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Journal of Chromatography B, 743 (2000) 339–348

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Partitioning of xylanolitic complex from *Penicillium janthinellum* by an aqueous two-phase system

Silgia Aparecida Costa[a], Adalberto Pessoa Jr.[b,*], Inês Conceição Roberto[a]

[a]*Department of Biotechnology, Faculty of Chemical Engineering of Lorena, Rod. Itajubá-Lorena, Km 74,5. 12.600-000, Lorena/SP, Brazil*

[b]*Biochemical and Pharmaceutical Department/FCF/USP, CEP 05315-970, P.O. Box 66083, São Paulo-SP, Brazil*

Abstract

This work evaluates the influences of five parameters (pH, PEG molecular mass, PEG concentration, concentration of buffer K_2HPO_4–KH_2PO_4 and NaCl concentration) on xylanolitic complex partitioning produced by *P. janthinellum* in aqueous two-phase systems, using a 2^5 factorial experimental design. A mathematical model to quantify the influence of these parameters was attained and statistically tested. The optimum point for total protein extraction was obtained under the following conditions: pH 7.0, PEG 10 000, 3.67% PEG, 10% potassium phosphate and 12.4% NaCl. The partition coefficient (K) value experimentally obtained was 5.25 and that predicted by the model was 5.89.

Keywords: Partitioning; Aqueous two-phase systems; *Penicillium janthinellum*; Experimental design; Xylanolitic complex

1. Introduction

The development of techniques and methods for the separation and purification of proteins has been an important prerequisite for many of the advances made in the biotechnology industry [1–4]). Aqueous two-phase systems (ATPS), either polymer–polymer–water or polymer–salt–water, provide a method which has several advantages, including biocompatibility, and it can be easily scaled up to the industrial level [5]. Proteins partition unevenly in the two phases, depending on the nature of the polymer molecular mass, polymer and salt concentration, ionic composition and strength, and pH of the system, together with the size, charge and hydrophobicity of the biomolecule, which are the principal factors influencing the partition coefficient [6,7]. Factors and mechanisms that cause the uneven distribution of proteins between the two phases are little understood, but empirical rules have been developed. In practice, the technique requires further experimentation to find an adequate system for each particular application [8].

Biodegradation of xylan, a major component of the plant cell wall, requires the combined action of several enzymes [9]. The enzymatic complex produced by *P. janthinellum* is composed of endoxylanases that cleave internal xylosidic linkages on the xylan backbone and β-xylosidases that release xylosyl residues by endwise attack of xylooligosaccharides. The recent interest in xylan-degrading enzymes comes from their potential use in the pulp and paper industry for biobleaching, thus reducing the amount of toxic bleaching chemicals in plant effluents. According to Durán et al. [10] xylanases

*Corresponding author. Fax: +55-11-815-6386.
E-mail address: pessoajr@usp.br (A. Pessoa Jr.)

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00212-7

produced by *P. janthinellum* can be used for reducing the chlorine charge in eucalyptus-pulp bleaching with a simultaneous gain in brightness. These enzymes are also important in clarification of beer and juice, and in partial xylan hydrolysis in animal feed [11].

In this study, we identified some important variables to the process of liquid–liquid extraction of a xylanolitic complex from *P. janthinellum* using an aqueous two-phase system.

2. Experimental

2.1. Microorganism and culture media

The microorganism *P. janthinellum*, isolated from decaying wood by Milagres [12], was identified by the Biosystematic Research Center of Canada and deposited in their collection under the designation of CRC 87 M-115. The strain was initially maintained in silica stocks and later on agar slants. The fungus was cultivated at 30°C for 5 days in medium containing 1% (w/v) glucose, 0.1% (w/v) yeast extract, 2% (v/v) concentrated salt solution based on Vogel's medium [13] and 2% (w/v) agar–agar. The medium was autoclaved at 121°C for 15 min. The spore inocula were obtained by suspending spores in water, filtering through gauze and pouring the filtrate into Erlenmeyer flasks. The final concentration of spores was 10^5/ml.

2.2. Preparation of sugar cane bagasse acid hydrolysate

In order to prepare the hydrolysate for fermentation, 800 g of dry milled bagasse was mixed with 8 l of sulphuric acid solution (0.25%) and autoclaved for 45 min at 121°C. The liquid fraction was separated by filtration and the pH adjusted to 5.5 with NaOH.

2.3. Enzyme production and determination

The cultivation medium for enzyme production was composed of the sugar cane bagasse hemicellulosic hydrolysate described above supplemented with 2% (v/v) concentrated salt solution based on Vogel's medium [12] and 1% yeast extract. The medium was then autoclaved for 15 min at 121°C. Shake-flask cultures were grown in Erlenmeyer flasks (125 ml) containing 25 ml of medium. Standard cultivation conditions were: temperature 30°C; initial pH 5.5 (uncontrolled); cultivation time 96 h. The medium was then filtered and the cells collected. Xylanase activities were determined by incubating 0.5 ml of diluted culture filtrate with 0.5 ml of an Birchwood xylan suspension (10 $g\,l^{-1}$) in 0.05 *M* phosphate buffer (pH 5.5) for 5 min at 50°C. The released reducing equivalents were measured by a colorimetric assay [14] based on the absorbance at 540 nm using xylose solution as a standard reference. Activity units were expressed as micromoles of reducing equivalents released per minute.

2.4. Protein determination and analytical electrophoresis

The amount of total protein was determined according to the Coomassie Blue method described by Bradford [15] using bovine serum albumin (BSA) as a protein concentration standard. Sodiumdodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed according with method described by Weber et al. [16].

2.5. Preparation of phase systems

The extraction studies were carried out without cells. Phase systems were prepared from PEG, phosphate (KH_2PO_4–K_2HPO_4) and NaCl in solid form. A 3-g amount of medium containing xylanase was added to the systems (total mass=10 g) and deionized water was used to adjust the concentrations (expressed as %, w/w) of the components to the desired values. By varying the proportion between KH_2PO_4 and K_2HPO_4 the pH of the system was adjusted. Centrifugation (2500 *g*, 10 min) to speed up phase separation was used after thorough vortex-mixing of the system components and the phase volume ratios were determined using graduated centrifuge tubes. Samples of the top and bottom phases were then assayed as for enzyme activity. During all partition experiments the temperature was ~25°C. All component (PEG, NaCl and

Table 1
Factors and levels in the five-factor three-level response surface design used for optimizing the xylanase partition coefficient by ATPS

Run number	Factors	Inferior level (−1)	Superior level (+1)	Center point (0)
X1	pH	5.0	8.0	6.5
X2	MM PEG	600	6000	4000
X3	% PEG	10.0	35.0	22.5
X4	% Phosphate	10.0	25.0	17.5
X5	% NaCl	0.0	10.0	5.0

phosphate) concentrations in this work were expressed as % (w/w).

2.6. Experimental designs and statistical analysis

A 2^5 experimental design [17] was used to evaluate the effect of the pH (X_1), PEG MM (X_2), PEG concentration (% w/w) (X_3), phosphate potassium concentration (% w/w) (X_4) and NaCl concentration (% w/w) (X_5) on the partition coefficient variable. For each of the five factors, high (coded value: +1) and low (coded value: −1) set points were selected (Table 1). Table 2 presents all the 32 (2^5) set point combinations that were made, and the partition coefficients, *K*, obtained. A statistical model for an optimized partition coefficient employing the significant variables was determined by the response surface regression procedure. The model is expressed by Eq. (1)

$$\hat{Y}_i = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} b_{ii} X_i^2 + \sum_{i=1 j=i+1}^{n=1} \sum^{n} b_{ij} X_i X_j \qquad (1)$$

where Y_i = response variable, b_0, b_i, b_{ii} and b_{ij} = regression coefficients; X_i and X_j = variables under study.

2.7. Chemicals

Birchwood 4-O-methyl-β-D-glucoroxylan (90% xylose) was obtained from Sigma (St. Louis, MO, USA), and poly(ethylene glycol) (PEG) from Merck (Darmstadt, Germany). All the other chemicals were of analytical grade.

3. Results and discussion

Table 2 presents the design matrix of the variables (pH, MM-PEG, PEG concentration, phosphate potassium concentration and NaCl concentration) and the partition coefficients obtained after total protein extraction by ATPS. The results of the partition coefficients of total proteins varied in the range of 0 and 9 as a function of the conditions employed. Those equal to zero indicate that there was no phase separation, mainly due to the low concentration of the components or to the saturation of the phase systems. The treatment of the interaction effects on the partition-coefficient (*K*) is shown in Table 3. As can be seen the molecular mass of PEG (X_2), % PEG (X_3) and % NaCl (X_5) exerted a significant influence on the partition coefficient at the 5% level. The variables MM PEG (X_2) and % NaCl (X_5) both presented positive signal as main effects, whereas the variable % PEG (X_3) presented a negative signal. The second-order effects for the variables X_2X_3 and X_3X_5 were of negative signal. This indicates that the value of *K* can be increased with the increase in variable X_2 and decrease in variable X_3. The variables X_3X_5 presented the same effect, i.e. their partition coefficient values can be increased by decreasing and increasing X_3 and X_5 levels, respectively. The interaction effect of variables X_2X_5 gave a positive signal, suggesting that an increase in any one these variables can increase the value of *K*. No significant effect of the pH (X_1) was observed in this study. On the contrary, in a study of xylanase extraction, Costa et al. [18] reported that the effect of pH was significant, while the effect of MM PEG was insignificant. However, the later exerted influence on the extraction of total proteins.

Table 2
Protein partition coefficient (K) from 2^5-full factorial design with center point under different treatments

Assay no.	Assay sequence	Factors[b]					K[a]
		X_1	X_2	X_3	X_4	X_5	
1	25	−1	−1	−1	−1	−1	0
2	36	+1	−1	−1	−1	−1	0
3	20	−1	+1	−1	−1	−1	0
4	28	+1	+1	−1	−1	−1	1.08
5	22	−1	−1	+1	−1	−1	0
6	4	+1	−1	+1	−1	−1	0.23
7	34	−1	+1	+1	−1	−1	0
8	23	+1	+1	+1	−1	−1	0.39
9	8	0	0	0	0	0	0.40
10	14	−1	−1	−1	+1	−1	0
11	26	+1	−1	−1	+1	−1	0.56
12	6	−1	+1	−1	+1	−1	1.05
13	3	+1	+1	−1	+1	−1	0.68
14	12	−1	−1	+1	+1	−1	0
15	19	+1	−1	+1	+1	−1	0.18
16	7	−1	+1	+1	+1	−1	0
17	35	+1	+1	+1	+1	−1	0
18	16	0	0	0	0	0	0.38
19	9	−1	−1	−1	−1	+1	0
20	30	+1	−1	−1	−1	+1	0.35
21	17	−1	+1	−1	−1	+1	7.9
22	15	+1	+1	−1	−1	+1	1.89
23	5	−1	−1	+1	−1	+1	0
24	21	+1	−1	+1	−1	+1	0
25	29	−1	+1	+1	−1	+1	0
26	1	+1	+1	+1	−1	+1	0
27	24	0	0	0	0	0	0.44
28	2	−1	−1	−1	+1	+1	0
29	33	+1	−1	−1	+1	+1	0.42
30	31	−1	+1	−1	+1	+1	9.47
31	11	+1	+1	−1	+1	+1	2.16
32	18	−1	−1	+1	+1	+1	0
33	13	+1	−1	+1	+1	+1	0.11
34	27	−1	+1	+1	+1	+1	0
35	10	+1	+1	+1	+1	+1	0
36	32	0	0	0	0	0	0.43

[a] $K = (K_1 + K_2)/2$, Average partition coefficient.
[b] X_1 = pH, X_2 = PEG molecular mass, X_3 = % PEG, X_4 = % potassium phosphate, X_5 = % NaCl.

As a function of these results, an analysis of variance (ANOVA) was made considering all the significant effects shown in Table 4. The ANOVA showed that the determination coefficient value ($R^2 = 0.587$) was low, and that the P value for the lack-of-fit ($P < 0.05$) was significant, which indicates that the linear model is not adequate for this study. A new region of work was investigated. Initially a path of steepest ascent was proposed to alter the work region and to obtain higher partition coefficient (K) values. According to the estimated effects shown in Table 3, X_3 and X_5 (negative and positive signals, respectively) suggested that the displacement of the experimental region should be made by decreasing the X_3 level and increasing X_5 level. X_2 was not coded because the value of PEG molecular mass commercially available does not adjust to the values calculated by the mathematical equations. Considering that the experimental design suggests an increase in PEG molecular mass and that PEG-10 000 solution

Table 3
Estimated effects, standard errors and Student's *t*-test of 2^5 factorial design with four center points

Variables[b]	Estimated effects	Standard error	*t* Values
Average	0.761	±0.227	–
X_1	−0.648	±0.482	1.34
X_2	1.388	±0.480	2.89[a]
X_3	−1.540	±0.482	3.19[a]
X_4	0.174	±0.482	0.36
X_5	1.133	±0.482	2.35[a]
X_1X_2	−0.879	±0.482	1.82
X_1X_3	0.761	±0.482	1.58
X_1X_4	−0.153	±0.482	0.32
X_1X_5	−0.906	±0.482	1.88
X_2X_3	−1.439	±0.482	2.98[a]
X_2X_4	0.088	±0.482	0.18
X_2X_5	1.144	±0.482	2.37[a]
X_3X_4	−0.215	±0.482	0.45
X_3X_5	−1.219	±0.482	2.53[a]
X_4X_5	0.078	±0.482	0.16

[a] Significant at the 5% level ($t=2.08$).
[b] X_1 = pH, X_2 = PEG molecular mass, X_3 = %PEG, X_4 = % potassium phosphate, X_5 = % NaCl.

is too viscous, we decided to vary only X_3 and X_5 and to fix the PEG molecular mass at 10 000. This decision can explain the reason why the *K* value did not reach its highest value ($K=9.47$) shown in Table 2, even at the optimized extraction conditions (Fig. 1). PEG-10 000 has the advantage of being easily handled because it is in the form of a very fine powder. The centre point was used to start the displacement of the experimental region. Table 5 shows the displacement levels, coded variables and

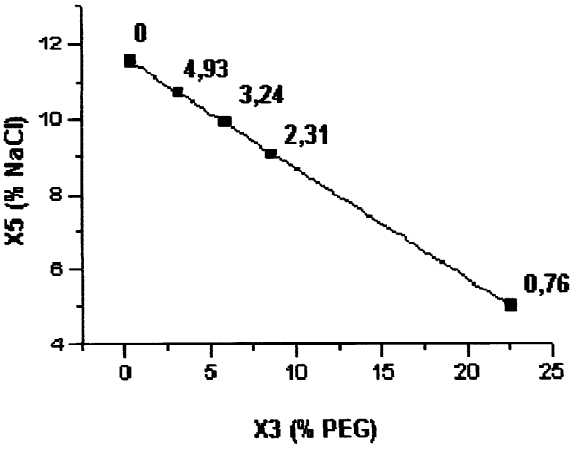

Fig. 1. Trajectory of planning of a path of steepest ascent proposed in Table 5.

partition coefficient values. Regarding the displacement levels, it was observed that for each 0.22 retreated unity in the value of X_3 (%PEG) it is necessary to increase 0.17 unity in the value of X_5 (%NaCl). Substituting the displacement levels (Table 5) in Eqs. (2) and (3) the coded values of X_3 and X_5 were obtained

$$X_3 = (\%\ \text{PEG} - 22.5)/12.5 \quad (2)$$

$$X_5 = (\%\ \text{NaCl} - 5)/5 \quad (3)$$

The displacement of a path of steepest ascent using the coded variables can be seen through the trajec-

Table 4
Analysis of variance (ANOVA) for the significant variables (X_2, X_3 and X_5) that represent the liquid–liquid extraction of protein in the aqueous two-phase system quadratic model[a]

Source of variations[b]	Sum of squares	Degrees of freedom	Mean square	*F* value	*P* value
Factor X_2	15.544	1	15.544	8.94	0.0059
Factor X_3	18.988	1	18.988	10.92	0.0027
Factor X_5	10.271	1	10.271	5.91	0.0220
X_2X_3	16.574	1	16.574	9.53	0.0046
X_2X_5	10.476	1	10.476	6.03	0.0208
X_3X_5	11.895	1	11.895	6.84	0.0144
Lack of fit	11.952	2	5.976	3.44	0.0468
Pure error	46.936	27	1.738		
Total (corr.)	142.639	35			

[a] $R^2=0.587$.
[b] X_1 = pH, X_2 = molecular mass, X_3 = % PEG, X_4 = % potassium phosphate, X_5 = % NaCl.

Table 5
Values of displacement levels, coded variables and results of partition coefficients (K) of total protein obtained altering the work region

Treatment	Displacement levels		Coded variables		$K_{protein}$
	X_3 (%PEG)	X_5 (%NaCl)	X_3 (%PEG)	X_5 (%NaCl)	
1	0	0	22.50	5.00	0.76
2	−1.11	0.81	8.62	9.05	2.31
3	−1.33	0.98	5.87	9.90	3.24
4	−1.55	1.14	3.12	10.70	4.93
5	−1.77	1.30	0.37	11.70	0

tory presented in Fig. 1. Fig. 1 also shows that the K values of total proteins increased when the percentage PEG decreased from 22.5 to 3.12% and when the percentage NaCl increased from 5 to 10.7%. The increase in K value was observed until the 4th test. Starting from the 5th test K value decreased from 4.93 to 0. When K reached the maximum value ($K=4.93$) it was necessary to stop the displacement and investigate the region around this test. A 2^3 factorial design with centered face was proposed to study the region around the 4th test (Table 6).

Table 6 shows the matrix of the experimental design and the results of the partition coefficients (K). Substituting the negative and positive signals in Eqs. (4) and (5) it was possible to code the values of X_3 and X_5. The results of the partition coefficients obtained ranged between 0 and ~7.

$$X_3 = (\%\,\mathrm{PEG} - 3.12)/2.75 \quad (4)$$

$$X_5 = (\%\,\mathrm{NaCl} - 10.7)/1.7 \quad (5)$$

Based on these results, an ANOVA for K response variable was carried out considering all the effects (Table 7). This analysis revealed that X_3 and X_5 and the quadratic effect of X_3 had significant influence ($P<0.05$) on K value. From the lack-of-fit ($P>0.05$) it can be seen that the quadratic model presents a good fit for this study.

Table 8 shows the ANOVA of model regression. The model is significant at 5% probability level, giving a determination coefficient $R^2=0.91$. The percentage of variance, explained by the regression, that is the ratio between the sum of squares due to the regression and the total sum of squares, is 94%. This value can not be compared with 100% owing to the contribution of lack-of fit to pure error. In this study 99.66% of the model can be explained. The mathematical model to represent the process of liquid–liquid extraction of protein in aqueous two-phase system considering the significant variables is described by the following Eq. (6)

$$\hat{y} = 4.94 + 1.5X_3 + 0.79X_5 - 3.44X_3^2 \quad (6)$$

where $\hat{y}$ = partition coefficient, X_3 = coded % PEG level, X_5 = coded % NaCl level.

Table 6
Values of X_3 and X_5 variables coded by Eqs. (2) and (3) and the partition coefficients obtained

Treatment	% PEG	% NaCl	% PEG	% NaCl	K
1	−1	−1	0.37	9.0	0[a]
2	+1	−1	8.62	9.0	2.19
3	−1	+1	0.37	12.4	0[a]
4	+1	+1	8.62	12.4	3.63
5	−1	0	0.37	10.7	0[a]
6	+1	0	8.62	10.7	3.19
7	0	−1	3.12	9.0	3.45
8	0	+1	3.12	12.4	6.79
9	0	0	3.12	10.7	4.50
10	0	0	3.12	10.7	4.87
11	0	0	3.12	10.7	5.09

[a] No phase separation.

Table 7
ANOVA for the significant variables (X_3 and X_5) that represent the total protein liquid–liquid extraction in aqueous two-phase system

Source of variations[b]	Sum of squares	Degrees of freedom	Mean square	*F* value	*P* value
Factor X_3	13.530	1	13.530	152.19	0.0065[a]
Factor X_5	3.808	1	3.808	42.84	0.0226[a]
X_3X_5	0.518	1	0.518	5.83	0.1371
X_3^2	30.268	1	30.268	340.47	0.0029[a]
X_5^2	0.011	1	0.011	0.13	0.7534
Lack of fit	2.436	3	0.812	9.14	0.1003
Pure error	0.177	2	0.088		
Total (corr.)	52.725	10			

[a] $P<0.05$; $R^2=0.95$.
[b] X_3 = %PEG, X_5 = % NaCl.

The response surface for the model is shown in Fig. 2 and can be expressed by Eq. (6) showing the values of partition coefficient as a function of X_3 (% PEG) and X_5 (% NaCl). Deriving Eq. (6) and making it equal to zero, we can find the variable levels that you provide the maximum value for partition coefficient. The maximum total protein partition coefficient value predicted by the model was 5.89 (system: PEG-10 000, 3.67% PEG, 10% potassium phosphate and 12.4% NaCl) and the value experimentally obtained was 5.25. This value is considered to be good, when the standard error is taken into account.

To conclude this work an electrophoretic analysis was carried out to show the profile of the total proteins obtained after extraction carried out under the conditions defined by the experimental design. Electrophoresis was also carried out under the conditions optimized by Costa et al. [18] to extract xylanases from *P. janthinellum* by ATPS and compared in this work.

Fig. 3 shows the electrophoretic profile of the protein bands of the initial broth obtained from the cultivation of *P. janthinellum* in sugar cane bagasse hemicellulosic hydrolysate (line 3) and also from the top (line 4) and bottom phases (line 5) after the extraction using the optimum conditions of the xylanase experimental design (pH 7.0, PEG 4000, 8.83% PEG, 10% phosphate potassium and 6.02% NaCl). The initial broth contained 42.12 U/ml of xylanases and 269.86 mg/ml of total proteins. After concentration by lyophilization, 63.67 U/ml of xylanases and 407.55 mg/ml of total proteins were obtained. Line 3 (fraction of initial broth) shows the presence of nine bands of proteins with relative molecular masses in the range of $10\cdot10^3$, $20\cdot10^3$, $30\cdot10^3$, $45\cdot10^3$, $60\cdot10^3$, $80\cdot10^3$ and $100\cdot10^3$ Da. After the liquid–liquid extraction in aqueous two-phase system, the enzymes partitioned between the phases, those of lower molecular mass stayed on the top phase and those of higher molecular mass on the bottom phase. Line 4 (top phase obtained at the optimum point of xylanase extraction) shows the presence of three bands with relative molecular

Table 8
ANOVA for the total regression of the model that represents the liquid–liquid extraction process of total protein in aqueous two-phase system quadratic model[a]

Source of variations	Degrees of freedom	Sum of squares	Mean square	*F* value	*P* value
Model	3	49.580	16.526	36.79	0.0001
Lack of fit	5	2.968	0.593	6.67	
Pure error	2	0.177	0.088		
Total (corr.)	10	52.724			

[a] $R^2=0.91$; % of explained variance: 94%; % of explainable variance: 99.66%.

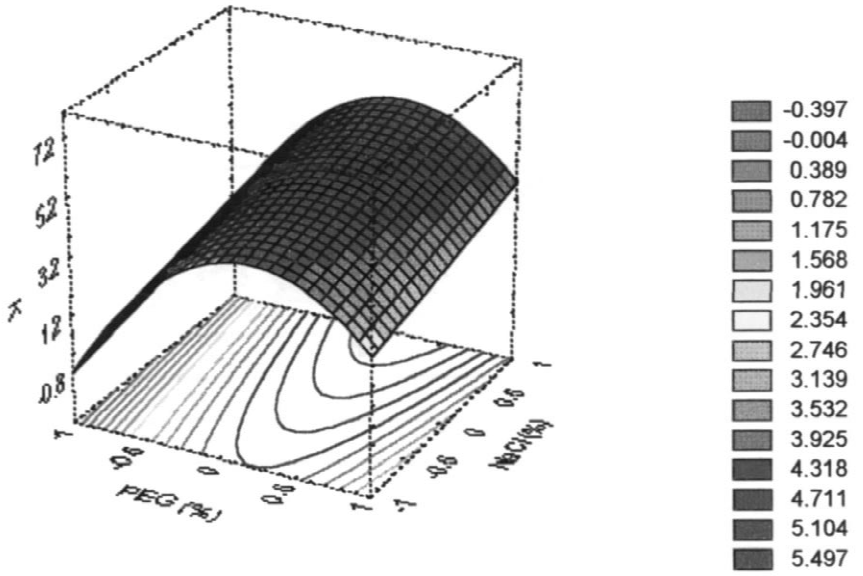

Fig. 2. Response surface for the partition coefficient (K) of total protein as a function of the variables X_3 (% PEG) and X_5 (% NaCl).

masses close to $14\cdot10^3$, $20\cdot10^3$ and $24\cdot10^3$. By comparing lines 3 (initial broth) and 4 it was observed that the initial broth presented several bands and after the extraction step only three of them were present. The results obtained by Rodrigues [19], using the technique of liquid–liquid extraction by reverse micelle, and by Cortez and Pessoa [20], using ethanol precipitation, suggest that the bands of 20 and $24\cdot10^3$ Da correspond to endoxylanases. With the optimized conditions 80% of xylanases was recovered and an enrichment factor of 1.34 times on the top phase was obtained. Concerning the band of $14\cdot10^3$ Da, it was found that this value is very close to the results obtained by Filho et al. [21], who purified an endoxylanase of $16\cdot10^3$ Da rel. mol. mass produced by *Myrthecium verrucaria,* and to the results obtained by Donnelly and Crawford [22], who obtained a p-coumaric esterase of $11\cdot10^3$ Da rel. mol. mass. Therefore, it is not possible to ascertain that the $14\cdot10^3$ Da rel. mol. mass band corresponds to one of these two enzymes.

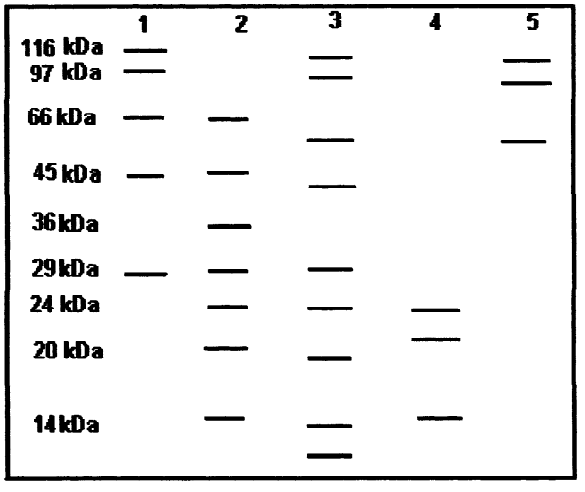

Fig. 3. Electrophoresis (SDS–PAGE) for the optimized conditions of xylanase liquid–liquid extraction in aqueous two-phase system. Lines: 1=markers of molecular mass ($116\cdot10^3$, $97\cdot10^3$, $66\cdot10^3$ and $45\cdot10^3$); 2=markers of low molecular mass ($66\cdot10^3$, $45\cdot10^3$, $36\cdot10^3$, $24\cdot10^3$, $20.1\cdot10^3$ and $14\cdot10^3$); 3=initial broth; 4=top phase of system; 5=bottom phase of system.

On line 5 an electrophoretic run was made using the bottom phase of the extraction system. The presence of three bands with molecular masses of $\approx60\cdot10^3$, $80\cdot10^3$ and $100\cdot10^3$ Da rel. mol. masses was observed. The band of $60\cdot10^3$ Da is probably an endoxylanase. Studies of precipitation with ethanol conducted by Cortez and Pessoa [20] showed bands of $104/110\cdot10^3$ Da rel. mol. mass, whereas Milagres [23] used a S-300 Sephacryl column and ultrafiltration, and obtained a band of $74.5\cdot10^3$ Da rel. mol.

mass. These authors concluded that these bands correspond to the β-xylosidase enzyme, which is present in the xylanolitic complex produced by *P. janthinellum*. The literature [24] reports that *Fusarium oxysporum* has produced endoxylanases with a relative molecular mass of $80 \cdot 10^3$ Da. A comparison between line 3 and lines 4 and 5 reveal that three enzymes whose relative molecular masses were $10 \cdot 10^3$, $30 \cdot 10^3$ and $50 \cdot 10^3$ Da were present in the initial broth but did not appear on lines 4 and 5. It is difficult to explain the absence of these bands. However, two hypotheses that can be suggested are the adherence of these enzymes to the interface of the system and the low protein concentration after partition, which impedes the detection of both the enzymes and the proteins. Fig. 4 shows the electrophoretic analysis of the optimum point of total protein extraction (pH 7.0, PEG 10 000, 3.67% PEG, 10% potassium phosphate and 12.4% NaCl). Line 3 (fraction of initial broth) presents the reproducibility of the bands shown in Fig. 3. Line 4 represents the top phase sample obtained after the extraction made at the optimum point of the total protein planning. The presence of varied bands in a range of $10 \cdot 10^3$, $20 \cdot 10^3$, $24 \cdot 10^3$, $30 \cdot 10^3$ and $10 \cdot 10^3$ Da rel. mol. masses can be observed. The $20 \cdot 10^3$ and $24 \cdot 10^3$ Da bands possibly correspond to the endoxylanase purified by Rodrigues [19]. Line 5 presents the bottom phase sample after the liquid–liquid extraction in aqueous two-phase system. The presence of bands in the range of $30 \cdot 10^3$, $40 \cdot 10^3$, $60 \cdot 10^3$, $80 \cdot 10^3$ and $100 \cdot 10^3$ Da rel. mol. masses was observed. The bands of $30 \cdot 10^3$, $40 \cdot 10^3$ and $60 \cdot 10^3$ Da probably correspond to the endoxylanases, since a significant quantity of studies on these enzymes report the same molecular masses [24]. Possibly the bands of $80 \cdot 10^3$ and $100 \cdot 10^3$ Da are β-xylosidases, for they correspond to the bands obtained in Fig. 3, line 5. Figs. 3 and 4 are included as electrophoretic chart to facilitate their understanding and to dispel possible doubts raised by the poor quality of the picture.

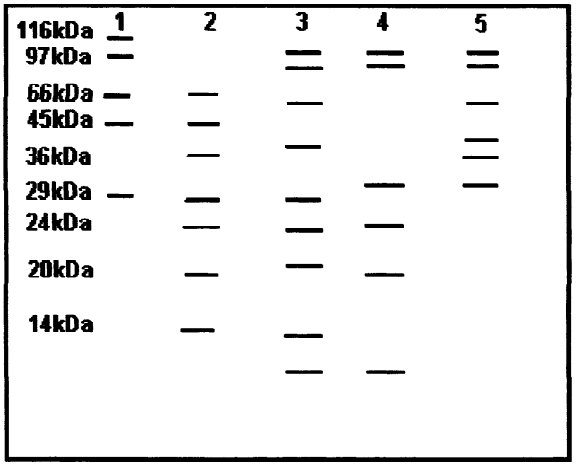

Fig. 4. Electrophoresis (SDS–PAGE) for the optimized conditions of total protein liquid–liquid extraction in aqueous two-phase system. Lines: 1=markers of molecular mass ($116 \cdot 10^3$, $97 \cdot 10^3$, $66 \cdot 10^3$ and $45 \cdot 10^3$); 2=markers of low molecular mass ($66 \cdot 10^3$, $45 \cdot 10^3$, $36 \cdot 10^3$, $24 \cdot 10^3$, $20.1 \cdot 10^3$ and $14 \cdot 10^3$); 3=initial broth; 4=top phase of system; 5=bottom phase of system.

4. Nomenclature

K	Partition coefficient (ratio of biomolecule concentration in the top phase to that in the bottom phase)
MM PEG	Poly(ethylene glycol) molecular mass
ATPS	Aqueous two-phase systems

Acknowledgements

S.A.C. acknowledges the financial support of CAPES/Brazil, in the form of a Master of Science fellowship, and FAPESP/São Paulo, Brazil. Thanks are also due to Adriane M.F. Milagres for her valuable suggestions, and to Maria Eunice M. Coelho for revising this text.

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