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β-Xylosidase Recovery by Reversed Micelles

Use of Cationic Surfactant

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Abstract

β-Xylosidase, an enzyme produced by *Penicillium janthinellum* fungus, was prepurified by fractionated precipitation with ethanol and extracted by reversed micelles of *N*-benzyl-*N*-dodecyl-*N*-*bis*(2-hydroxyethyl) ammonium chloride (BDBAC) cationic surfactant. A 2^{5-1} fractional factorial design was employed to evaluate the influence of the following factors on the enzyme extraction: pH (A), conductivity (B), surfactant concentration (C), cosolvent concentration (D) and temperature (E). A statistical analysis of the results revealed that, of the five variables studied, pH, surfactant concentration, and temperature had significant main effects ($p < 0.05$) on the recovery of β-xylosidase (Y). However, the interactions between pH and temperature, conductivity and cosolvent concentration, conductivity and temperature, BDBAC concentration and temperature also had significant influences. A first-order model ($R^2 = 0.98$) expressed by the equation $Y = 7.73 – 5.55A – 0.67B + 3.49C – 0.41D + 5.26E + 2.05AB – 3.26AC + 0.96AD – 7.07AE – 3.93BD – 2.19BE + 0.99CD + 0.78CE$ was proposed to represent the enzyme recovery as a function of the effects that were really significant. This model predicts a recovery value of about 40%, which is similar to that obtained experimentally (39.5%).

Index Entries: Reversed micelles; liquid-liquid extraction; β-xylosidase; cationic surfactant; downstream processing.

Introduction

Xylans are major components of the hemicellulosic fraction of lignocellulosic biomass, and their hydrolysis can be performed using xylanases *(1)*. The enzyme complex is composed of endoxylanases that cleave internal xylosidic linkages on the xylan backbone and β-xylosidase, which releases

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xylosyl residues by endwise attack of xylooligosaccharides *(1,2)*. The principal industrial application of this enzyme complex is in kraft pulp bleaching *(3)*. The xylanases produced by *Penicillium janthinellum* can be used to reduce the chlorine charge in *Eucalyptus* pulp bleaching with a simultaneous gain in brightness *(4)*. Some studies on xylanolytic complex recovery have been conducted employing techniques such as ethanol and salt precipitation, which have industrial applications *(5)*, and liquid-liquid extraction by aqueous two-phase systems *(6)*. However, these purification techniques did not improve the purification factor of the enzyme satisfactorily.

Reverse micellar systems have been extensively studied as a technique for the extraction and purification of proteins *(7–10)*. This technique allows the recovery and concentration of proteins from a dilute aqueous solution containing other bioproducts *(8)*. A reverse micellar system consists of aggregates of surfactant molecules containing an inner water core dispersed in an organic solvent medium. The polar microenvironment inside the reverse micelle permits the solubilization of protein while maintaining its native structure. The overall liquid-liquid extraction process by reverse micelles is conducted in two fundamental steps: a forward extraction, by which a protein is transferred from an aqueous solution to a reverse micellar organic phase; and a back extraction, by which the protein is released from the reversed micelles and transferred to an aqueous phase, so that it can be subsequently recovered *(10)*. The extraction process is mainly governed by electrostatic interaction between the charged protein and the micellar wall, and protein transfer can take place only during the forward extraction when the value of the aqueous phase pH is such that the net surface charge of the protein is electrically opposite to that of the surfactant head groups. Protein can also be extracted by hydrophobic interaction between the apolar regions of the molecule and surfactant tail *(11)*. In the back extraction, however, the pH value must be such that the protein has the same charge as the surfactant molecules, and the ionic strength is increased by adding salts; in this way repulsion forces are created and the micelles' diameter is diminished, causing the release of protein from the reverse micelles. Low ionic strength favors protein transfer to reverse micelles whereas high values promote protein release *(8,10,12)*. The reverse micellar technique is therefore particularly interesting for the recovery of extracellular enzymes.

The extraction and recovery of β-xylosidase have been investigated with a particular reference to the yield of the extraction process and the recovery of enzymic activity. The common approach of studying one variable at a time, while keeping the others constant within a set of selected values, has the drawbacks of requiring a large number of experiments and missing the interaction among the variables. An alternative is the use of experimental designs *(13)*. This methodology allows not only study of the variables but also verification of the effects of the factors individually and their interactions. The selective separation of the xylanolitic enzymes can

favor the hemicellulose hydrolysis, since this process can be performed later in two stages under the most appropriate conditions for each enzyme.

In this article, we describe the transfer of extracellular β-xylosidase from the xylanolitic complex produced by *P. janthinellum* to a reversed micellar phase of *N*-benzyl-*N*-dodecyl-*N*-*bis*(2-hydroxyethyl) ammonium chloride (BDBAC) cationic surfactant. We chose this surfactant because in previous studies on extracellular inulinase extraction, it promoted high recovery yields (~90%) *(10)*. We evaluated the influence of the following factors on the extraction: pH, temperature, solvent/cosolvent volume ratio, electrical conductivity, and surfactant concentration.

Materials and Methods

Preparation of Sugar Cane Bagasse Acid Hydrolysate

To prepare the hydrolysate for cultivation, 800 g of dry milled bagasse was mixed with 8 L of sulfuric 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 1.0 *N* NaOH.

Cultivation Medium and β*-Xylosidase Production*

P. janthinellum was isolated from decaying wood *(14)*. This microorganism was identified by the Biosystematic Research Center in Canada and deposited in its collection under the designation of CRC 87M-115. The strain was initially stored in silica and then transferred to agar slants. The fungus, used for the inoculum production, was cultivated at 30°C for 5 d in medium containing 2% glucose, 0.25% yeast extract, 2% (v/v) concentrated salt solution based on Vogel's medium *(15)*, and 2% agar-agar. The medium was sterilized at 121°C for 15 min. The inoculum (at a final concentration of 10^5 spores/mL) was obtained by suspending the spores in water and filtering the mixture through gauze placed on Erlenmeyer flasks.

The cultivation medium for enzyme production consisted of sugar cane bagasse hemicellulosic hydrolysate supplemented with 2% (v/v) concentrated salt solution based on Vogel's medium and 0.1% yeast extract. The medium was autoclaved for 15 min at 121°C. The cultivation was carried out in Erlenmeyer flasks (125 mL) containing 25 mL of medium at an initial pH of 5.5 (uncontrolled). The flasks were agitated for 96 h (60 rpm) at 30°C.

β*-Xylosidase Precipitation*

The enzyme was precipitated with 20 and 60% (v/v) ethanol. The pH value of the initial medium was adjusted to 4.5 by adding 1 *M* acetate buffer (pH 4.0) at a ratio of 9:1 (v/v). The ethanol was slowly mixed with the medium in a refrigerated bath (HETO-Holten, model CB15-25, Allerod, Denmark) at –4°C. The mixture was centrifuged (2000*g*) at 2°C for 15 min (Jouan Centrifuge Model 1812, Saint-Herblain, France). To prepare the

aqueous phase (first aqueous phase), samples of the precipitate were separately dissolved in acetate buffer (0.03 *M*) to obtain pH 3.0 and in Tris-HCl buffer (0.03 *M*) to obtain pH 9.0. β-Xylosidase activities in the aqueous phases and in the initial medium were measured by the method described in ref. *16*.

Liquid-Liquid Extraction

Liquid-liquid extraction was performed using an experimental design. The enzyme previously precipitated with ethanol was extracted from the first aqueous phase by BDBAC–reversed micelles in isooctane, by a two-step procedure. In the first step (forward extraction), 5.0 mL of the first aqueous phase (containing β-xylosidase) was mixed with an equal volume of micellar phase (BDBAC in isooctane/hexanol/water). This mixture was agitated on a vortex for 1 min, to obtain the equilibrium phase, and again separated into two phases by centrifugation at 1677*g* for 10 min (Jouan Centrifuge Model 1812). Two milliliters of the micellar phase (containing β-xylosidase) were mixed with 2.0 mL of fresh aqueous phase (1.0 *M* acetate buffer at pH 5.5 with 1.0 *M* NaCl), to transfer the enzyme from the micelles to this fresh aqueous phase, called the second aqueous phase (backward extraction), which was finally collected by centrifugation (1677*g* for 10 min). Both aqueous phases (first and second) were assayed to determine enzyme activity. The extraction results are reported in terms of total activity recovered (%) in the second aqueous phase using the β-xylosidase content of the first aqueous phase as a reference.

Enzyme Activity

β-Xylosidase activity was measured by the amount of *p*-nitrophenol released after the enzyme action in a *p*-nitrophenyl-β-D-xylopyranoside water solution *(16)*. The reaction medium containing 250 μL of 2 m*M* *p*-nitrophenyl-β-D-xylopyranoside (in water) and 250 μL of fermented medium at pH 5.5 (adjusted with 50 m*M* acetate buffer) was incubated at 50°C for 30 min. The reaction was stopped by adding 1 mL of 2 *M* sodium carbonate solution. The absorbance of the released *p*-nitrophenol after the β-xylosidase action was measured spectrophotometrically at 400 nm and compared with a standard reference. Activity units were expressed as micromoles of *p*-nitrophenol/(min · mL) at 50°C.

Factorial Design and Optimization of the System

The fraction of enzyme recovered (Y%), which is the response factor, was measured as a function of pH (A), electrical conductivity (B), BDBAC concentration (C), hexanol concentration (D), and temperature (E). For each of the five factors, high and low set points (coded values +1 and –1, respectively) were selected (Table 1); Table 1 presents the actual variable values. Extractions representing all the 16 (2^{5-1}) set-point combinations were made twice *(13)*. The assays were made by allotment to minimize eventual systematic errors. A statistical examination of the results and a response sur-

Table 1
Variables, Levels, and β-Xylosidase Recovery Using a 2^{5-1} Statistical Factorial Design

	Coded variables					Experimental conditions					
Assay number	pH	Conductivity (mS/cm)	BDBAC (*M*)	Hexanol (%)	Temperature (°C)	pH	Conductivity (mS/cm)	BDBAC (*M*)	Hexanol (%)	Temperature (°C)	Recovery (Y[%])
1	–1.0	–1	–1	–1	+1	3.0	4.0	0.1	5.0	26	19.3
2	+1.0	–1	–1	–1	–1	9.0	4.0	0.1	5.0	4	0.2
3	–1.0	+1	–1	–1	–1	3.0	10.0	0.1	5.0	4	2.8
4	+1.0	+1	–1	–1	+1	9.0	10.0	0.1	5.0	26	0.2
5	–1.0	–1	+1	–1	–1	3.0	4.0	0.2	5.0	4	0.0
6	+1.0	–1	+1	–1	+1	9.0	4.0	0.2	5.0	26	0.0
7	–1.0	+1	+1	–1	+1	3.0	10.0	0.2	5.0	26	36.4
8	+1.0	+1	+1	–1	–1	9.0	10.0	0.2	5.0	4	6.1
9	–1.0	–1	–1	+1	–1	3.0	4.0	0.1	10.0	4	1.0
10	+1.0	–1	–1	+1	+1	9.0	4.0	0.1	10.0	26	0.4
11	–1.0	+1	–1	+1	+1	3.0	10.0	0.1	10.0	26	3.0
12	+1.0	+1	–1	+1	–1	9.0	10.0	0.1	10.0	4	7.0
13	–1.0	–1	+1	+1	+1	3.0	4.0	0.2	10.0	26	43.7
14	+1.0	–1	+1	+1	–1	9.0	4.0	0.2	10.0	4	2.6
15	–1.0	+1	+1	+1	–1	3.0	10.0	0.2	10.0	4	0.0
16	+1.0	+1	+1	+1	+1	9.0	10.0	0.2	10.0	26	0.9

face study were carried out using the STATGRAPH 6.0 statistical program package.

Chemicals

Birchwood 4-*o*-methyl-β-D-glucoroxylan (90% xylose) was obtained from Sigma (St. Louis, MO). The cationic surfactant BDBAC (purity >99%) was purchased from Merck (Darmstadt, Germany) and used without further purification. All the other chemicals were of analytical grade.

Results and Discussion

Table 1 shows the recovery (Y[%]) of β-xylosidase obtained from the BDBAC reversed micelle extractions performed according to a 2^{5-1} fractional factorial design. As can be seen, changes in the pH and temperature affect the β-xylosidase recovery by reversed micelles. All the experiments performed at pH 9.0 provided recovery values lower than 7%, independently of the other factors. At pH 9.0, the enzyme is denatured because it presents low stability. Low enzyme recovery values were also found in the experiments conducted at 4°C. At this temperature the radius of the micelles is small (Table 2), and, consequently, their capacity for enzyme encapsulation is reduced. Micelle size, as well as its capacity and stability, strongly depends on temperature *(8,10)*. The high molecular weight (~110 kDa) of the β-xylosidase obtained from *P. janthinellum* may be responsible for its low extraction.

The highest enzyme recovery values (19.3, 36.4, and 43.7%) were obtained in assays 1, 7, and 13, respectively (*see* Table 1). The simultaneous increases in conductivity and BDBAC concentration (assays 1 and 7) from 4 to 10 mS/cm and from 0.10 to 0.20 *M*, respectively, provided an enzyme recovery augmentation of 88.5%. An even greater increase (126.1%) was observed in assay 13 in comparison with assay 1 (the BDBAC and the hexanol concentrations increased from 0.10 to 0.20 *M* and from 5 to 10%, respectively). This direct analysis of the results shows different combinations and interactions between the variables studied. Thus, the main effects of the factors and their interactions (pH, BDBAC, hexanol concentration, electrical conductivity, and temperature) can be better evaluated by statistical analysis.

Table 3 presents the estimated effects, standard errors, and Student's *t*-test values for the 2^{5-1} factorial design. Statistical analysis of the results confirms the effects of the factors and their interactions, as previously suggested. According to Table 3, pH and temperature had the highest effects on enzyme recovery: –11.10 and +10.52, respectively. A significant interaction between these two factors, with a high effect (–14.13), was also observed. This negative interaction means that the enzyme recovery can be enhanced by lowering the pH value to 3.0 and raising the temperature to 26°C (Fig. 1A).

Table 2
Water Content, w_o, and Micellar Radius, R_m, of BDBAC Reversed Micelles in Micellar Phase

	Micellar phase[a]			
	After forward extraction		After backward extraction	
	4°C	26°C	4°C	26°C
W_o	26.2	84.2	7.1	11.4
R_m (nm)	4.3	13.8	1.2	1.8

[a]Extraction conditions: pH = 3.0; conductivity = 4.0 mS/cm; BDBAC = 0.20 *M*; hexanol = 10%; and temperature = 4 and 26°C.

Table 3
Estimated Effects, Standard Errors, and Student's *t*-Test Values for the 2^{5-1} Factorial Design

Effect	Estimated effect	Standard error	Student's *t*-test value
Average	7.73	±0.39	19.49
Factor A (pH)	–11.10	±0.79	14.00[a]
Factor B (conductivity)	–1.34	±0.79	1.70
Factor C (conc. BDBAC)	6.99	±0.79	8.81[a]
Factor D (% hexanol)	–0.81	±0.79	1.03
Factor E (temperature)	10.52	±0.79	13.26[a]
AB	4.09	±0.79	5.16[a]
AC	–6.51	±0.79	8.21[a]
AD	1.92	±0.79	2.42[a]
AE	–14.13	±0.79	17.82[a]
BC	0.62	±0.79	0.78
BD	–7.86	±0.79	9.92[a]
BE	–4.38	±0.79	5.53[a]
CD	1.98	±0.79	2.50[a]
CE	7.55	±0.79	9.52[a]
DE	–1.17	±0.79	1.48

[a]Significant at the 5% level with 16 degrees of freedom (t = 2.12).

Figure 1A–D show the surface responses of the most significant interactions obtained after statistical analysis. According to the significant main effect shown in Table 3, the higher the BDBAC concentration the higher the β-xylosidase recovery. However, the interactions between this factor and all the other variables do not allow its isolated analysis. Figure 1B,C show response surfaces that correlate the BDBAC concentration (factor C) with temperature (factor E) and pH (factor A). All the interactions with factor C show that the enzyme recovery increases directly as a function of the BDBAC concentration. Nevertheless, the most evident effects of interaction were observed at 26°C (Fig. 1B) and pH 3.0 (Fig. 1C).

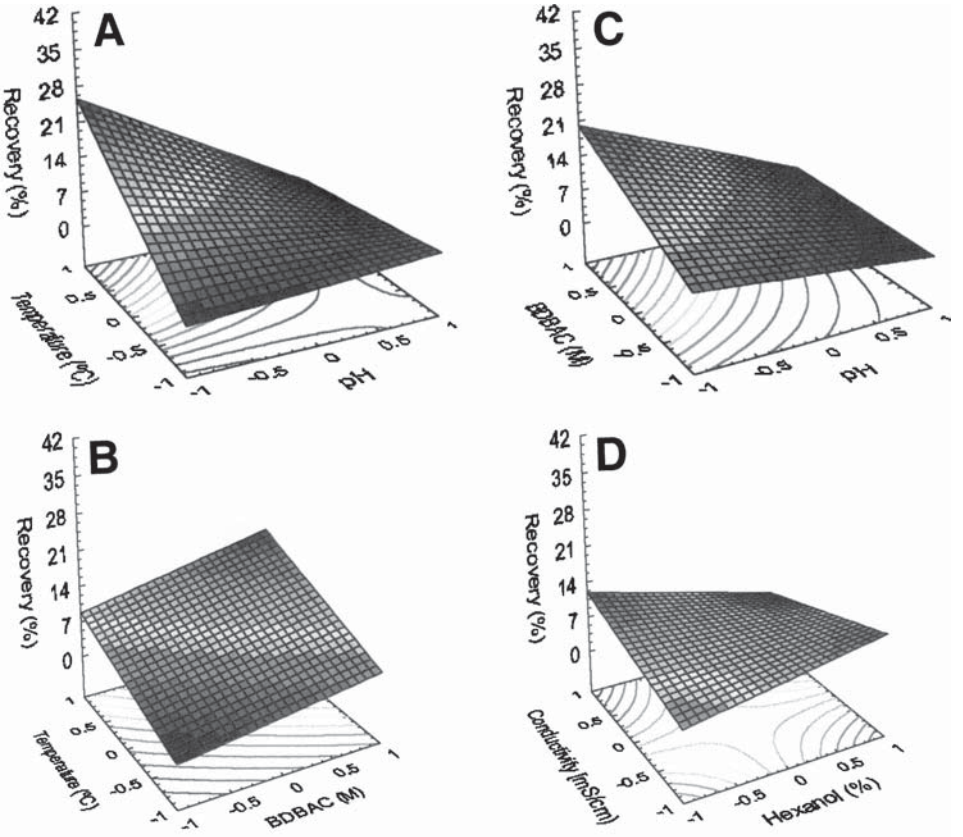

Fig. 1. **(A)** Effect of temperature and pH on β-xylosidase extraction by reversed micelles. BDBAC = 0.15 *M*; electrical conductivity = 7.0 mS/cm; hexanol = 7.5%. **(B)** Effect of temperature and BDBAC concentration on β-xylosidase extraction by reversed micelles: pH = 6.0; electrical conductivity = 7.0 mS/cm; hexanol = 7.5%. **(C)** Effect of BDBAC concentration and pH on β-xylosidase extraction by reversed micelles. electrical conductivity = 7.0 mS/cm; hexanol = 7.5%; temperature = 15°C. **(D)** Effect of electrical conductivity and hexanol concentration on β-xylosidase extraction by reversed micelles. BDBAC = 0.15 *M*; pH = 6.0; temperature = 26°C.

The highest β-xylosidase recovery value was attained at higher temperature and BDBAC concentration values (Fig. 1B), because this enzyme has a high molecular weight (~110 kDa), thus requiring larger reversed micelles for its encapsulation *(5,17)*. The diameter of the reversed micelles can be increased by increasing the BDBAC concentration and/or temperature *(8,10)*.

Figure 1C shows the β-xylosidase recovery as a function of the pH value and of the BDBAC concentration. The enzyme recovery increased at a low pH value (3.0). For a cationic surfactant such as BDBAC, a good extraction is expected at pH values higher than the enzyme p*I*, which for β-xylosidase ranges from 4.9 to 6.0 *(17,18)*. In general, under these conditions, the net charge of the enzyme is negative and its electrostatic inter-

action with the surfactant is favored. In the present work, however, this was not observed, since the highest extraction yields were obtained at pH values lower than p*I*. This behavior can be explained by the hydrophobic interaction between the enzyme and the surfactant *(11,19)*.

Significant interactions could also be observed between pH and conductivity (AB), pH and hexanol concentration (AD), conductivity and hexanol concentration (BD), and BDBAC and hexanol concentration (CD). The highest interaction effect (–7.86) occurred between factors B and C. The surface response for this interaction is shown in Fig. 1D. As can be seen, the recovery increases at the levels +1 for hexanol concentration (maximum level = 10%) and –1 for conductivity (minimum level = 4.0 mS/cm). There is no clear explanation for such behavior, but this antagonistic effect can occur owing to the addition of cosolvents to the cationic reversed micellar system to increase the micelle size, which is also affected by the high ionic strength *(8)*. The effect of the ionic strength is primarily to mediate the electrostatic interactions between the protein surface and the surfactant head groups. The Debye screening effect determines the electrical double-layer properties adjacent to any charged surface, and affects the range above which electrostatic interactions can overcome thermal motion of the soluble molecules. The characteristic distance for these electrostatic interactions is the Debye length, which is inversely proportional to the square root of the ionic strength. Thus, increased ionic strength will decrease this interaction distance, and hence inhibit the solubilization of the protein.

In this work, the ionic strength also had an effect on the micelle formation and its size, although the extraction occurred at a pH value lower than the p*I* value and depended only on hydrophobic interactions. Likewise the electrostatic interactions influenced the formation and the size of the micelle, but not their capacity for protein encapsulation. The results indicate that the apolar region of the enzyme interacted with the surfactant tail and was transferred to the micellar phase. Nevertheless, as can be seen in Table 2, water content, w_o, values varied according to the temperature (extractions performed at 4°C provided a micelle radius smaller than that obtained at 26°C). As expected, the reversed micelles in the first micellar phase were higher than in the second micellar phase. The water content of the reversed micelles (w_o) or size of the reversed micelles strongly increased, as also reported by Regalado et al. *(20)* for AOT/isooctane microemulsion and by Pessoa and Vitolo *(10)* for BDBAC/isooctane-hexanol microemulsion.

After an analysis of variance (ANOVA) of the significant factors (Table 4), a linear mathematical model was adjusted to the results (Eq. 1):

$$\begin{aligned} Y = {} & 7.73 - 5.55A - 0.67B + 3.49C - 0.41D + 5.26E + 2.05AB \\ & - 3.26AC + 0.96AD - 7.07AE - 3.93BD - 2.19BE + 0.99CD + 0.78CE \end{aligned} \quad (1)$$

where Y = β-xylosidase recovery (%); A = pH; B = electrical conductivity (mS/cm); C = BDBAC concentration (*M*); D = hexanol concentration (%); and E = temperature (°C).

Table 4
ANOVA of Significant Factors[a]

Variation	Sum of squares	Degrees of freedom	Mean square	*f* Value	*p* Value
Factor A	987.01	1	987.01	189.74	0.0000
Factor B	14.47	1	14.47	2.78	0.1136
Factor C	390.88	1	390.88	75.14	0.0000
Factor D	5.33	1	5.33	1.02	0.3256
Factor E	885.36	1	885.36	170.19	0.0000
AB	133.91	1	133.91	25.74	0.0001
AC	339.17	1	339.17	65.20	0.0000
AD	29.49	1	29.49	5.67	0.0292
AE	1598.10	1	1598.10	307.21	0.0000
BD	494.87	1	494.87	95.13	0.0000
BE	153.91	1	153.91	29.59	0.0000
CD	31.52	1	31.52	6.06	0.0248
CE	456.17	1	456.17	87.69	0.0000
Block	6.27	1	6.27	1.2	0.2877
Total error	94.71	18	5.03		
Total	5614.90	31			

[a]$R^2 = 0.98$.

Table 5
ANOVA for Total Regression[a]

	Sum of squares	Degrees of freedom	Mean square	*f* Value	*p* Value
Model	5520.20	13	424.631	80.7106	0.0000
Total error	94.70	18	5.26115		
Total	5614.90	31			

[a]$R^2 = 0.98$.

The coefficient of correlation between the data and the model (R^2) was 0.98, and the validity of the model was verified by the ANOVA for the total regression (Table 5). This model was shown to be highly significant, according to the *f*-test ($f_{model} = 80.71 >> f_{table} = 2.3$). In addition, it presents no lack of fit, since the differences between the values experimentally obtained and the values predicted by the model can be explained by the experimental errors.

Equation 1 predicts 40% of β-xylosidase recovery (Y) under the following conditions: pH = 3.0; electrical conductivity = 4.0 mS/cm; BDBAC concentration = 0.20 *M*; hexanol = 10%; and temperature = 26°C. Using these conditions, an assay was performed and the result was 39.5% of enzyme recovery, which confirms the validity of the model.

In conclusion, a BDBAC/isooctane/hexanol reversed micellar system was efficiently used as a recovery method of β-xylosidase from *P. janthinellum*.

The recovery was controlled by hydrophobic interactions. The response surface methodology was useful for understanding the enzyme extraction.

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