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Journal of Chromatography A, 944 (2002) 217–224

JOURNAL OF CHROMATOGRAPHY A

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Preparative chromatography of xylanase using expanded bed adsorption

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Abstract

Expanded bed adsorption was used to purify a marketable xylanase often used in the kraft pulp bleaching process. Experiments in packed and expanded beds were carried out mainly to study the adsorption of xylanase on to a cationic adsorbent (Streamline SP) in the presence of cells. In order to study the presence of cells, a *Bacillus pumilus* mass (5% wet mass) was mixed with the enzyme extract and submitted to an expanded bed adsorption system. One xylanase was purified to homogeneity in the packed bed. However, the 5% cell content hampered purification. © 2002 Published by Elsevier Science B.V.

Keywords: Kraft pulp; Expanded bed adsorption; Adsorption; Preparative chromatography; Xylanase; Enzymes

1. Introduction

Xylanases are enzymes able to hydrolyse xylan, an abundant biopolymer mainly composed of heteropolysaccharides, which can be found in plant tissues as a major component of the cell wall. A variety of applications in the bioconversion and food industries have been suggested for xylanases, and one of their major potential applications is in the pulp and paper industry. In this case, commercial xylanases have been used in the bleaching process under alkaline conditions showing great potential by reducing the quantity of polluting organic chlorine compounds required. Xylanase prebleaching technology is now in use at several mills around the world; the main motivating factors for this technology are the economic and environmental advantages that xylanase offers to the bleach plant [1]. The optimum enzyme for this process should be active at high temperatures and under alkaline pH conditions, hence it could be introduced at different stages of the bleaching process without requiring changes in pH or temperature [2–4].

However, the characteristics of biotechnological systems make purification the most expensive part of biomaterials process production. Spalding [5] has commented that values as high as 50% of overall costs in the biotechnology industries are related to downstream processing. Thus, the development of new and economically advantageous purification methods is a challenging area. Purification of target proteins requires their separation from the media or from the raw extract used for the maintenance of the biomolecules. Expanded bed adsorption (EBA) [6–10] is quite a good alternative to be used in initial steps of downstream processing since it allows that particulate-containing feedstock to be submitted directly to the column without need for a prior

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0021-9673/02/$ – see front matter
PII: S0021-9673(01)01368-1

clarification step (centrifugation or filtration). Hence, it generally leads to a reduction in processing time, preventing harmful molecules like proteases, nucleic acids, etc., can interact with the target biomolecule, thereby reducing its biological function. Moreover, EBA offers an overall cost reduction, since there is no need for clarification equipment.

Even though EBA is playing an important role in downstream processing, some questions are presently arising, such as for instance, what is the effect of cells on EBA performance? Is there a cell content limit for using EBA without hampering in any way bed hydrodynamics?

The aim of this work was to purify a xylanase from a commercial extract (Pulpzyme HC, Novonordisk) used in the kraft pulp bleaching process using EBA as well as to study the effect of cells on this purification process.

2. Experimental

2.1. Materials

2.1.1. Pulpzyme HC and reagents

Pulpzyme HC was a kind gift from Novonordisk (São Paulo, Brazil). All other chemicals were purchased from Sinthy (São Paulo, Brazil) and were of analytical grade.

2.1.2. Microorganism

In order to simulate the presence of cells, a mass of *Bacillus pumilus* (5% wet mass) previously isolated in our laboratory was mixed with the Pulpzyme HC extract and submitted to a EBA system. The wild strain was maintained on slants containing xylan from corn.

2.1.3. Adsorbent

Streamline SP, a strong cation exchanger, was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Streamline SP particles are spherical with a particle density of 1180 kg m^{-3} and a particle size of 194.5 μm, composed of a crystalline quartz core covered by 6% cross-linked agarose.

2.2. Equipment

An acrylic custom-made column (25.0 cm×2.5 cm I.D.) was fitted with an adjustable piston in order to minimise headspace over the fluidised bed. A perforated distributor was put at the bottom of the column. It was made of stainless steel with five orifices 1.0 mm in diameter giving a 0.8% open area ratio. A 60-μm mesh was used both below the perforated plate distributor and on the inner piston surface in order to avoid loss of adsorbent by backflow or by elutriation, respectively. A ruler was put on the column wall in order to help the bed height record. Fig. 1 illustrates a diagram of the column used in this work. The column was coupled to a Gradifac system (Amersham Pharmacia

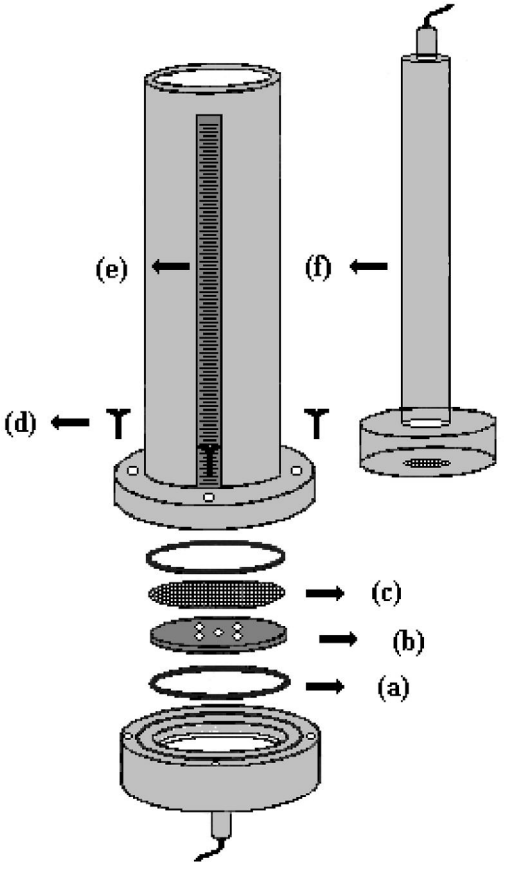

Fig. 1. Drawing of the column used in this work: (a) rubber gasket, (b) distributor (perforated plate), (c) screen (60 μm), (d) screw, (e) ruler, (f) piston.

Biotech). This system consists basically of a mixer, a UV monitor, a chart record and a controller as well as a peristaltic pump (P-50) that allows flow rates from 0.1 to 50.0 ml min^{-1} to be obtained.

2.3. Procedures

2.3.1. Residence time distribution (RTD)

RTD experiments for the bed expanded without cells were carried out in order to determine the axial liquid dispersion coefficient for the range of flow rates used in this work. In our case, 0.5% (v/v) acetone in Tris–HCl (50 m*M*, pH 6.9) at 25°C was used as a tracer in a negative-step input UV signal in order to obtain the number of theoretical plates (N), as described in Ref. [11]. The procedure for RTD determination is described below.

The buffer (50 m*M* Tris–HCl, pH 6.9) was pumped upwards through the bed at a chosen velocity until bed expansion had stopped. Then the piston was moved down to about 1.0 cm above the expanded bed surface. When the signal from the UV monitor was stable, the solution was changed to buffer–acetone (0.5%, v/v), i.e. the positive-step input signal. The solution was then changed to buffer when the UV signal was stable at maximum absorbance (100%), i.e. the negative-step input signal. The change was marked on the chart recorder paper and the UV signal was allowed to stabilise at the baseline level (0%). The number of theoretical plates (N) was calculated from the negative-step input signal as:

$$N = t^2/\sigma^2$$

where t is the mean residence time, i.e. the distance from the ''mark'' on the chart recorder paper to 50% of the maximum absorbance, and σ is the standard deviation, i.e. half the distance between the points for 15.85 and 84.15% of the maximum absorbance. The relation between the axial liquid dispersion coefficient (D_L) and N is:

$$D_L = (UH_{EXP})/(2\varepsilon N)$$

where U is the superficial velocity, H_{EXP} is the expanded bed height, and ε is the bed voidage in expanded mode, calculated as $\varepsilon = 1-[(1-\varepsilon_o).(H_o/H_{EXP})]$. In the packed bed, the bed voidage (ε_o) is approximately 0.4. H_o is the packed bed height.

2.3.2. Preparation of cells containing extract

B. pumillus was grown in Erlenmeyer flasks containing xylan medium according to Duarte et al. [12]. Inoculated flasks were shaken continuously at 200 rpm and 40°C for 24 h. After centrifugation supernatant was discarded and the whole cells were weighed and shaken overnight with Tris–HCl buffer (50 m*M*, pH 6.9) followed by addition of the Pulpzyme HC extract.

2.3.3. Packed bed experiment

In this experiment, the column contained a packed bed 5.0 cm in height, i.e. a column volume (CV) of 25.0 ml of Streamline SP. The column was equilibrated with 5 CVs of Tris–HCl (50 m*M*, pH 6.9). Both the loading (20.1 mg ml^{-1} total protein and 94 726 U ml^{-1} total activity) and the washing steps were carried out at 4.9 ml min^{-1} (60 cm h^{-1}) using 1.8 and 3.0 CVs, respectively. Linear gradient elution was carried out using 2.6 CVs at 2.5 ml min^{-1} (30 cm h^{-1}) with Tris–HCl (50 m*M*, pH 6.9) as buffer A and 1.0 *M* NaCl in Tris–HCl (50 m*M*, pH 6.9) as buffer B.

2.3.4. Expanded bed experiments with and without cells

In these experiments, the column contained a packed bed 5.0 cm in height ($H_o = 5.0$ cm; 25.0 ml) of Streamline SP. Both the loading (70.4 mg ml^{-1} total protein and 111 796 U ml^{-1} for the experiment without cells; 19.6 mg ml^{-1} total protein and 192 738 U ml^{-1} for the experiment with cells) and the washing steps were carried out at 8.2 ml min^{-1} (100 cm h^{-1}) using 1.8 CV and 3.0 CVs, respectively. Linear gradient elution was carried out using 2.6 CVs at 4.9 ml min^{-1} (60 cm h^{-1}) with Tris–HCl (50 m*M*, pH 6.9) as buffer A and 1.0 *M* NaCl in Tris–HCl (50 m*M*, pH 6.9) as buffer B. In both cases, a degree of expansion (H_{EXP}/H_o) of 1.3 was observed.

2.4. Assays

2.4.1. Assay of xylanase activity

Xylanase activity was assayed according to Bailey et al. [13] with 1% birchwood xylan solution in

glycine–NaOH buffer (100 m*M*, pH 10.0) at 55°C. The amount of reducing sugars was determined according to Miller [14]. One unit of xylanase activity was defined as 1 μmol of xylose produced per min under the given conditions.

2.4.2. Determination of protein

Determination of total protein was carried out according to the Sedmak and Grossberg [15] modified method, which is based on the drop in absorbance at 595 nm compared to the maximum dye absorbance recorded (465 nm) due to protein–dye binding. Using the modified method, 100 μl of the sample (or any value up to 1500 μl) was added to 1400 μl (or any value depending on sample volume) of water and 1500 μl of Coomassie blue solution in a cuvette. Then, the contents of the cuvette were mixed well and the A_{595}/A_{465} ratio was recorded using a spectrophotometer (UV–Vis 911A, GBC Scientific Equipment, Victoria, Australia). A A_{595}/A_{465} protein curve had previously been obtained for several protein contents using bovine serum albumin (BSA) as the standard protein.

2.4.3. Purification factor and yield

Purification factor (PF) is defined as the mass (specific activity) of xylanase pooled in the elution step divided by the mass (specific activity) of xylanase in the feedstock, i.e. purification factor = $(\text{specific activity of xylanase})_{\text{elution}}/(\text{specific activity of xylanase})_{\text{feedstock}}$.

Yield (%) is defined as the concentration of xylanase pooled in the elution step divided by the concentration of the xylanase in the feedstock, i.e. yield = $(\text{activity of xylanase})_{\text{elution}}/(\text{activity of xylanase})_{\text{feedstock}}$.

2.4.4. Electrophoresis

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in 12% homogeneous gel [16]. The gels were stained with Bio-Rad silver (Hercules, CA, USA). The molecular mass (M_r) markers were phosphorylase *b* (94 000), BSA (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000) and α-lactalbumin (14 400), available as a standard kit (Amersham-Pharmacia Biotech).

3. Results and discussion

3.1. Packed bed experiment

The experiment in packed bed mode was carried out in order to study the behaviour of Pulpzyme HC adsorption on to Streamline SP under *plug-flow* conditions. Fig. 2 shows that two peaks showed xylanasic activity (probably, iso-enzymes) when a linear salt gradient was used. The first one was eluted with about 0.18 *M* NaCl and the second one was eluted with 0.85 *M* NaCl. This means that the latter rather than the former interacted strongly with the adsorbent. However, only the first peak was purified to homogeneity, as can be observed by SDS–PAGE (Fig. 3). In fact, 6.6 mg of total protein and 930 013 U of xylanasic activity were present in the tubes that showed the purified enzyme (peak 1) with a purification factor of 30 and a 21.8% yield (Table 1); 129.4 mg of total protein and 2 834 684 U of xylanasic activity with a purification factor of 4.7 and a 66.5% yield were found for peak 2.

3.2. Expanded bed experiments with and without cells

The previous experiment, using a packed bed in a *plug-flow* mode, was successful in purifying a xylanase from a commercial extract. However, one knows that the hydrodynamics in the EBA is mainly influenced by the axial liquid dispersion coefficient [17–20], as well as the column distributor [21]. This means that previous knowledge of the RDT is sufficient to know whether a *plug flow* or *a back-mixing* mode is present in the bed. Fig. 4 illustrates the typical axial liquid dispersion coefficient values for our system [using Tris–HCl (50 m*M*, pH 6.9) as fluidising liquid], including the flow rate used in this work. It can be seen that for the 100 cm h^{-1} (8.2 ml min^{-1}) linear velocity used there is a low axial liquid dispersion coefficient (5.3×10^{-6} m^2 s^{-1}), i.e. there is no significant deviation from *plug flow*. However, there are few papers dealing with the effect of cells on purification in EBA. Fernández-Lahore et al. [22] have studied the effect of cells on the hydrodynamics of EBA. It has been shown that a 5% cell content is enough to alter the hydrodynamics of an expanded bed as well as that an anionic

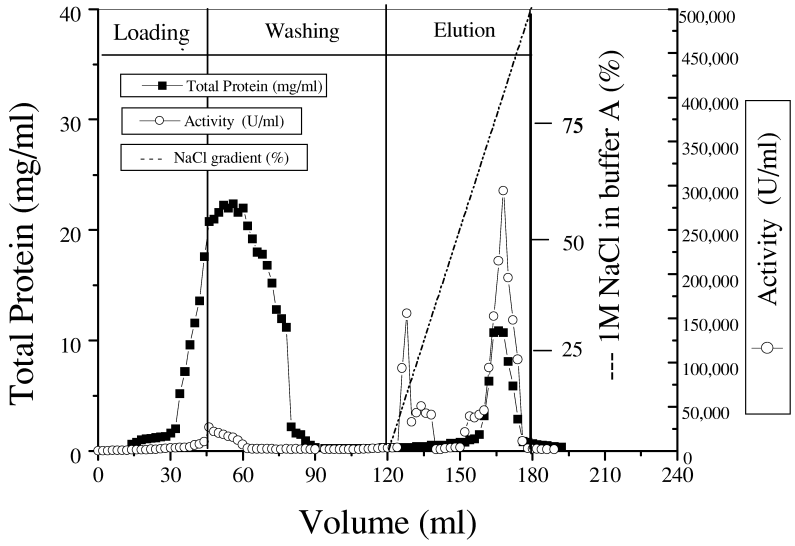

Fig. 2. Xylanase purification using packed bed. Dotted line shows the salt linear gradient as the percentage of the 1 *M* NaCl in Tris–HCl (50 m*M*, pH 6.9) (buffer B).

exchanger rather than a cationic one can interact strongly with cells due to its surface charges (negative charges are present on the cell surface). Based on this fact, we have chosen to study the effect of a 5% cell content (wet mass) on the purification of Pulpzyme HC.

Chromatograms for the experiments without and with cells are shown in Figs. 5 and 6, respectively. For the expanded bed without cells, 76.2 mg of total protein and 1 667 440 U of xylanasic activity were found, giving a 13.8-fold purification factor and a 21.8% yield for peak 1. Peak 2 showed 232.8 mg of total protein and 1 193 318 U of xylanasic activity with a 3.2-fold purification factor and a 23.7% yield (Table 2).

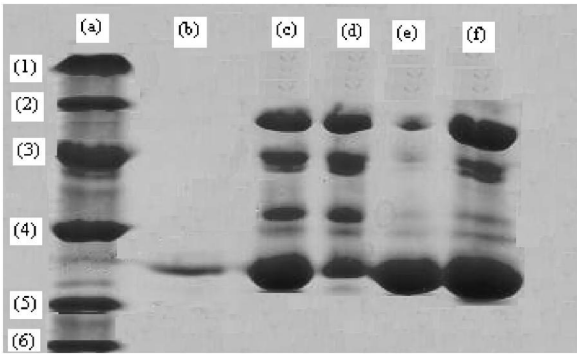

Fig. 3. SDS–PAGE stained with Coomassie blue for the tube that showed the xylanase purified (first peak) in packed bed. (a) Markers, (b) purified xylanase (peak 1), (c) total elution, (d) total washing, (e) total loading, (f) feedstock. (1) Phosphorylase *b* (94 000), (2) BSA (67 000), (3) ovalbumin (43 000), (4) carbonic anhydrase (30 000), (5) soybean trypsin inhibitor (20 000) and (6) α-lactalbumin (14 400).

The effect of the presence of cells in the bed can be observed in Fig. 6. It can be seen that, in fact, the presence of cells itself is enough to change the chromatogram completely. Even though a cationic adsorbent was used in this experiment, a non-uniform performance was obtained. This fact becomes most evident when salt is applied to the column. This may be the reason why it is quite difficult to obtain with confidence a DTR curve using a salt (e.g. NaCl, KCl) as tracer [11], mainly for a high cell content. In this case, other properties like fluorescence are helpful. We would like to highlight that as a *plug flow* mode exists under the conditions of the experiment, only the presence of cells justifies the difference between both experiments. When a 5% (wet mass) cell content was present in the bed there was a decrease in both purification factor and the yield (Table 3) with some xylanase activity leaving the column in the washing step (peak 1). Xylanase

Table 1
Purification in packed bed

Step	Volume (ml)	Protein (mg)	Activity (U)	Sp. act. (U/mg)	Purification factor	Yield (%)
Feedstock	45	906	4 262 428	4705	1.0	100
Loading	45	171	109 755	641	–	–
Washing	75	591	388 872	658	–	–
Peak 1	16.4	6.6	930 013	140 911	29.9	21.8
Peak 2	29.1	129.4	2 834 684	21 906	4.7	66.5

activity of the second enzyme (peak 2) was widely distributed in the elution. In this case, just a 1.7-fold purification factor and a 21.8% yield were obtained for this peak.

4. Conclusions

We have studied the effect of cells on the purification of a commercial xylanase applied to kraft pulp bleaching. At least one iso-xylanase was purified to homogeneity in the packed bed. In this case, it was possible to obtain a 30-fold purification factor as well as a 21.8% yield for peak 1 and a 4.7-fold purification factor as well as a 66.5% yield for peak 2. For the experiment in the expanded bed without cells, a 13.8-fold purification factor and a 21.8% yield were obtained for peak 1. A 3.2-fold purification factor and a 23.7% yield were obtained for peak 2. However, a 5% cell content (wet mass) was enough to alter the bed hydrodynamics even though a cationic adsorbent had been used. In this case, peak 1 was eluted in the washing step (a 2.3-fold purifica-

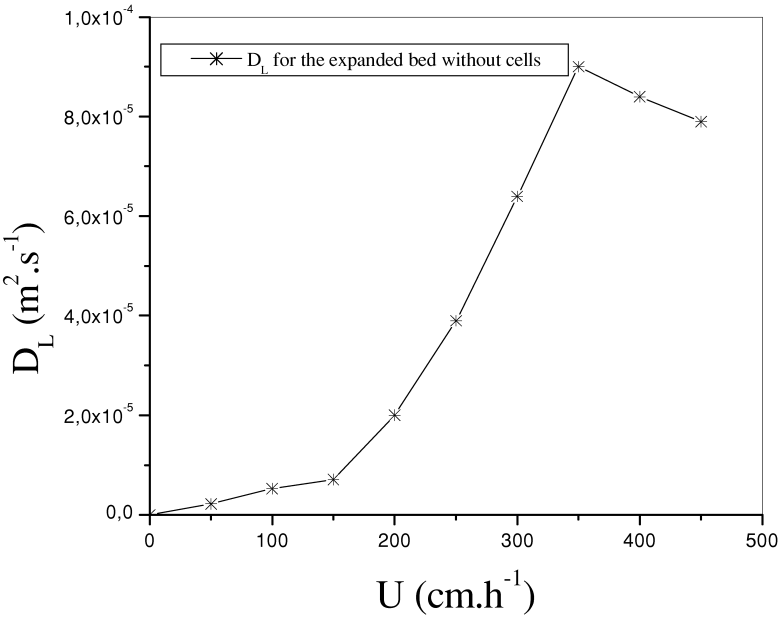

Fig. 4. Axial liquid dispersion coefficient for different linear velocities.

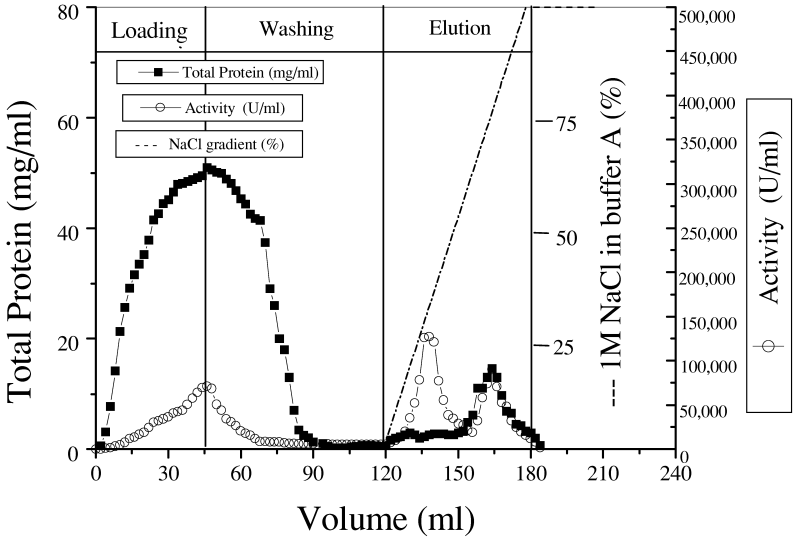

Fig. 5. Xylanase purification using expanded bed without cells. Dotted line shows the salt linear gradient as the percentage of the 1 *M* NaCl in Tris–HCl (50 m*M*, pH 6.9) (buffer B).

tion factor and a 13.2% yield) and peak 2 was widely distributed during the elution step, in this case a 1.7-fold purification factor and a 21.8% yield were obtained.

Acknowledgements

The authors would like to thank Novonordisk (Brazil), FAPESP (Fundação de Amparo à Pesquisa

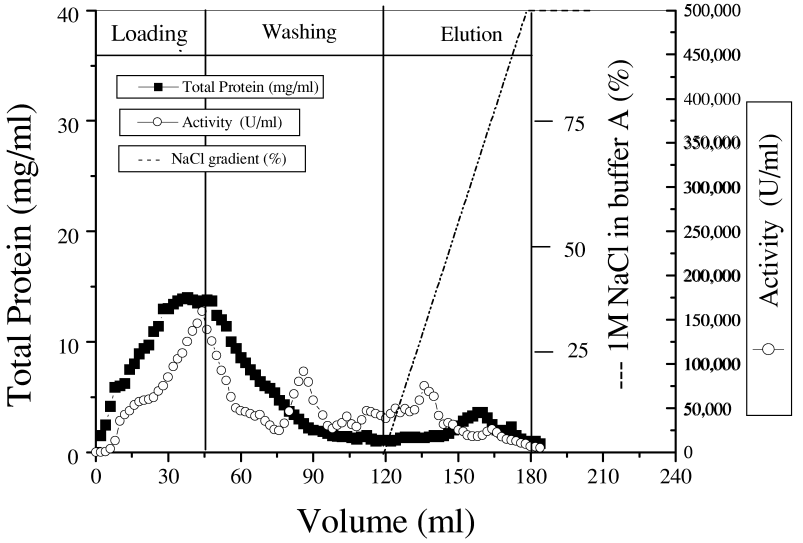

Fig. 6. Xylanase purification using expanded bed with cells. Dotted line shows the salt linear gradient as the percentage of the 1 *M* NaCl in Tris–HCl (50 m*M*, pH 6.9) (buffer B).

Table 2
Purification in expanded bed without cells

Step	Volume (ml)	Protein (mg)	Activity (U)	Sp. act. (U/mg)	Purification factor	Yield (%)
Feedstock	45	3169	5 030 838	1587	1.0	100
Loading	45	1458	1 137 845	780	–	–
Washing	75	1402	1 032 235	736	–	–
Peak 1	29.0	76.2	1 667 440	21 882	13.8	33.1
Peak 2	29.1	232.8	1 193 318	5126	3.2	23.7

Table 3
Purification in expanded bed with cells

Step	Volume (ml)	Protein (mg)	Activity (U)	Sp. act. (U/mg)	Purification factor	Yield (%)
Feedstock	45	883	8 673 210	9822	1.0	100
Loading	45	414	2 928 180	7073	–	–
Washing	75	354	3 853 150	10 885	1.1	–
Peak 1 (in washing)	24.2	50.7	1 148 180	22 646	2.3	13.2
Peak 2	65	115	1 891 880	16 451	1.7	21.8

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