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Production, recovery and purification of a recombinant β-galactosidase by expanded bed anion exchange adsorption

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ABSTRACT

β-Galactosidase is a hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides; its major application in the food industry is to reduce the content of lactose in lactic products. The aim of this work is to recover this enzyme from a cell lysate by adsorption onto Streamline-DEAE in an expanded bed, avoiding, as much as possible, biomass deposition onto the adsorbent matrix. So as to achieve less cell debris–matrix interaction, the adsorbent surface was covered with polyvinyl pyrrolidone. The enzyme showed to bind in the same extent to naked and covered Streamline-DEAE (65 mg β-gal/g matrix) in batch mode in the absence of any biomass. The kinetics of the adsorption process was studied and no effect of the polyvinyl pyrrolidone covering was found. The optimal conditions for the recovery were achieved by using a lysate made of 40% wet weight of cells, a polyvinyl pyrrolidone-covered matrix/lysate ratio of 10% and carrying out the adsorption process in expanded bed with recirculation over 2 h in 20 mM phosphate buffer pH 7.4. The fraction recovered after the elution contained 65% of the initial amount of enzyme with a 12.6-fold increased specific activity with respect to the lysate. The polyvinyl pyrrolidone content in the eluate was determined and found negligible. The remarkable point of this work is that it was possible to partially purify the enzyme using a feedstock containing an unusually high biomass concentration in the presence of polyvinyl pyrrolidone onto weak anion exchangers.

1. Introduction

β-Galactosidase (β-gal) is widely found in nature and it is one of the most important enzymes used in dairy processing [1]. This enzyme catalyzes the hydrolysis of β-galactosides into monosaccharides, its major application is in the food industry to reduce the content of lactose in lactic products [2–6]. The hydrolysis of lactose by β-galactosidase can be used to improve digestibility, solubility, and sweetness of dairy products [7–9]. β-Gal was traditionally purified from a variety of sources by ammonium sulfate precipitation and different chromatography techniques [10–17]. These methods involve a large number of steps, which make them time consuming and difficult to scale up. In order to establish a competitive biotechnological process for protein purification, expanded bed adsorption (EBA) has been proposed as a single-unit operation combining separation, concentration and capture of the target protein [18,19]. The use of this technique simplifies the clarification steps and produces a concentrate, including partially pure product, ready for the next purification step, usually packed-bed chromatography. Actually, the use of expanded bed simplifies the downstream-processing flow sheets for the recovery of a wide variety of proteins, with concomitant savings in equipment and operating costs [20–22].

EBA allows the adsorption of target proteins from unclarified feedstock by an increase in bed voidage due to the fluidization of the support with an upward flow. Several authors have reviewed the characteristics of the technique [23–25]. Its particularity consists in the stabilization of the fluidized bed by using adsorbents with an appropriate distribution in density and/or particle size combined with a well-designed fluid distributor at the column inlet. This is supposed to produce a performance comparable to protein capture in packed bed mode. Numerous examples have been described in the literature for the purification of proteins from different types of sources [20,21,26–29]. During EBA, all bioparticles contained in the feed should freely pass through the expanded bed while the target protein is bound.

Abbreviations: β-gal, β-galactosidase; PVP, polyvinyl pyrrolidone; EBA, expanded bed adsorption.

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Unfortunately, cells or cell debris tend to interact with the adsorptive supports [30–34], particular in the case of ion exchangers, leading to the formation of aggregates of cells and adsorbent beads, which alters the bed stability and thereby dramatically decreases the adsorption efficiency. Cells may bind to the surface of the beads, clogging the pores and consequently reducing the static binding capacity of the stationary phase [7]. Harsh washing and regeneration conditions may be necessary and the life expectancy of the support may decrease as a result of irreversible fouling of the beads. Cell/support interactions can even lead to the aggregation of the solid particles until the bed collapses and the column is blocked [35].

As the common limitations encountered during the direct recovery of bioproducts from an unclarified feedstock are related to the presence of biomass, in a previous work [36], a number of chemical additives were screened for their ability to inhibit either biomass deposition, cell aggregation, or a combination of both effects. Results indicated that the coating of anion-exchanger beads with the synthetic polymer polyvinyl pyrrolidone 360 (molecular mass 360) alleviated biomass deposition and consequently restored EBA process performance [37–40]. The use of this non-toxic polymer could help to avoid detrimental biomass deposition during expanded bed adsorption of bioproducts and other direct contact sequestration methods [41].

The aim of the present study was to develop a fast method for the large-scale purification of β-gal from recombinant *Saccharomyces cerevisiae* by expanded-bed adsorption using PVP-covered Streamline-DEAE. First, the adsorption process of β-gal onto Streamline-DEAE and PVP-covered Streamline-DEAE was characterized. Next, β-gal was separated by EBA using PVP-covered Streamline-DEAE.

2. Materials and methods

2.1. Materials

Adsorbent and column were purchased from GE Healthcare, Munich, Germany. Water was of ultrapure quality. Polyvinyl pyrrolidone 360 (PVP) was obtained from Sigma–Aldrich Chemie GmbH, Steinheim, Germany. Yeast extract and soy peptone were Difco quality (Becton Dickinson GmbH, Heidelberg, Germany). All other chemicals were of analytical grade.

2.2. Saccharomyces cerevisiae strain

The *S. cerevisiae* W303-1A strain transformed with the plasmid pBIVU02-1 codifies β-galactosidase. The transformed yeast strain was maintained at −80 °C in 50% glycerol (v/v) [42].

2.3. Inoculum and enzyme production media

YNB medium (0.67% yeast nitrogen base and 2% glucose) supplemented with auxotrophic amino acids (50 mg/L histidine; 20 mg/L adenine; 70 mg/L tryptophan; 20 mg/L uracil) and sterilized by microfiltration was used to start *S. cerevisiae* culture. The strain was grown for 16 h in 25-mL flasks containing 5 mL of YNB medium maintained in an orbital shaker (200 rpm) at 30 °C. Afterwards, 5 mL of this preinoculum were transferred into 500-mL flasks containing 100 mL of YPD medium (1% yeast extract, 2% bactopeptone, and 2% glucose). The inoculum was grown for 24 h in an orbital shaker (200 rpm) at 30 °C and then transferred into the bioreactor containing the expression medium YPGal (1% yeast extract, 2% bactopeptone, and 4% galactose) [42,43].

2.4. Enzyme production in bioreactor

Fermentation was carried out in a 20 L bioreactor with a working volume of 15 L at pH 5.5 and 30.0 °C. YPGal medium and the reaction vessel were sterilized at 121 °C for 20 min. The fermenter was inoculated at 5% (v/v) ratio. Oxygen tension was measured as the percentage of dissolved oxygen in the culture medium in relation to air saturation using an oxygen electrode. We calibrated 100% dissolved oxygen at a flow rate of 15 L/min (1000 rpm), at 30 °C before inoculation. During fermentation, air was supplied at a constant flow rate of 5 L/min. Mechanical agitation, produced by three Rushton turbines, was automatically controlled by setting the percentage of dissolved oxygen in the culture medium at 55%. Samples were collected under aseptic conditions to control fermentation performance and monitor biomass, protein concentration, and enzymatic activity.

2.5. Lysate preparation

After the fermentation, the sample was centrifuged at 4000 rpm during 10 min to separate the cells. The cells were re-suspended in buffer and were lysated in a bead mill [44].

2.6. PVP-covering of Streamline-DEAE

In order to coat the adsorbent beads with PVP, two covering steps were applied: (1) overnight incubation of the beads in batch in a 1% PVP 360 media; (2) a mobile phase containing 1% PVP 360 was pumped through the matrix in the column for about 3 column volumes. Subsequently, the bed was washed with plain mobile phase.

2.7. Stream line β-gal interaction

2.7.1. Column adsorption experiments

the breakthrough curve for β-gal using naked and PVP-covered Streamline-DEAE was determined by loading a 2 mg/mL β-gal solution in a column containing 1 mL of matrix. The effect of different flowrates (from 0.1 mL/min to 2 mL/min) was assayed. The dynamic binding capacity of the bed is determined when the β-gal concentration in the column effluent reached 10% of the initial concentration ($C/C_0 = 0.1$) in order to reduce the loss of the target product in the flow.

2.7.2. Batch adsorption experiments

A β-gal stock solution was prepared from which the solutions for equilibrium tests were prepared to the desired concentrations through successive dilutions. An accurately weighed quantity of the matrix (100 mg) was mixed with each dilution in eppendorf tubes. These solutions were stirred on a thermostatic mechanical shaker operating at a constant agitation speed; 3 h was found to be enough to reach adsorption equilibrium. The matrix was then separated and the supernatant was analyzed for the final β-gal concentration.

Blanks containing no β-gal or adsorbent were conducted in similar conditions as controls to evaluate possible color change and/or precipitation processes. The amount of β-gal adsorbed at equilibrium time by the adsorbent (q_e) was calculated from the mass balance equation given by Eq. (1) where C_o and C_e are the initial and final β-gal concentrations in liquid phase (mg L^{-1}), respectively, V is the volume of enzyme solution (L) and m the mass of adsorbent used (g).

$$q_e = \frac{V(C_o - C_e)}{m} \quad (1)$$

Plotting solid-phase concentration against liquid-phase concentration graphically depicts the equilibrium adsorption isotherm. In

this study, experimental data were fitted to the Langmuir isotherm equation [21,45,46].

2.8. Expanded bed adsorption

A glass column (10 mm diameter) was used for all expanded bed experiments. The bottom inlet was packed with glass beads to distribute the inlet flow. During the experiments, the top plunger in the column was lowered to be just above the surface of the expanded adsorbents to minimize the dead volume of the system. EBA experiments were conducted at room temperature. A volume of 10.2 mL Streamline-DEAE was packed in the expanded bed to give a settled bed height of 13.0 cm. Following conditioning, the bed was equilibrated with buffer at a rate of 2 mL/min to give a stable height, where $H/H_0 = 2.0$. Adsorption of β-gal was performed by recirculating the lysate in the expanded bed. After adsorption, the bed was washed with buffer upwardly and then eluted using buffer with different NaCl concentration.

2.9. Analytical methods

2.9.1. Biomass

Biomass was determined by measuring the optical density (OD) at 600 nm of a 1:100 dilution of the samples using a spectrophotometer.

2.9.2. Protein concentration

Protein concentration was determined according to the Bradford method, using Coomassie reagent (Bio-Rad Laboratories, Hercules, CA, USA). Bovine serum albumin (BSA) (Sigma, Munich, Germany) was used as a standard. Absorbance was measured at 595 nm using a plate reader.

2.9.3. Enzymatic activity

Enzymatic activity was evaluated according to Lederberg [47] using orthonitrophenyl-β-D-galactopyranoside as the substrate. Released o-nitrophenol (ONP) was measured spectrophotometrically at 420 nm. One arbitrary unit (AU) of β-galactosidase activity was defined as the amount of enzyme that increases 0.001 absorbance units at 420 nm in 1 min at 30 °C [48].

2.9.4. PVP determination

The PVP concentration was determined spectrophotochemically at 217 nm. Spectra of a 0.1% PVP solution was carried out to determine the peak position and the absortivity coefficient. A standard curve was done.

2.10. Polyacrylamide gel electrophoresis

SDS-PAGE was carried out according to standard procedures: 8% (w/v) acrylamide (Bio-Rad Laboratories) was used for stacking gel and 13% (w/v) for resolving gel. The enzyme samples tested were the lysate and the fractions obtained after the ion-exchange adsorption. 20 μL of each sample and 10 μL of markers were applied to the gel (broad-range markers: myosin, 200,000 MW; β-galactosidase, 116,000 MW; phosphorylase, 97,000 MW; serum albumin, 66,200 MW; ovalbumin, 45,000 MW; carbonic anhydrase, 31,000 MW; trypsin inhibitor, 21,500 MW; lysozyme, 14,400 MW; aprotinin, 6500 MW) (Bio-Rad Laboratories).

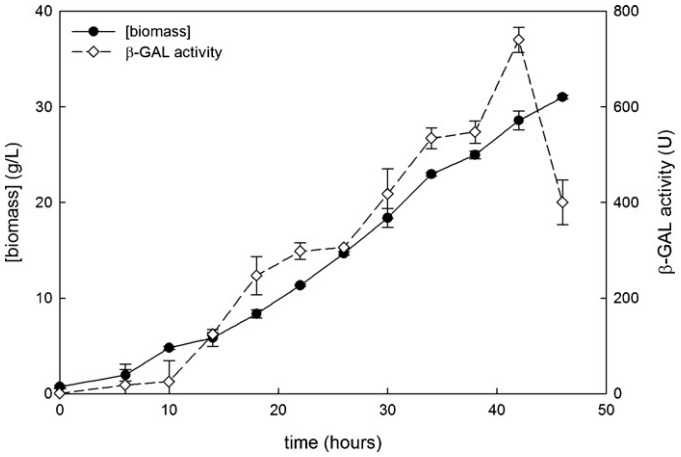

Fig. 1. Profile of biomass production and β-gal enzimatic activity during the fermentation process. Medium conditions: see Section 2.

3. Results and discussion

3.1. β-Galactosidase production

The exponential growth occurred during 40 h and the maximum biomass value (31 g/L) was attained after 46 h of fermentation as shown in Fig. 1.

The level of protein secretion to the culture medium increased continuously up to 42 h of fermentation, obtaining a total protein concentration of 28 g/L and a recombinant β-gal activity of 740 AU/L. The cytoplasmic enzymatic activity increases throughout cell growth, indicating that the levels of expression of recombinant β-gal increase with biomass production. However, after 42 h of fermentation, the β-gal activity decreased, which may be due to the presence of proteolytic enzymes in the media [49–51].

3.2. PVP release determination from the PVP-covered Streamline-DEAE

The washing of the PVP-covered matrix was assayed in batch mode and in a column, in continous mode. The PVP concentration determined after the washing was plotted vs. the volume of the washing buffer/matrix volume (data not shown). The same profiles of PVP release were obtained in both modes: after washing 1 volume of matrix with 3 volumes of plain buffer, the concentration released of PVP was less than 0.1%. Moreover, after 6-fold volume washing, the PVP was indetectable in the solution.

3.3. Determination of the Steamline-DEAE–β-gal binding capacity

In order to obtain the breakthrough curve, a standard solution of β-gal was loaded into a column packed with Streamline-DEAE. Several experimental conditions (flow rate, enzyme concentration, matrix volume) were assayed. However, it was not possible to obtain a typical breakthrough curve: the protein was released in the flow trow and the shape of the curve was not the typical sigmoideal one. We assumed that the differences were due to the lack of time for the solid–liquid phase to reach equilibrium. Since the dynamic binding capacity of β-gal to Streamline-DEAE could not be determined, we proceeded to determine the equilibrium binding capacities. The equilibrium adsorption isotherm of β-gal on the naked and PVP-covered Streamline-DEAE matrix using the

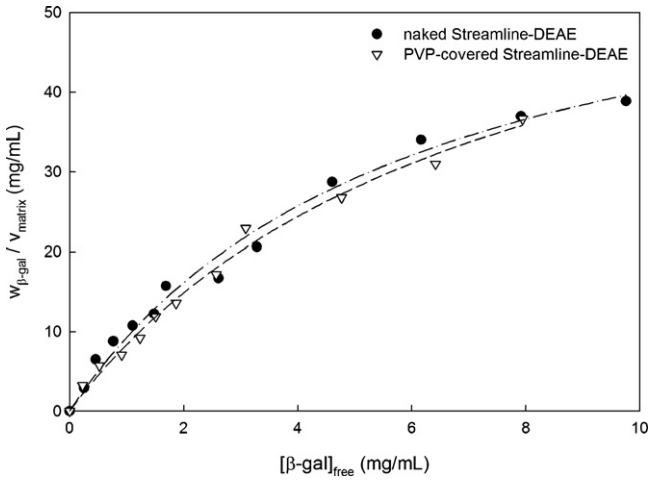

Fig. 2. Binding isotherm of β-gal to Streamline-DEAE and Streamline-DEAE–PVP. Medium: 20 mM buffer phosphate, pH 7.4, and temperature 20 °C.

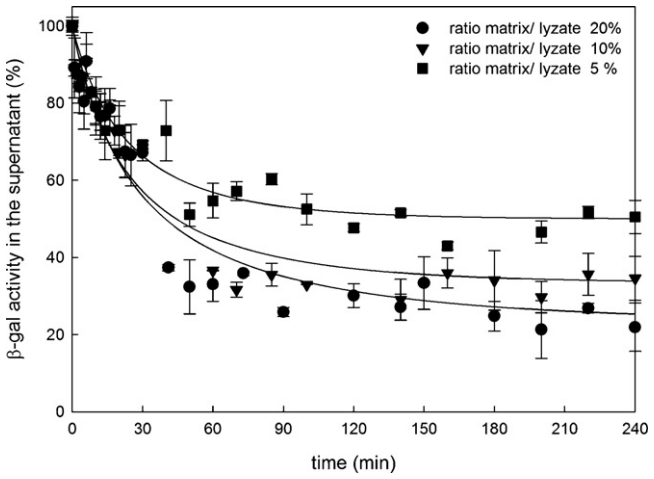

Fig. 3. Kinetics of the adsorption of β-gal onto naked (top) and PVP-covered (bottom) Streamline-DEAE. Medium: 20 mM buffer phosphate, pH 7.4. Cell content in the lysate: 40% (w/w).

commertially available enzyme was obtained as shown in Fig. 2. Data were fitted according to type Langmuir equation:

$$\frac{w_E}{w_M} = \frac{(w_E/w_M)_{\max} Kc}{1 + Kc} \quad (2)$$

being (w_E/w_M) the enzyme to matrix mass ratio, $(w_E/w_M)_{\max}$ the enzime to matrix mass ratio when the matrix is saturated, K the binding equilibrium constant and c the free enzyme concentration in equilibrium.

The naked Streamline-DEAE showed a binding maximal capacity of 63 ± 4 mg β-gal/mL and PVP-covered Streamline-DEAE bound 68 ± 5 mg β-gal/mL. These values are suggesting that the adsorptive capacity of Streamilne-DEAE is not modified when it is covered by PVP. These maximal capacity values of Streamline-DEAE for β-gal, can be considered a good value compared to the capacity observed for this matrix for other proteins [14]. The equilibrium constant (K) was also determined: (0.17 ± 0.02) mL/mg for naked Steamline-DEAE and (0.14 ± 0.01) for PVP-covered Streamline-DEAE, which suggests a slight decrease in the matrix affinity for the enzyme due the presence of PVP. This can be attributed to a change in the polarizability and dielectric constant of the micro-environment due to the presence of PVP near the matrix surface.

3.4. Steamline-DEAE β-gal adsorption kinetics in batch mode

The kinetic adsorption of β-gal from the cell lysate onto naked and PVP-covered Streamline-DEAE was determined by measuring the enzymatic activity remaining in the supernatant over the time in batch mode. The matrix/lysate ratio was assayed, as shown in Fig. 3. After 120 min, the β-gal remaining in the supernatant reached a constant value. The amount of β-gal adsorpted onto the matrix was highly dependent on the matrix/lysate ratio. The lower ratio was not effective enough since there was around 60% of β-gal in solution. The enzyme concentration remaining in the supernatant was similar in the case of the 10% and 20% matrix/lysate ratio. Taking this into account, the subsequent experiments were carried out using a 10% matrix/lysate ratio since a higher amount of matrix did not improve the adsorption process; therefore, there was no need to consume a bigger amount of matrix.

The data were fitted assuming a first-order decrease of the activity in time from Eq. (3):

$$A_{ct} = A_{ct}^{o} e^{-kt} \quad (3)$$

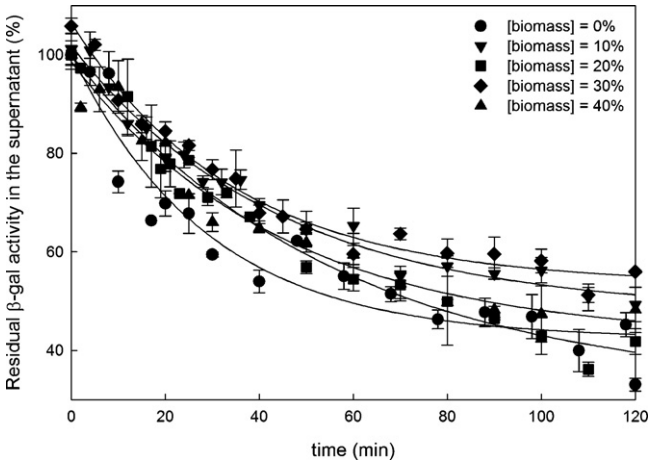

Fig. 4. Biomass effect in the adsorption of β-gal onto PVP-covered Streamline-DEAE. Medium: buffer phosphate 20 mM, pH 7.4, and temperature 20 °C.

where A_{ct} and A_{ct}^{o} are the β-gal activities at any time and at the begining, respectively, and k is the uptake kinetic constant as shown in Table 1.

No differences can be observed in the uptake kinetic constant at low biomass concentration, however, for concentrations of 10 and 20%, the uptake kinetics was faster in the absence of PVP than in its presence. This finding is consistent with a steric hindrance mechanism between the matrix and the β-gal due to the PVP presence.

3.5. Purification of β-gal from the cell lysate in expanded bed mode with recirculation

The adsorption of β-gal onto PVP-covered and naked Streamline-DEAE was assayed in column mode with recirculation. The outlet was collected in a reservoir and the column was loaded

Table 1
β-Gal uptake kinetic constants of the adsorption in batch mode.

Biomass content in the lysate (%, w/w)	Naked Streamline-DEAE k (10^{-2} min^{-1})	PVP-covered Streamline-DEAE k (10^{-2} min^{-1})
5	3.06 ± 0.06	3.19 ± 0.02
10	3.08 ± 0.04	2.50 ± 0.03
20	2.54 ± 0.03	2.30 ± 0.03

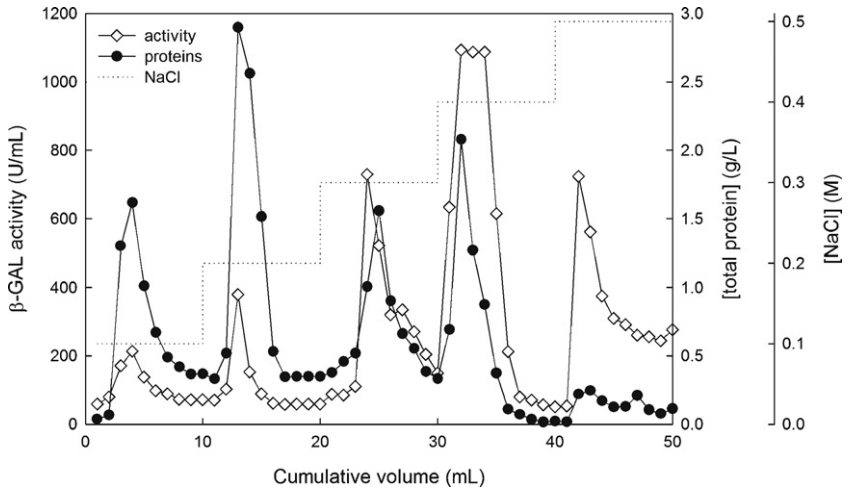

Fig. 5. The elution profile of β-gal after the adsorption of the lysate. Medium: buffer phosphate 20 mM, pH 7.4, and temperature 20 °C.

Table 2
β-Gal uptake kinetic constants of the adsorption in columne with recirculation.

Biomass content in the lysate (%, w/w)	kc (10^{-2} min^{-1})
0	3.4 ± 0.7
10	2.2 ± 0.2
20	1.8 ± 0.2
30	2.8 ± 0.3
40	2.3 ± 0.4

with that solution. The adsorption process onto the PVP-covered matrix results more suitable for purification since the performance of the bed was not disturbed by the cell debris content in the lysate. There was no channel formation or matrix clogging in this case. The effect of the cell debris content was assayed. A 40% cell lysate was prepared and was divided into two fractions. One of the fractions was centrifuged to obtain a lysate with no cell debris. By mixing in the appropiate proportion, 40% cell lysate and the 0% cell lysate, lysates with intermediate cell debris content between (0 and 40%) were obtained. All the lysates were analyzed to make sure they contained the same initial β-gal activity and conductance before carrying out the adsorption processes. Adsorption kinetics curves were obtained as shown in Fig. 4. The β-gal activity still remaining in the supernatant was around 30% in all the cases, after 120 min of recirculation.

The data of the remaining activity in the suspension vs. time were fitting assuming a first-order kinetics following an equation similar to Eq. (3). The uptake kinetic constant (kc) value was calculated from the experimental data, as shown in Table 2.

It can be seen that the kc values are affected by a great uncertainty. Statistical tests showed that there is no significant difference among them, except for the kc value obtained in the lysate in the absence of biomass. In this case, the kc suggests that the presence of biomass decreases the enzyme uptake kinetic, but no difference between 10 and 40% of biomass was observed. As the amount of β-gal absorbed onto the matrix seems to be the same using the different lysates, we decided to continue working with 40% cell lysate to avoid a centrifugation step.

The elution profile of β-gal after the adsorption of 40% cell lysate is shown in Fig. 5. After exhaustive washing of the matrix, the elution was carried out with increasing NaCl concentration buffers. The biggest amount of β-gal was eluted at 400 mM NaCl, reducing 5 times the total protein concentration. The best purification was obtained at 500 mM NaCl since there is a lower amount of total protein content. Table 3 shows the purification table considering a pool of 1 mL-fractions (number 21–50). A recovery of 65% of the

Table 3
Purification table.

	40% cell lysate	Eluate (fractions 21–50 mL)
Volume (mL)	80	30
β-Gal activity (AU/mL)	164.1	284.5
Total activity (AU)	13,132	8536
Protein concentration (mg/mL)	1.77	0.244
Protein amount (mg)	141.9	7.32
Specific activity (AU/mg)	92.54	1166
Recovery (%)	100	65
Fold purification	1	12.6

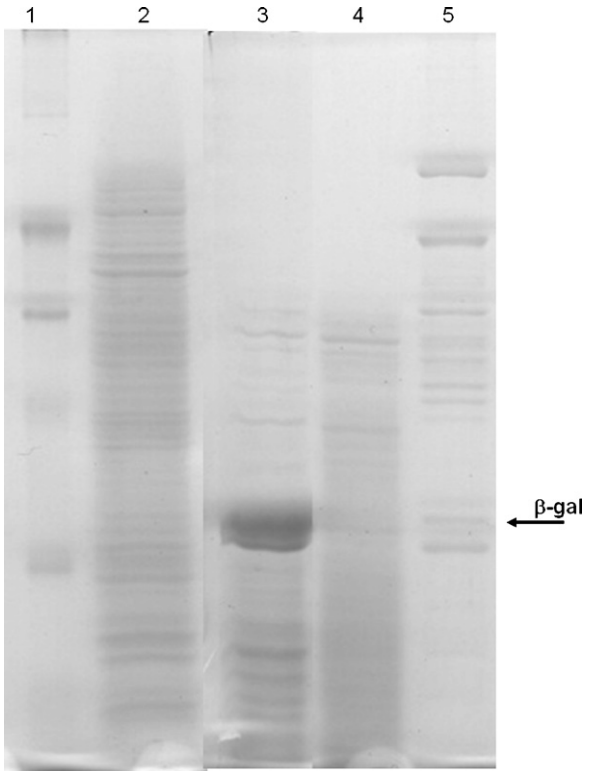

Fig. 6. SDS-PAGE in the gel, Lane 1: molecular weight standard; Lane 2: lysate; Lane 3: fraction conteining partialy purified β-Gal (eluted with NaCl 0.4 M + 0.5 M); Lane 4: fraction eluted with NaCl 0.3 M; Lane 5: commertial β-galactosidase.

initial enzyme concentration with a purification factor of 12.6 was obtained.

The purity of recombinant β-galactosidase obtained after the purification process was evaluated by SDS-PAGE as shown in Fig. 6.

4. Conclusions

The aim of the present study was to improve the application of the EBA, based on ionic exchange, to the recovery of a slightly acid protein, β-gal, by covering the matrix with PVP.

We have demonstrated that the PVP-covering of Streamline-DEAE does not affect the adsorption capacity of the matrix. However, the presence of PVP covering the matrix induced a slight decrease in the adsorption kinetic and affinity, which is consistent with a steric hindrance of this polymer on the matrix active site.

The covering of the matrix with PVP avoided the clogging, the channel formation and the bed stability was significantly improved. This allowed us to apply the PVP-covered Streamline-DEAE to the purification of β-gal, obtaining a recovery of 65%, increasing 12.6 times the purity of the sample in one single operation and avoiding thereby centrifugation. It is remarkable that it was possible to work with a lysate containing an unusually high amount of cell debris, improving the capacity of the process.

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