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CHEMICAL SCIENCES

Recovery of β-galactosidase produced by *Kluyveromyces lactis* by ion-exchange chromatography: Influence of pH and ionic strength parameters

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Abstract: The use of β-galactosidase in food products has been a major focus of the industry. Therefore, the development of efficient and inexpensive methodologies to purify it is essential. Thus, this study aimed to recover the enzyme β-galactosidase (β-gal) by ion-exchange chromatography in a fixed-bed column. Batch adsorption tests were performed using four types of adsorbents. The β-gal adsorption capacity in batch mode using Streamline DEAE resin presented the best performance, with a retention capacity of 18.77 ± 0.14 U/g at pH 6.0. A 2^2 experimental design was applied to optimize the β-gal recovery using an AKTA Start system, evaluating the ionic strength and the pH as process parameters. The results showed that ionic strength exerted a greater influence on fold purification (FP). The β-gal fraction in elution using 0.1-0.4 M of NaCl showed a yield of 51.65 ± 0.17% and FP of 2.00 ± 0.43. Electrophoresis confirmed the β-gal recovery, where an evident band with a molecular weight between 60 and 120 kDa was observed. These results point to the recovery of a stable β-gal of *K. lactis* with potential industrial applications.

Key words: β-galactosidase, adsorption, *Kluyveromyces lactis*, ion-exchange chromatography, recovery.

INTRODUCTION

The global generation of cheese whey (CW) by the dairy industry is currently around 200 million tons per year (Treu et al. 2019). Since CW is a by-product with significant polluting potential, due to its high biological oxygen demand (Andrade et al. 2017), several biotechnological strategies have been investigated regarding the further use of this industrial waste as feedstock for the production of value-added molecules, such as β-galactosidase (β-gal) (Carota et al. 2017, Carvalho et al. 2020). β-gal is an enzyme widely used in the food industry, as it acts as a catalyst in the hydrolysis of lactose in milk and its derivatives, and in the synthesis of galacto-oligosaccharides (GOS) with prebiotic properties (Silva et al. 2020, Zhao et al. 2018). Currently, β-gal is mainly obtained by submerged fermentation using *Kluyveromyces lactis* because the yeast has high enzyme production ability and adapts broadly to the dairy environment (You et al. 2017). As in most biotechnological processes, this process involves the recovery of the β-gal using different strategies (Machado et al. 2015).

The chromatography using an ion-exchange matrix is widely used for protein adsorption, in which the concept is based on the attraction between proteins molecules and the resin that presents opposite charges. One of the advantages of this process is that it provides smooth separation conditions, allowing proteins to maintain their conformation (Medeiros et al. 2012, Braga et al. 2014). It is essential to highlight that the costs of this operation can reach up to 90% of the total production costs, and the reduction of purification steps implies directly on costs and improves the yields of the targeted molecule (Sousa Junior et al. 2016), which is crucial for development of new alternative biotechnological strategies regarding the use of industrial wastes. Thus, the objective of the present work was to investigate the use of ion-exchange chromatography as a straightforward, simple strategy to recover β-gal enzyme produced by *Kluyveromyces lactis* yeast using raw cheese whey as lactose substrate. The influence of pH and ionic strength parameters was evaluated to obtain a high purification factor without impairment in yield, in a simple one-step process.

MATERIALS AND METHODS

Microorganism and inoculum production

Kluyveromyces lactis NRRL Y-8279 was obtained from the ARS Culture Collection (NRRL Culture Collection, United States Department of Agriculture, Peoria, IL, USA). For inoculum preparation, *K. lactis* was cultivated in YEPD medium (10 g/L of yeast extract, 20 g/L of dextrose, 20 g/L of peptone, 20 g/L of agar) for 24 h, at 30 °C. Three isolated colonies were transferred into 50 mL of culture medium containing 10 g/L of lactose, 5.0 g/L of KH_2PO_4, 1.2 g/L of $(NH_4)_2SO_4$, 1.0 g/L of yeast extract and 0.4 g/L of $MgSO_4.7H_2O$, prepared in potassium phosphate buffer (0.2 M, pH 5.5) according to Lima et al. (2013). The inoculums were cultivated under agitation of 180 rpm, at 30 °C, for 16 h in a rotating incubator.

Production of β-gal

The enzyme β-gal was produced through submerged cultivation using rotary incubator (Tecnal, model TE-241), with 250 mL Erlenmeyer flasks containing 50 mL of culture medium (20 g/L of cheese whey, 1.3 g/L of $(NH_4)_2SO_4$, 12 g/L of yeast extract, 5.0 g/L of KH_2PO_4 and 0.4 g/L $MgSO_4.7H_2O$), in 0.2 M potassium phosphate buffer at pH 5.5 (Braga et al. 2014). The fermentation was performed with 10% (v/v) of inoculum. The culture medium was previously sterilized and the lactose from the cheese whey was filtered in a 0.22 µm membrane. The cultivations in rotary incubators were performed at 30 °C, 180 rpm, for 20 h. A detailed description on the β-gal production from cheese whey is given elsewhere (Carvalho et al. 2020).

The enzymatic extract was obtained from the yeast cell disruption after the fermentation. The procedure was performed in centrifuge tubes of 50 mL, containing 25 mL of the cell suspension and 27.5 g of glass pearls (diameter ranging from 0.95 to 1.05 mm) under vortex agitation for 5 min, followed by 1 minute in an ice bath (Braga et al. 2014). The enzymatic extract was collected and stored at -20 °C.

Determination of adsorption conditions for β-gal recovery

In order to determine the initial adsorption conditions of β-gal, four types of adsorbents (Multimodal Capto MMC, Streamline DEAE, Streamline SP and Amberlite XAD polymeric-XDA 7) and three pHs (6.0, 7.0 and 8.0) were tested. The assays were performed in batch, at 25 °C and 150 rpm, in a rotary incubator for 60 minutes. In 25 mL Erlenmeyer flasks, 0.2 g

of resin, 2 mL of sodium phosphate buffer 200 mM in the respective pH, and 2 mL of enzymatic extract were added (Padilha et al. 2017). The enzymatic activity retained in the solid phase was calculated according to Eq. 1:

$$q(U/g) = \frac{V(C_0 - C)}{M_{ads}} \quad (1)$$

where q is the amount of enzyme adsorbed on the resin, V is the volume of the enzymatic extract, C_0 is the value of the initial activity, C is the value of activity in equilibrium, and M_{ads} is the adsorbent mass.

Optimization of β-gal recovery using experimental design

The resin that presented the best adsorption capacity to the target molecule was selected for the recovery assays using the AKTA purification system (GE Healthcare Bio-Science, New Jersey, USA) using a fixed-bed column HR 16/5 with a bed volume of 6 mL (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The influence of the factors (independent variables) ionic strength and pH on dependent variables fold purification (FP) and yield (Y) of β-gal was analyzed through a 2^2 experimental design with three central points, resulting in 7 runs. The factors and levels chosen for the design are presented in Table I.

The process was performed with a superficial velocity of the mobile phase of 100 cm/h. The column containing the DEAE streamline resin was initially equilibrated for 30 minutes with buffer A (sodium phosphate). A load of 4.5 mL of the crude extract was injected into the system. The non-bonded or weakly bonded molecules were removed from the system in the washing step (7.5 mL of buffer A). Then, for the elution stage, a mobile phase ionic strength was linearly increased by adding the buffer B (sodium phosphate 50 mM, 125 mM and 200 mM at pH 6.0, 6.5 and 7.0 and NaCl 1 M) from 0 to 100%, aiming to separate the proteins according to the strength with which they were adsorbed in the resin. Samples were collected at each 1.5 mL. The runs were performed in duplicates, for the sake of data validation and system reproducibility.

Analytical methods

To determine the hydrolytic activity of the β-gal enzyme, ortho-nitrophenyl-β-D-galactoside (ONPG) was used (Freitas et al. 2020). The β-gal hydrolytic activity was determined at 37 ºC and pH 6.6 for 10 minutes and expressed in U/mL. A unit (U) of enzymatic activity corresponds to the amount of enzyme that catalyzes a reaction with the formation velocity of 1.0 µmol of ortho-nitrophenol (o-nitrophenol) for 1 minute, in the conditions of the assay. The total protein concentration was determined using the Bradford method (Bradford 1976). Bovine serum albumin (BSA) was used as standard protein for a calibration curve. The protein concentration was expressed in mg/mL and used to calculate the specific activity (U/mg).

From the enzymatic activity of the crude and recovered extract, the purification yield was obtained according to Eq. 2 and the fold

Table I. Factors and levels used in the 2^2 experimental design.

Factor	Levels		
	-1	0	+1
Ionic strength (mM)	50	125	200
pH	6.0	6.5	7.0

purification calculated from the specific activities of the crude and recovered extract according to Eq. 3 (Leitão et al. 2018).

$$\text{Yield (\%)} = \frac{\textit{Enzymatic activity of purified extract}}{\textit{Enzymatic activity of crude extract}} \times 100 \quad (2)$$

$$\text{FP} = \frac{\textit{Specific activity of purified extract}}{\textit{Specific activity of crude extract}} \quad (3)$$

The β-gal enzyme fractions of the best recovery range were subjected to qualitative analysis using polyacrylamide gel electrophoresis (PAGE) at 9% (Laemmli 1970).

Statistical analysis

The results obtained for the initial adsorption conditions of the β-gal were submitted to analysis of variance (ANOVA) followed by Tukey post-hoc test. A p-value <0.05 was adopted to determine significant differences, and the statistical analyses were performed in software Statistica 7.0 (StatSoft Inc, Tulsa, USA). All the experiments of the experimental design were performed randomly. The data were analyzed using the software Statistica 7.0 and the statistical significance of the model was evaluated by the coefficient of determination (R^2) and F-test (ANOVA).

RESULTS AND DISCUSSION

Determination of adsorption conditions for β-gal recovery

Figure 1 shows the results obtained in the adsorption experiments of β-gal in different adsorbents. In this assays, it was observed that the Streamline DEAE and Amberlite XAD7HP resins present the best selectivity to the target molecule at pH 6.0 ($p < 0.05$), which promotes a better performance of the chromatographic system with a β-gal enzyme retention capacity of 18.77 ± 0.14 U/g, and 20.97 ± 0.41 U/g, respectively. The isoelectric point of β-gal (5.0) gives the enzyme a negative net charge under the studied conditions, which favors the association with positively charged adsorbents such as the DEAE (diethylaminoethyl) resin (Mazi et al. 2016). The negative charges in the enzyme can also explain the low recovery obtained with the Streamline SP (sulfopropyl) that present negative groups. The better β-gal adsorption in Amberlite XAD7HP

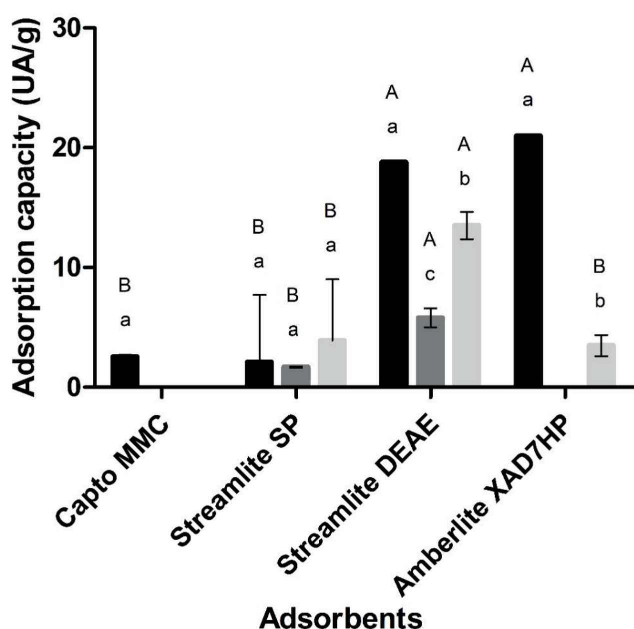


Figure 1. Adsorption of β-gal on four resins in the pH range of 6.0 to 8.0. Different lowercase letters (a, b) indicate statistical differences (p <0.05) for different pH with the same adsorbent. Different uppercase letters (A, B) indicate statistical differences (p <0.05) for adsorbents considering the same pH.

resin, a polymeric matrix formed by acrylic ester monomers, can be explained by hydrogen bonding between adsorbate and adsorbent (Chaubal & Payne 1995, Padilha et al. 2019).

Optimization of β-gal recovery using experimental design

After identification of the best adsorbent and pH for the β-gal adsorption, an experimental design 2^2, with three central points, was carried out to optimize the enzyme recovery. The DEAE Streamline resin was tested in the automated chromatography system FPLC AKTA Start. It was investigated the influence of the factors (independent variables) pH and ionic strength (IS) on the fold purification (FP) of the enzyme and yield (Y) of the process (dependent variables). The experimental conditions and the responses for linear experimental design are presented in Table II. It can be seen that the results related to FP ranged from 0.56 (Run 2) to 2.00 (Run 3), while those obtained for the Y ranged from 49.08% (Run 1) to 91.07% (Run 2).

The use of statistical methods and mathematical models contributed to the development and optimization of processes over time. The response surface methodology (RSM) is a tool to evaluate and model experimental data to identify independent and combined influences of independent variables in the output variables of the process (Câmara Junior et al. 2016). Based on the adjusted regression coefficients, a statistical model that correlates the responses yield (Y) and fold purification (FP) with the variables ionic strength and pH were constructed, as observed in Eqs. 4 and 5, in which X_1 is the pH and X_2 the ionic strength.

$$Y (\%) = 67.427 - 2.142*X_1 - 17.576*X_2 + 3.422*X_1*X_2 \quad (4)$$

$$FP = 1.114 + 0.186*X_1 + 0.512*X_2 + 0.148*X_1*X_2 \quad (5)$$

The Pareto charts obtained for Y and FP responses present the influences by both linear effects of the selected variables (Figure 2). The concept of interaction terms indicates that changes in a combination of process variables had a synergistic effect on the experimental response (Zhao et al. 2018). The change from the lower level (-1) to the higher (+1) of the ionic strength and pH increases the purification degree of the β-gal enzyme to 2.00, but the ionic strength exerts a greater influence on the FP.

Table II. Matrix of the 2^2 experimental design showing the coded and real (in parenthesis) values of variables used to recovery the β-gal.

Run	pH	Ionic strength (mM)	Fold Purification[a]	Yield[a] (%)
1	-1 (6.0)	+1 (200)	1.29 ± 0.29	49.08 ± 0.75
2	-1 (6.0)	-1 (50)	0.56 ± 0.52	91.07 ± 0.12
3	+1 (7.0)	+1 (200)	2.00 ± 0.42	51.64 ± 0.35
4	+1 (7.0)	-1 (50)	0.64 ± 0.13	79.95 ± 0.45
5[b]	0 (6.5)	0 (125)	1.21± 0.01	65.73 ± 0.45
6[b]	0 (6.5)	0 (125)	1.05 ± 0.85	66.47 ± 0.77
7[b]	0 (6.5)	0 (125)	1.08 ± 0.01	68.11 ± 0.41

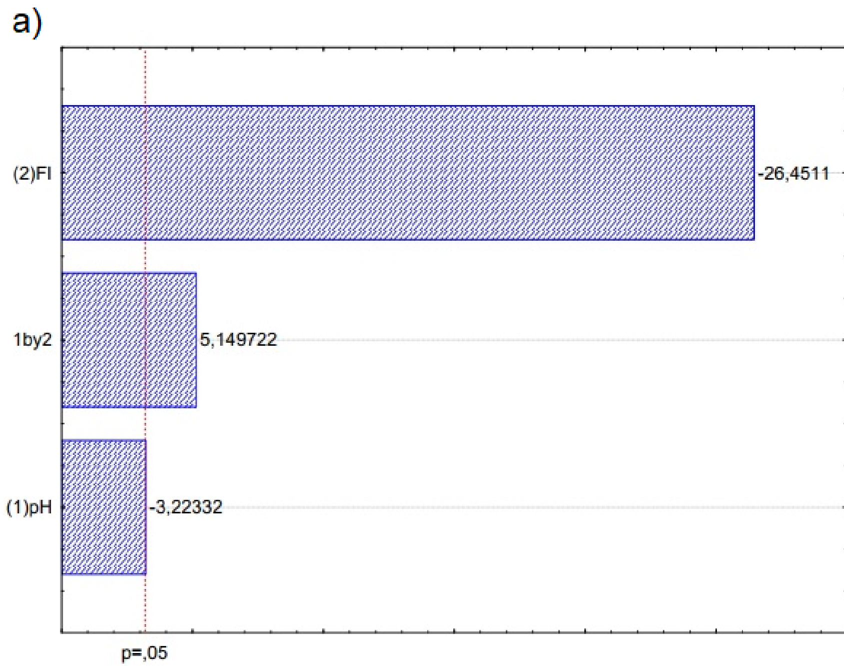
[a]**Means ± standard deviation of triplicate assays.**
[b]**Central points.**

The analysis of variance (ANOVA) performed in the obtained models confirmed the significance (p <0.05) evidenced in Table III and the lack-of-fit test (LoF) was not significant (p >0.05), suggesting the capacity to predict the models. High correlation coefficients (R^2; 0.98858 and 0.99594, for FP and Y respectively), indicate a strong concordance of the adjusted data with the empirical results, responsible for robust empirical models (Câmara Junior et al. 2016). Through the F test, it can be observed that the models proposed in Eqs. 4 and 5 are statistically significant at 95% confidence level. For the Y, the calculated F value (245.192) was 26.42 times higher than the listed F value ($F_{3,3}$ = 9.28) and for the FP, the calculated F value (141.666) was 15.26 times higher than the listed F value (Table III).

The 3D representations of the adjusted models (Eqs. 4 and 5) are shown in Figure 3. The highest region found on the response surfaces indicates the ideal conditions for maximizing the responses of the recovery process of β-galactosidase. It is observed in Figure 3a that lower values of ionic strength (IS) and pH favor the degree of recovery of the enzyme, reaching 91.07 ± 0.12% of yield. In contrast, in these conditions, the fold purification for the biomolecule of interest was 0.56 ± 0.52 (Figure 3b). It is also evident that higher values of IS and pH increase the degree of purity for the enzyme reaching a FP of 2.00 ± 0.43 with a yield of 51.64 ± 0.35%. The results of Y and FP obtained in this work are promising and superior to those reported by Lima et al. (2016), who used the technique of multimodal chromatography for recovery and purification of β-gal and reached around 48% of recovery with a FP of 1.17. In the case of Medeiros et al. (2012), the authors used Sepharose Q resin and obtained recovery values close to 88%. However, it is important to highlight that only commercial enzymes were used in this study, which favors the process, given the higher degree of purity of samples.

Figure 4 shows the variation of protein concentration and enzymatic activity throughout the chromatography stages (load, elution and washing) of the run with the best performance. The variation of both responses analyzed during the entire run was observed. At the first moment, during the loading stage, there was a slight concentration of protein, with little significant enzymatic activity. In the course of the washing stage, it can be identified a slight increase in the amount of enzyme present in the mobile phase, considering that the function of this stage is to carry out the molecules not adsorbed to the matrix of the column and due to the high hydrophilic character of the biomolecule of interest. During the elution phase, it was evidenced that the total protein and enzyme concentrations did not increase proportionally to the ionic strength of the mobile phase, showing a purification scale with four peaks of enzymatic activity (0.1, 0.2, 0.3 and 0.4 M of NaCl), presenting similar results to Lima et al. (2016). The enzyme recovery from the crude broth is considered significant, even with the presence of some contaminants.

It was possible to observe, according to Table IV, that the fraction of β-gal in the best elution range using between 0.1 and 0.4 M NaCl had a yield of 51.65 ± 0.17% and FP of 2.00 ± 0.43. These results were similar to the study performed by Braga et al. (2014) that performed the purification of the β-gal enzyme and initially reached 48% of recovery compared to the initial extract. The ion-exchange chromatography is well used in protein adsorption. One of the advantages of this process is that it provides smooth separation conditions allowing proteins to maintain their conformation (Medeiros et al. 2012, Braga et al. 2014).



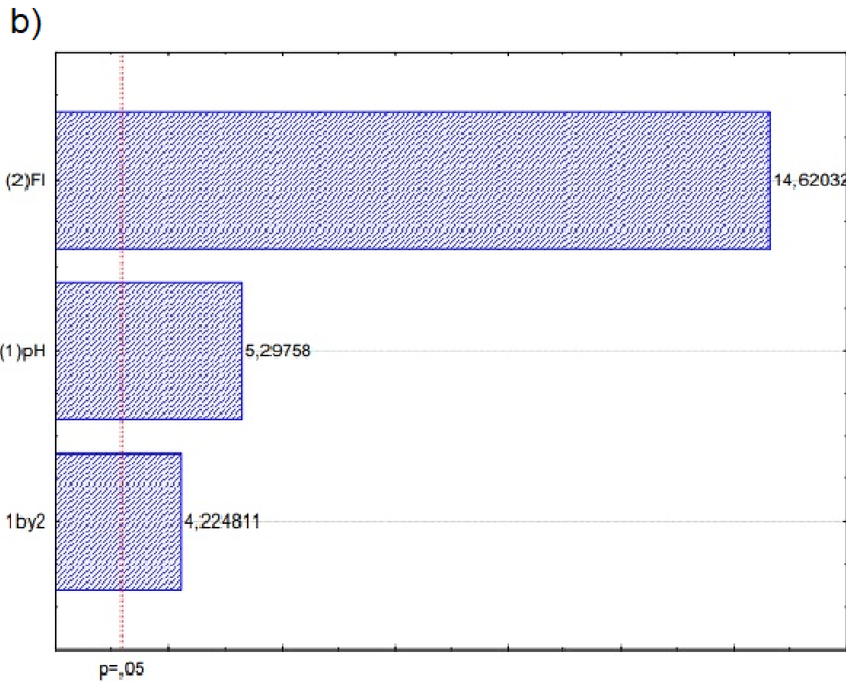


Figure 2. Pareto charts of the standardized effects for yield (a) and fold purification of β-gal (b).

Polyacrylamide gel electrophoresis (PAGE)

The fraction of the β-gal enzyme in the best elution range using 0.1 to 0.4 M NaCl in the optimum recovery condition with IS of 200 mM and pH 7.0 was subjected to qualitative analysis employing a 9% PAGE under non-denatured conditions as shown in Figure 5.

The wide-band marker protein used contained molecular mass ranging from 66 to 669 kDa, serving as a standard to identify the molar mass of the β-gal enzyme used

in this research. The β-gal enzyme may vary its properties according to its source. The molecular mass may range from 201 to 850 kDa, of β-gal produced by *Escherichia coli* to those produced by *K. marxianus*, respectively (Gekas & Lopez-Leiva 1985). The literature reports that β-gal molecules originating from *K. lactis* have a molecular mass varying between 120 and 140 kDa, values calculated by particle size exclusion chromatography (Boeris et al. 2012, Carminatti 2001). It is observed in Figure 4 an evident band with molecular weight in the range between 60 and 120 kDa was observed, configuring the enzyme of interest.

In conclusion, these results suggest that a single-stage ionic chromatography is a potential recovery strategy of a stable β-galactosidase produced from *K. lactis* with potential industrial applications from the low-cost residue, which is a crucial aspect to the development of new alternative biotechnological processes from industrial waste.

Table III. Analysis of variance (ANOVA) to validate the models (Eq. 4 and Eq. 5).

Source of variation	Square sum	Degrees of freedom	Mean square	F value*
Yield (Eq. 4)				
Regression	1300.497	3	433.499	245.192
Residual	5.305	3	1.768	
Lack of fit	2.327	1	2.327	
Pure error	2.978	2	1.489	
Total	1305.802	6		
R^2	0.9858			
Fold purification (Eq. 5)				
Regression	1.274	3	0.425	141.666
Residual	0.015	3	0.003	
Lack of fit	0	1	0	
Pure error	0.015	2	0.007	
Total	1.289	6		
R^2	0.99594			

***Listed $F_{3,3}$ value (95%) = 9.28.**

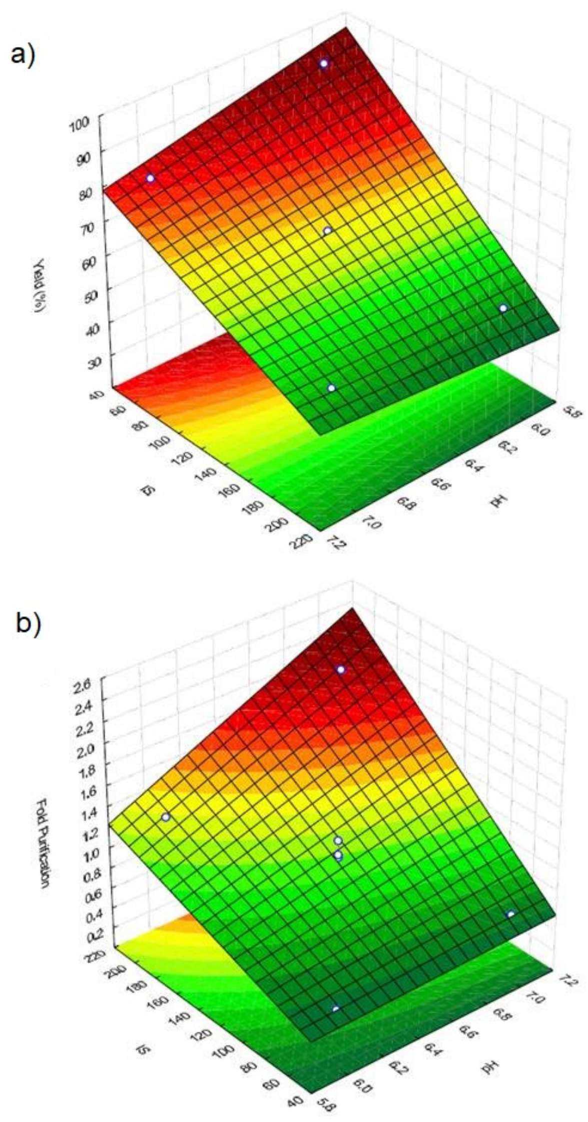


Figure 3. Response surface for yield (a) and fold purification (b) of β-gal as function of pH and ionic strength (IS).

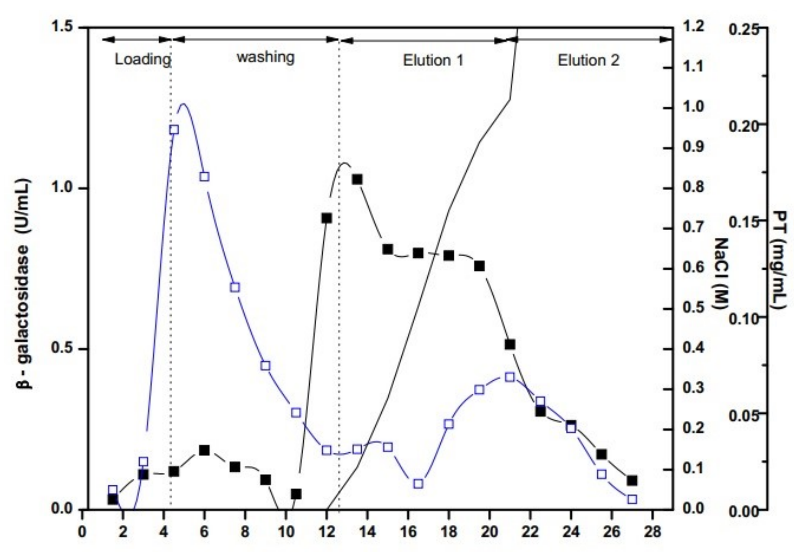


Figure 4. Chromatogram of β-gal recovery in a fixed-bed, submitted to a NaCl gradient through elution, where (□) protein concentration and (■) β-gal activity.

Table IV. Mass balance in each stage in the chromatographic run in function of total protein and enzymatic activity of β-gal produced by *K. lactis*[a].

Stage	Volume (mL)	Total Protein (mg)	Enzymatic activity (U)	Yield (%)	Fold Purification
Loading	4.5	0.19 ± 0.02	0.27 ± 0.03	3.93 ± 0.11	0.08 ± 0.12
Washing	7.5	0.73 ± 0.04	0.41 ± 0.10	16.67 ± 0.18	0.12 ± 0.10
Elution 1 (0.1-0.4 M NaCl)	6.0	0.16 ± 0.03	3.54 ± 0.09	51.65 ± 0.17	2.00 ± 0.43
Elution 2 (0.5-1.0 M NaCl)	9.0	1.56 ± 0.19	3.68 ± 0.10	31.70 ± 0.03	0.58 ± 0.02

[a]Means ± standard deviation of duplicate assays.

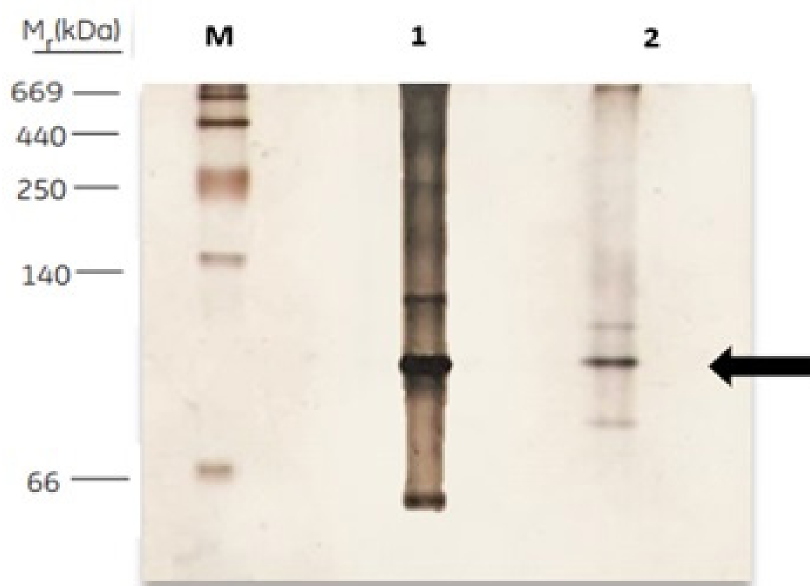


Figure 5. Native polyacrylamide gel electrophoresis 9%. (M) Maker protein, (1) crude extract and (2) elution sample of 0.1 to 0.4 M of NaCl.

Acknowledgments

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