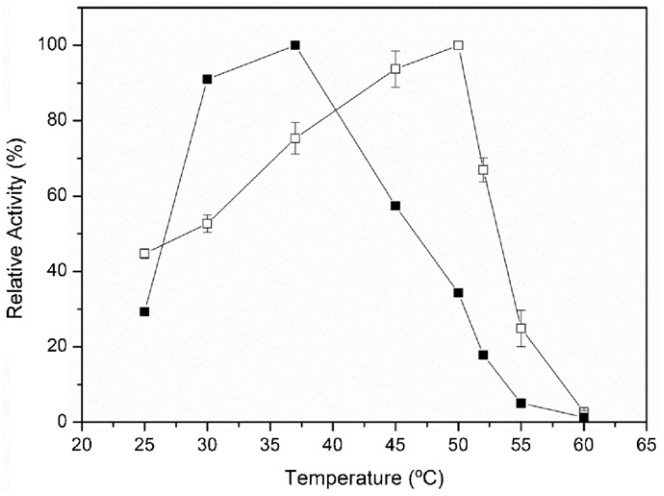


**Fig. 3.** Temperature effects on the hydrolytic activity of soluble (□) and immobilized (■) β-gal when hydrolyzing a 1.25 mM ONPG solution in 50 mM of potassium phosphate buffer supplemented with 0.1 mM $MnCl_2$ at pH 6.6.

optimal operating temperature for the immobilized enzyme was only 37 °C. This result is comparable to what has been published in a different study [8], where the immobilized enzyme is better at maintaining its activity at lower temperatures but loses it faster when subjected to temperatures above 37 °C in comparison with the soluble enzyme. The change in the optimal temperature toward a lower value is unexpected, but this change has also been observed by other authors [8,41,42]. One possible explanation is that the impurities contained in the crude extract (soluble enzyme) may stabilize the enzyme molecule, protecting it against thermal inactivation. Those impurities may have been removed from the nearby of the enzyme after immobilization, probably reducing enzyme stability.

Fig. 4 shows the pH dependence of the immobilized enzyme activity at a constant temperature of 37 °C in comparison with the activity of the soluble enzyme. The soluble enzyme exhibited a low activity at the pH of approximately 5.5. The immobilized enzyme, on the other hand, retained 60% of its activity at the same pH, indicating that the immobilization helped to increase the stability of the enzyme. The higher stability of the immobilized β-gal may be explained by the new microenvironment provided by the chitosan

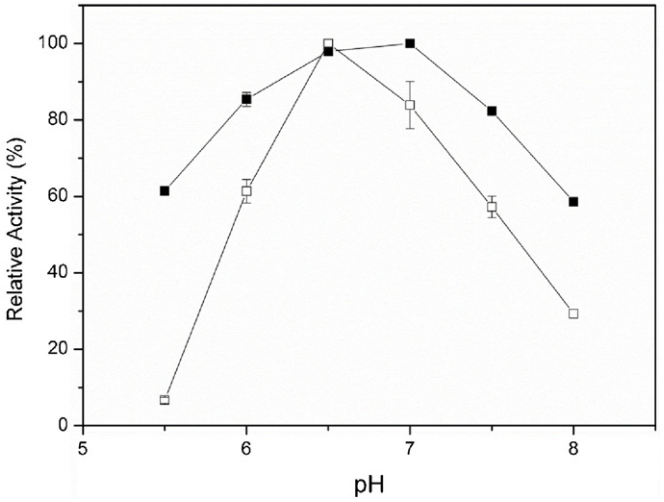


**Fig. 4.** pH effects on the on hydrolytic activity of soluble (□) and immobilized (■) β-gal at 37 °C when hydrolyzing a 1.25 mM ONPG solution in 50 mM of potassium phosphate buffer supplemented with 0.1 mM $MnCl_2$ at pH 6.6.

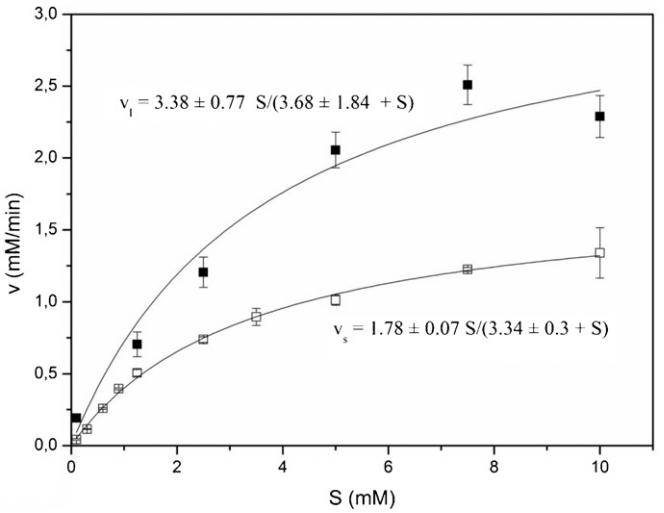


**Fig. 5.** Influence of substrate concentration on the initial rates of ONPG hydrolysis by (□) soluble and (■) immobilized β-gal from *K. lactis* at 37 °C. The lines represent the fitting of the non-linear Michaelis–Menten model to the experimental data. $v_s$ is the rate of ONPG hydrolysis catalyzed by soluble enzyme and $v_I$ is the rate of ONPG hydrolysis catalyzed by the immobilized enzyme.

matrix. At high $H^+$ concentrations, the amino groups of chitosan are protonated, attracting $OH^-$ ions, resulting in a higher local pH in comparison with the bulk solution [43]. Furthermore, immobilized enzyme has a broader optimum pH range when compared to the soluble enzyme. In the case of the soluble enzyme, the maximum activity was achieved at pH 6.5, while the immobilized enzyme exhibited a plateau of maximum activity from pH 6.5 to pH 7.0. The results from the temperature and pH changes on the enzymatic activity, suggests that the immobilized enzyme possesses a maximum catalytic activity at a lower temperature in comparison with the soluble enzyme, and it is believed that this change in the optimum temperature was neither promoted by interactions between the enzyme and the support nor any conformational changes of the enzyme molecules. However, it is highly possible that the microenvironment in the vicinity of the support may be very different from the bulk solution environment, which may explain the pH stabilization observed for the immobilized enzyme. Immobilization may promote the stability of enzymes against extreme reaction conditions that are deleterious to soluble enzymes [44].

### 3.6. The influence of immobilization on ONPG hydrolysis kinetics

$K_m$ and $V_{max}$ were estimated by using a non-linear regression of the Michaelis–Menten equation to fit the experimental data obtained for the initial rates of ONPG hydrolysis. The results obtained for soluble and immobilized β-gal are shown in Fig. 5. For the soluble enzyme, $K_m$ was approximately 3.34 ± 0.3 mM and $V_{max}$ was approximately 1.78 ± 0.07 mM/min with $R^2$ value of 0.997. For the immobilized enzyme, $K_m$ was approximately 3.68 ± 1.84 mM and $V_{max}$ was approximately 3.38 ± 0.77 mM/min with an $R^2$ value of 0.95. It can be observed that the $K_m$ values for the immobilized enzyme and the soluble enzyme are similar when taking into account experimental errors, indicating that immobilization did not reduce any substrate access to the enzyme and that there are no apparent diffusion limitations. The maximum velocity of the immobilized β-gal was double that of the soluble enzyme, although enzyme loads in the assays were similar, where there was 0.04 U of soluble enzyme and 0.03 U of immobilized enzyme. The catalytic efficiency of the immobilized enzyme in this work was approximately 130% that of the soluble enzyme. Some authors [8,45,46] have discussed the effect of immobilization on enzymes and have

concluded that the impact is not always negative. For instance, Goto et al. [46] observed that the immobilization of β-glucosidase caused a decrease in the $K_m$ value, but the $V_{max}$ value was increased.

The kinetic parameters estimated in this work are similar to those that have been reported by other researchers. Zhou and Chen [23] determined the $K_m$ and $V_{max}$ for β-gal from the *K. lactis* Maxilact LX 5000 strain in soluble form and when it was immobilized on graphite. For the soluble β-gal, $K_m$ was 1.74 mM and $V_{max}$ was 77.45 mmol ONP/min mg, while they were $K_m$ was 9.34 mM and $V_{max}$ was $8.75 \times 10^{-3}$ mmol ONP/min mg for the immobilized enzyme. The authors noted that an increase in $K_m$ is related to the difficulty experienced by the substrate when coming into contact with the enzyme, which was not observed in this work.

### 3.7. Thermal stability and estimation of inactivation energy

The thermal stability of β-gal was studied at temperatures of 40, 50 and 60 °C, and the results can be observed in Fig. 6. For each thermal stability assay, the thermal deactivation constant, the half-life ($t_{1/2}$) and the stabilization factor were determined as shown in Table 4.

In Fig. 6(A), it can be observed that the soluble and immobilized enzyme showed similar deactivation profiles at 40 °C. Both biocatalysts showed the same stability, retaining approximately 50% of their initial activities after 2 h of exposition. However, the immobilized enzyme showed an increased stability at 50 °C (Fig. 6(B)). The soluble enzyme lost all its activity even before it had been incubated for 50 min, while the immobilized β-gal still retained more than 70% of its initial activity. The soluble and immobilized enzymes showed a similar deactivation profile at 60 °C, where both types of enzymes lost all their activities before 20 min of incubation, as shown in Fig. 6(C).

A low stabilization factor (SF) was achieved at 40 °C as shown in Table 4, since half-lives of soluble and immobilized enzyme were very close, 203.87 and 223.60 min, respectively. Nevertheless, a higher SF was observed when the incubation temperature was increased. The immobilized enzyme showed the highest stabilization factor at 50 °C, being approximately 8.3 times more stable than the soluble enzyme. These results reinforce the idea that the immobilized β-gal may be stabilized better in the new microenvironment provided by the chitosan matrix, as has been discussed previously. Although immobilized enzyme was approximately 5.6 times more stable than the soluble enzyme at 60 °C, half-lives were reduced when the enzyme was exposed to drastic temperatures. For the immobilized enzyme, for instance, it decreased from 223.6 to 1.6 min, which means it is suffers a rapid deactivation at 60 °C.

According to the literature [25], the estimation of the deactivation thermodynamic parameters provides important information on the probable mechanism of enzyme denaturation. In this work, the deactivation energy calculated by the Arrhenius equation between the temperatures of 40–60 °C for soluble β-gal and immobilized β-gal were 285.7 kJ/mol and 213.6 kJ/mol, respectively. These values are in the range that has been estimated for many microbial enzymes (167–293 kJ/mol) [24].

Table 5 presents the changes in enthalpy ($\Delta H^*$) and entropy ($\Delta S^*$) calculated using the transition state theory for the thermal deactivation of the soluble β-gal and the immobilized β-gal at a temperature range of 40–60 °C. The thermodynamic parameter values estimated in this work are of the same order of magnitude as the values that have been previously reported [25]. It can be observed that $\Delta H^*$ slightly decreases with an increase in the temperature while $\Delta S^*$ remains constant. Furthermore, the $\Delta S^*$ values are close to zero, suggesting that thermal deactivation did not cause significant changes to the tertiary structure of the enzyme [25].

According to another study [47], a decrease in the $\Delta S^*$ and the $\Delta H^*$ values indicates the thermostabilization of the enzyme. By comparing the $\Delta S^*$ and the $\Delta H^*$ values of the soluble and immobilized enzymes as presented in Table 5, it can be observed that immobilization was able to promote the thermal stability of β-gal because the $\Delta S^*$ and the $\Delta H^*$ values are shown to decrease upon immobilization. The authors explain that the decrease in $\Delta S^*$ may be promoted by decreasing the flexibility of an external loop, thereby stabilizing the modified enzyme [47].

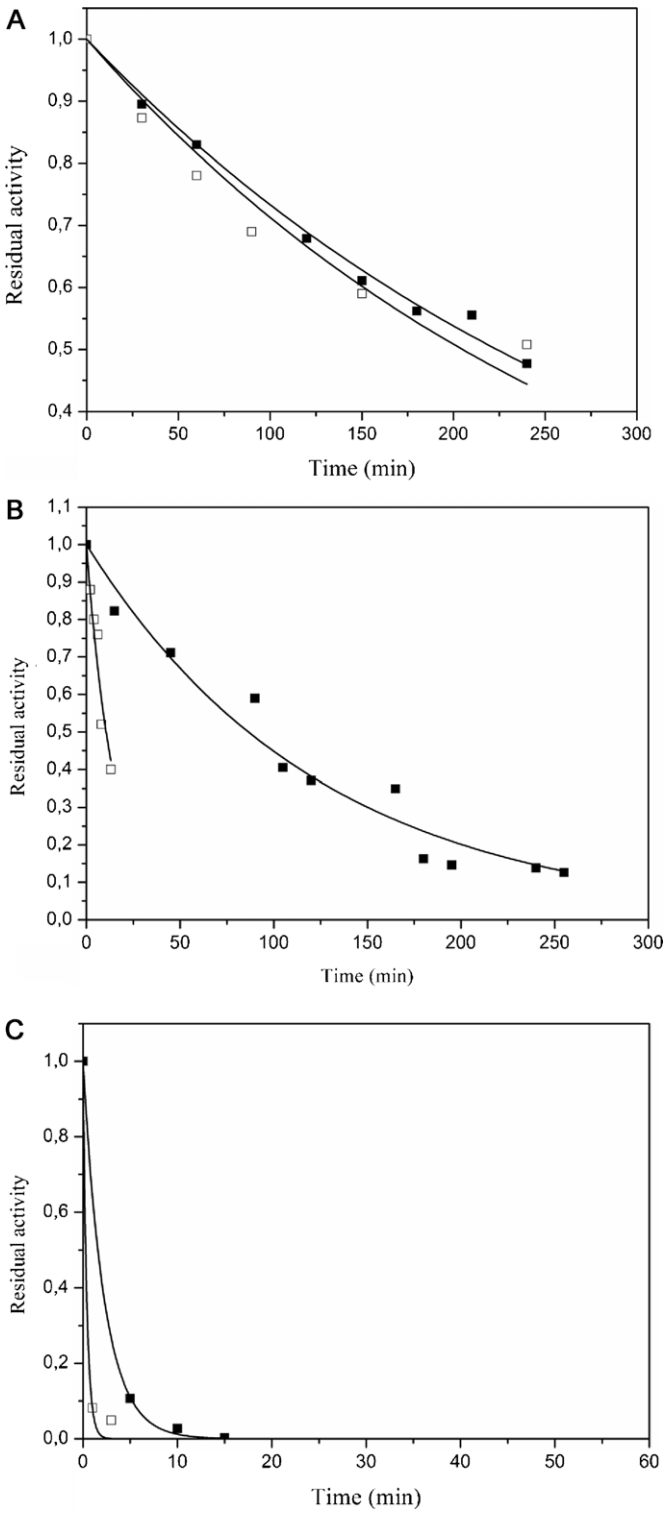


**Fig. 6.** Thermal deactivation profiles for soluble (□) and immobilized (■) β-gal at pH 6.6 at temperatures of (a) 40 °C, (b) 50 °C and (c) 60 °C. The lines represent the trend of the first-order deactivation model.

**Table 4**
Experimental values for the thermal deactivation constant ($K_d$), half-life ($t_{1/2}$) and the Stabilization Factor (SF) of soluble and immobilized β-gal.

| Temperature | β-Galactosidase | 1st-order model | | $t_{1/2}$ (min) | (SF) |
|---|---|---|---|---|---|
| | | $K_d$ | $R^2$ | | |
| 40 °C | Soluble | 0.0034 ± 0.000 | 0.9474 | 203.9 | 1.1 |
| | Immobilized | 0.0031 ± 0.000 | 0.9921 | 223.6 | |
| 50 °C | Soluble | 0.0661 ± 0.006 | 0.9447 | 10.5 | 8.3 |
| | Immobilized | 0.0080 ± 0.001 | 0.9650 | 86.6 | |
| 60 °C | Soluble | 2.4906 ± 0.412 | 0.9961 | 0.3 | 5.6 |
| | Immobilized | 0.4414 ± 0.015 | 0.9997 | 1.6 | |

**Table 5**
Thermodynamic parameter values of thermal inactivation of soluble and immobilized β-gal at 40–60 °C.

| $T$ (°C) | $\Delta H$ (kJ/mol) | | $\Delta G$ (kJ/mol) | | $\Delta S$ (kJ/mol/K) | |
|---|---|---|---|---|---|---|
| | Soluble | Immobilized | Soluble | Immobilized | Soluble | Immobilized |
| 40 | 283.09 | 211.00 | 91.62 | 91.86 | 0.61 | 0.38 |
| 50 | 283.01 | 210.92 | 86.66 | 92.33 | 0.61 | 0.37 |
| 60 | 282.92 | 210.84 | 79.37 | 84.16 | 0.61 | 0.38 |

### 3.8. Reuse of the immobilized β-gal

Fig. 7 shows a reuse study of the immobilized β-gal, using the subsequent hydrolysis of ONPG as a model reaction. In this work, the immobilized enzyme was reused for 10 cycles with a good stability as more than 70% of the initial activity was retained after the tenth cycle. In this work, one cycle is defined as a batch of ONPG hydrolysis at 37 °C and pH 7.3, lasting 5 min. The result achieved may be considered satisfactory in comparison with the results obtained by other researchers [48–50]. Verma et al. [48] immobilized β-gal from *K. lactis* on functionalized silicon dioxide nanoparticles and used the same model reaction to evaluate the immobilized enzyme at 40 °C over 11 cycles, where the biocatalyst retained approximately half of its initial activity at the end of the last cycle. Tardioli et al. [49] observed that the immobilized enzyme had a higher stability than the soluble enzyme. After four cycles of lactose hydrolysis lasting 20 min each at 40 °C and pH 7, the immobilized enzyme retained 83% of its initial activity. Other authors [50] used composite microspheres of tamarind gum and chitosan as a support but only retained 53% of its initial activity after 9 cycles of ONPG hydrolysis at 37 °C and pH 7.3.

### 3.9. Lactose hydrolysis by the soluble and immobilized β-gal

Fig. 8 shows the hydrolysis of lactose catalyzed by the soluble and immobilized β-gal performed in a batch reactor and at 40 °C. Soluble enzyme hydrolyzed lactose linearly until it reaches a value of 71% at 210 min. After that, the reaction slowed down until almost termination, reaching a conversion higher than 80% after 330 min. This result shows the capability of β-gal from *K. lactis* NRRL Y1564 to catalyze the lactose hydrolysis into galactose and glucose. When immobilized enzyme was used, a higher lactose conversion (79%) was achieved at the beginning of the assay (up to 210 min). Afterwards, the hydrolysis of lactose catalyzed by the immobilized β-gal also showed a slow down and lactose conversion reached a value over 80%, which was similar to the value achieved when using the soluble enzyme. The improvement of the reaction course when lactose hydrolysis was catalyzed by the immobilized β-gal was also observed by other authors [12]. They noticed that the immobilization of a thermophilic β-galactosidase on Sepabeads supports decreased product inhibition. They attributed the improvement of the reaction courses to higher inhibition constants of the immobilized enzyme.

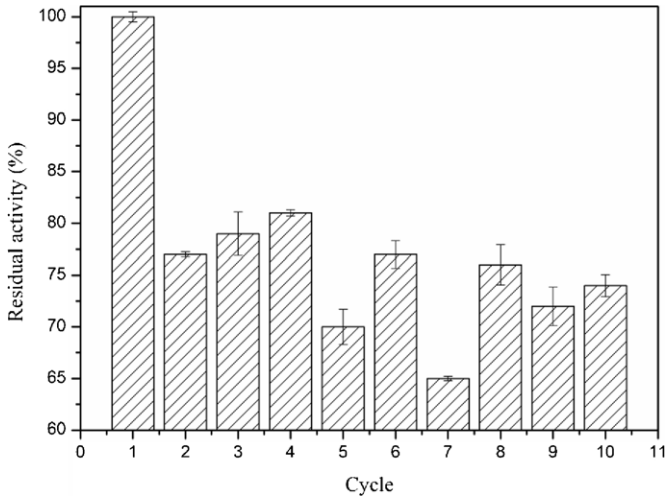


**Fig. 7.** Reuse and stability of immobilized β-gal when hydrolyzing ONPG at 37 °C.

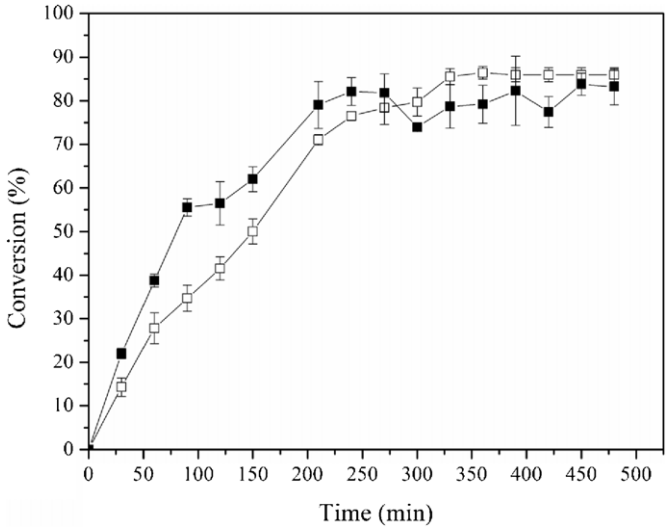


**Fig. 8.** Time course of lactose hydrolysis catalyzed by soluble (□) and immobilized (■) β-gal.

## 4. Conclusions

In this work, a culture medium prepared by using deproteinized whey supplemented with yeast extract was efficiently used for the production of β-gal through the cultivation of *Kluyveromyces* strains. Among the strains evaluated, the *K. lactis* NRRL Y1564 strain was selected because it showed the highest volumetric enzyme activity. Furthermore, the kinetic results suggest that the synthesis of β-gal by *K. lactis* NRRL Y1564 is associated with cell growth. Chitosan activated with glutaraldehyde is a suitable alternative low cost support for β-gal immobilization, providing the immobilized enzyme with higher thermal, operational and storage stabilities in comparison with the soluble enzyme. After immobilization, the optimal temperature was lowered and a broader optimal pH operating range was observed in comparison with the soluble enzyme. The kinetics of ONPG hydrolysis catalyzed by soluble and immobilized β-gal suggests that there is no conformational change experienced by the enzyme during immobilization. The higher stability of immobilized β-gal was most likely a result of the new microenvironment provided by the chitosan matrix. Last but not least, the immobilization of β-gal not only to improved enzyme stability, but also improved the performance of the biocatalyst in the hydrolysis of lactose, probably by altering the kinetic parameters of the enzyme (inhibition constants).

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