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Screening of Variables in β-Xylosidase Recovery Using Cetyl Trimethyl Ammonium Bromide Reversed Micelles

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Abstract

β-Xylosidase recovery by micelles using cetyl trimethyl ammonium bromide (CTAB) cationic surfactant was verified under different experimental conditions. A 2^{5-1} fractional factorial design with center points was employed to verify the influence of the following factors on enzyme extraction: pH (x_1), CTAB concentration (x_2), electrical conductivity (x_3), hexanol concentration (x_4), and butanol concentration (x_5). Statistical analysis of the results shows that of the five variables studied only hexanol and electrical conductivity did not have significant effects on the recovery of β-xylosidase. The other factors had significant effects in increasing order: $(x_1) > (x_2) > (x_5)$. The model predicts a recovery value of about 45%, which is similar to that obtained experimentally (43.5%).

Index Entries: Reversed micelles; β-xylosidase; liquid-liquid extraction; statistical design.

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, which cleave internal xylosidic linkages on the xylan backbone, and β-xylosidase, which releases xylosyl residues by endwise attack of xylooligosaccharides *(1,2)*.

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Plants are the source of renewable natural fibers used in the paper and textile industries. Normally, the cellulosic fibers are bleached by chemical processes, which are harmful to the environment. Environmental problems could be avoided by replacing the chemical bleaching processes with biologically oriented processes, such as the use of xylanase *(3–5)*. In fact, using xylanases facilitates pulp bleaching, lowers chlorine consumption, and reduces toxic discharges *(6,7)*. In addition, xylanases also can be employed in the clarification of beer and juice *(6–8)* and in baking processes, increasing the loaf volume *(8)*. At present, β-xylosidase is produced by *Penicillium janthinellum* from processed or refined substrates, such as sugars, cellulose, and xylan.

Some studies on xylanolytic complex recovery have been conducted employing techniques such as ethanol and salt precipitation, which have industrial applications, and liquid-liquid extraction by aqueous two-phase systems *(9–12)*. 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 *(13–15)*. This technique allows the recovery and concentration of proteins from a dilute aqueous solution containing other bioproducts *(13–16)*. 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 recovered subsequently *(13–15)*. The extraction process is mainly governed by electrostatic interaction between the charged protein and the micellar wall, and protein transfer only takes place during the forward extraction, when the value of the pH of the aqueous phase is such that the net surface charge of the protein is electrically opposite of that of the surfactant head groups. Protein can also be extracted by hydrophobic interaction between the apolar regions of the molecule and the surfactant tail *(13)*. In the back-extraction, however, the pH value must allow the protein to have the same charge as the surfactant molecules and the ionic strength to be increased by the addition of salts. In this way, repulsion forces are created and the micellar diameter is diminished, causing the release of protein from the reverse micelles. Low ionic strength favors protein transfer to reverse micelles, and high values promote protein release *(17)*. This technique is therefore particularly interesting for the recovery of extracellular enzymes *(13–17)*.

The present study describes the transfer of extracellular β-xylosidase from *P. janthinellum* to a reversed micellar phase of the cetyl trimethyl ammonium bromide (CTAB) cationic surfactant and the influence of the

following factors: pH, CTAB concentration, electrical conductivity, hexanol concentration, and butanol concentration. The extraction and recovery of β-xylosidase enzymic protein has been investigated with particular attention to the recovery of the enzymatic activity.

Materials and Methods

Chemicals

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

Preparation of Sugarcane Bagasse 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.

Microorganism and Growth Conditions

The isolation of *P. janthinellum* (CRC 87M-115) from decaying wood was as described by Milagres *(18)*. The fungi, initially maintained in silica stocks and then transferred to agar slants, were cultivated at 30°C for 5 d in medium containing 2% glucose, 0.25% yeast extract, 2% concentrated salt solution (v/v) based on Vogel's *(19)*, medium and 2% agar-agar. The medium was autoclaved at 112°C for 5 min. To obtain the inoculum, the spores were suspended in water and the suspension was filtered through gauze placed in Erlenmeyer flasks. The final spore concentration was 10^5/mL.

Cultivation Medium and β-*Xylosidase Production*

The cultivation medium for enzyme production consisted of sugarcane bagasse hemicellulose hydrolysate supplemented with 2% concentrated salt solution (v/v) 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 Activity and Precipitation*

β-Xylosidase activity was measured by the method described by Kumar and Ramón *(20)*. The enzyme was precipitated with ethanol at 20 and 60% (v/v). The pH value of the precipitation 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 at –4°C, and the mixture was centrifuged (2000*g* for 15 min) at 2°C. To prepare the aqueous

phase, samples of the precipitate formed were separately solubilized in acetate buffer (0.03 *M*) to obtain pH 3.0 and 4.0, and in glycine/HCl buffer (0.03 *M*) to obtain pH 2.0. The electrical conductivity of the solubilized samples was adjusted to 4.0 mS/cm by adding NaCl.

Reversed Micellar Liquid-Liquid Extraction

Different values of CTAB concentration, electrical conductivity, hexanol and butanol concentrations, and pH were used in the enzyme extraction, according to the method of reversed micelles described by Pessoa and Vitolo *(15)* using CTAB prepared with a solution containing hexanol, isooctane, and butanol.

Experimental Design and Statistical Analyses

A 2^{5-1} fractional factorial design with center points was employed to evaluate the influence of the following factors on the enzyme extraction: pH, CTAB concentration, electrical conductivity, and hexanol and butanol concentrations (Table 1). For each of the factors, high (coded value: +1), center (coded value: 0) and low (coded value: –1) set points were selected. Extractions representing all 16 (2^{5-1}) set point combinations were made, as well as three extractions representing the center point (coded value: 0). Assays were conducted randomly. To analyze the results, STATGRAPHIC® software version 6.0 was used.

Results and Discussion

Table 1 shows the factor levels and the recovery results (*Y*[%]) of β-xylosidase obtained from the CTAB reversed micelle extractions performed according to a 2^{5-1} fractional factorial design with center points. Experiments 4, 6, 10, 12, 14, and 16 (pH 8.0) provided recovery values >15%. On the other hand, all the experiments performed at pH 3.0 provided recovery values <8%, independently of the levels of the other factors. The highest enzyme recovery values (59.3, 32.3, and 35.3%) were obtained in experiments 4, 12, and 16, respectively, with a CTAB concentration of 0.2 *M*. Comparison of assays 12 and 4 shows that when butanol concentration was increased from 10 to 20% and hexanol concentration was decreased from 10 to 5%, the enzyme recovery increased by 84%.

The results show that different combinations among the variables studied (pH, CTAB concentration, hexanol and butanol concentrations, and electrical conductivity) can be better evaluated by statistical analysis.

Table 2 gives the estimated effects, standard errors, and student's *t*-test. According to the results, pH and butanol concentration had the most significant main effects (+20.26 and +8.86) on enzyme recovery ($p < 0.10$ and $R^2 = 0.95$). A significant interaction between the factors *AB*, with a high effect (+10.32), and *AE* (+10.92), can also be observed. The positive interaction means that the enzyme recovery was enhanced when high levels of pH (8.0), CTAB concentration (0.2 *M*), and butanol concentration (20%)

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

Assay no.	pH	CTAB (*M*)	Electrical conductivity (mS/cm)	Hexanol (%)	Butanol (%)	Recovery (%)
1	3.0	0.1	4.0	5.0	20	0.0
2	8.0	0.1	4.0	5.0	10	0.6
3	3.0	0.2	4.0	5.0	10	0.0
4	8.0	0.2	4.0	5.0	20	59.3
5	3.0	0.1	10.0	5.0	10	7.0
6	8.0	0.1	10.0	5.0	20	18.7
7	3.0	0.2	10.0	5.0	20	1.7
8	8.0	0.2	10.0	5.0	10	0.0
9	3.0	0.1	4.0	10.0	10	7.3
10	8.0	0.1	4.0	10.0	20	17.5
11	3.0	0.2	4.0	10.0	20	2.9
12	8.0	0.2	4.0	10.0	10	32.3
13	3.0	0.1	10.0	10.0	20	1.5
14	8.0	0.1	10.0	10.0	10	18.8
15	3.0	0.2	10.0	10.0	10	0.0
16	8.0	0.2	10.0	10.0	20	35.3
17	5.5	0.15	7.0	7.5	15	5.1
18	5.5	0.15	7.0	7.5	15	4.5
19	5.5	0.15	7.0	7.5	15	9.5

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

Variable	Estimated effect	Standard error	*t*-Value
A (pH)	20.26	±3.117	6.50[a]
B (CTAB)	7.50	±3.117	2.41[b]
C (electrical conductivity)	–4.61	±3.117	1.48
D (hexanol)	3.53	±3.117	1.13
E (butanol)	8.86	±3.117	2.84[b]
AB	10.32	±3.117	3.31[a]
AC	–4.61	±3.117	1.48
AD	2.79	±3.117	0.89
AE	10.92	±3.117	3.50[a]
BC	–9.77	±3.117	3.14[b]
BD	–1.17	±3.117	0.37
BE	7.88	±3.117	2.52[b]
CD	3.51	±3.117	1.12
CE	–1.02	±3.117	0.32
DE	–9.17	±3.117	2.95[b]
Average	11.68	±1.430	8.17

[a]Significant at the 5% level with 3 DF (t = 3.182).
[b]Significant at the 10% level with 3 DF (t = 2.353).

were used. A possible explanation for this is the electrostatic interaction between the enzyme and the surfactant *(13,21)*. Concerning the interaction between CTAB and electrical conductivity (*BC*), the negative effect (–9.77) shows that β-xylosidase recovery increased when a high CTAB concentration and a low electrical conductivity were used (0.2 *M* and 4 mS/cm, respectively).

Figure 1A–E shows the response surfaces of the significant interactions obtained after statistical analysis. Figure 1A, 1C, and 1D show response surfaces that correlate the CTAB concentration with pH, electrical conductivity, and butanol concentration, respectively. The highest β-xylosidase recovery value (59.3) was attained at the highest CTAB concentration (0.2 *M*), probably because this enzyme has a high molecular weight (~110 kDa), thus requiring reversed micelles with large diameters for its encapsulation. This can be explained by increasing the surfactant concentration *(16)*. The influences of pH and electrical conductivity on extraction suggest that the effect of the ionic strength primarily consists of mediating the electrostatic interactions between the protein surface and the surfactant head groups.

The interaction between butanol concentration (*E*) and pH (*A*) (Fig. 1B) shows that β-xylosidase recovery increased when pH and butanol concentration were maintained at the highest levels (8.0 and 20%, respectively). However, the interaction between butanol concentration (*E*) and hexanol concentration (*D*) suggests that an antagonistic effect occurred, owing to the addition of cosolvents to the cationic reversed micellar system to increase the micellar size, which is also affected by the high ionic strength *(16,21)*. 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 the present study, the ionic strength also had influenced the micellar formation and size. The extraction was conducted at a pH value higher than the enzyme p*I* (isoelectric) value and its extraction depended only on electrostatic interactions.

After analysis of variance (ANOVA) including the significant factors (Table 3), a mathematical model was adjusted to the results (Eq. 1), and the coefficient of correlation between the data and the model (R^2) was 0.90. The validity of the model was verified by the ANOVA for total regression (Table 4). According to the F test ($F_{model} = 11.86 >> F_{table} = 3.35$), the model was highly significant. In addition, it had no lack of fit, since the differences between the values experimentally obtained and the values predicted by the model were owing to experimental errors:

$$Y = 11.69 + 10.13A + 3.75B + 4.43E + 5.16AB + 5.46AE - 4.89BC - 3.94BE - 4.58DE \quad (1)$$

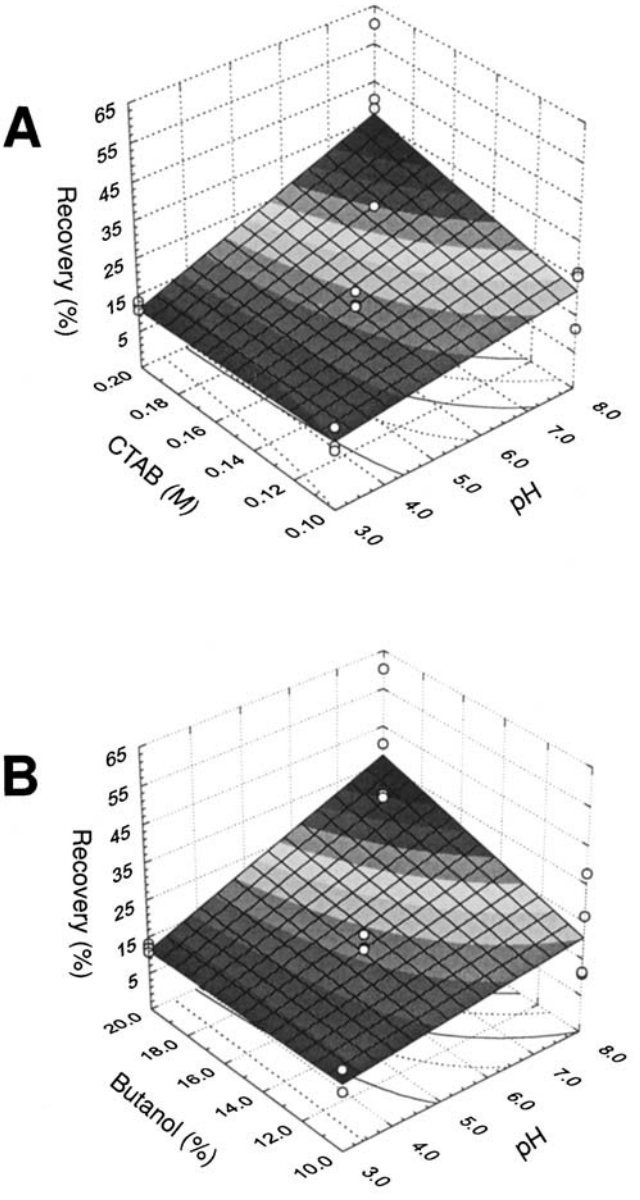

Fig. 1. **(A)** Effect of pH and CTAB concentration on β-xylosidase extraction by reversed micelles: electrical conductivity = 7.0 mS/cm, hexanol = 7.5%, and butanol = 15%; **(B)** effect of pH and butanol concentration on β-xylosidase extraction by reversed micelles: electrical conductivity = 7.0 mS/cm, hexanol = 7.5%, and CTAB = 0.15 *M*
(continued on next page).

in which Y is the β-xylosidase recovery (%), A is the pH, B is the CTAB concentration (M), C is the electrical conductivity (mS/cm), D is the hexanol concentration (%), and E is the butanol concentration (%).

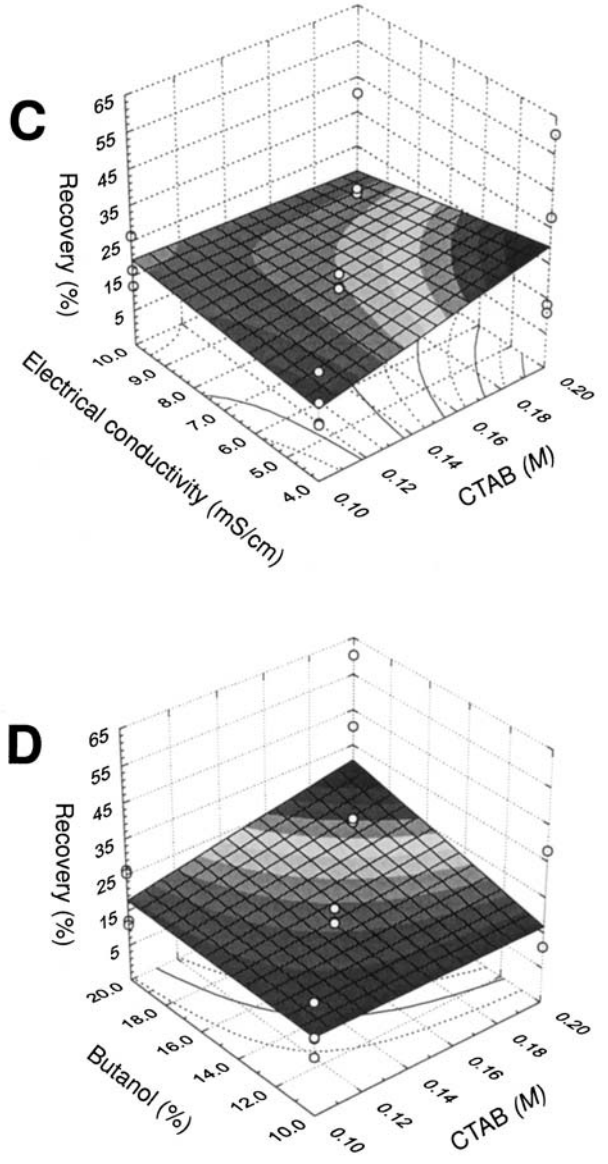

Fig. 1. *(continued)* **(C)** effect of electrical conductivity and CTAB concentration on β-xylosidase extraction by reversed micelles: pH = 5.5, hexanol = 7.5%, and butanol = 15%; **(D)** effect of CTAB concentration and butanol concentration on β-xylosidase extraction by reversed micelles: electrical conductivity = 7.0 mS/cm, pH = 5.5, and hexanol = 7.5% *(continued on next page)*.

Equation 1 predicts a 44% β-xylosidase recovery (Y) under the following conditions: pH = 8.0, electrical conductivity = 4.0 mS/cm, CTAB concentration = 0.2 M, hexanol concentration = 5%, and butanol concentration = 20%. The result experimentally obtained (about 39%) strengthens the promise of validity of the model for interpolations.

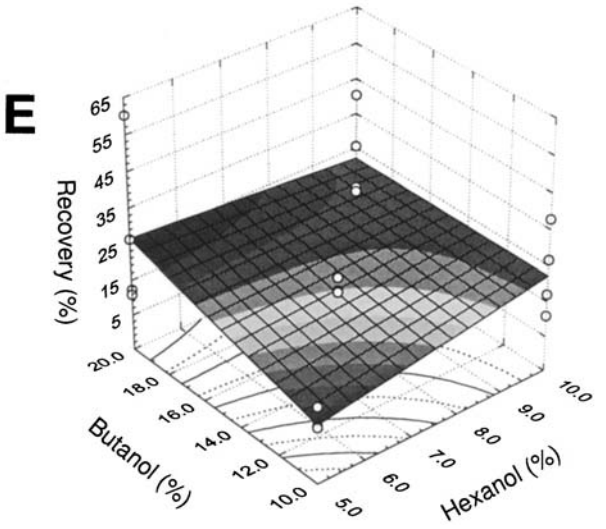

Fig. 1. *(continued)* **(E)** effect of butanol concentration and hexanol concentration on β-xylosidase extraction by reversed micelles: electrical conductivity = 7.0 mS/cm, pH = 5.5, and CTAB = 0.15 *M*.

Table 3
ANOVA of Significant Factors

Source of variation	Sum of squares	Degrees of freedom	Mean square	F Values	*p* Values
A (pH)	1642.48	1	1642.48	218.49	0.004[c]
B (CTAB)	225.53	1	225.53	30.00	0.032[c]
E (butanol)	313.91	1	313.91	41.76	0.023[c]
AB	426.32	1	426.32	56.71	0.017[c]
AE	477.31	1	477.31	63.50	0.015[c]
BC[a]	382.11	1	382.11	50.83	0.019[c]
BE	248.30	1	248.30	33.03	0.029[c]
DE[b]	336.26	1	336.26	44.73	0.021[c]
Lack of fit	411.98	8	51.50	6.85	0.133
Pure error	15.04	2	7.52		
Total	4479.23	18			

[a]C, electrical conductivity.
[b]D, hexanol.
[c]Significant at the 5% level $R^2 = 0.90$.

Table 4
ANOVA for Total Regression[a]

Variation	Sum of squares	Degrees of freedom	Mean square	F Value
Model	4052.22	8	506.53	11.86
Error	427.02	10	42.70	—
Total	4479.24	18		

[a]$R^2 = 0.90$; F = 3.35.

Conclusion

A CTAB/isooctane/hexanol and butanol reversed micellar system was efficiently used as a recovery method of β-xylosidase from *P. janthinellum*. The recovery was controlled by electrostatic interactions. The response surface methodology helped (qualitatively) us to understand the mechanism of enzyme extraction. The recovery value obtained experimentally (39%) was very close to one predicted by the model (44%). This demonstrates that the use of reversed micellar solutions as extractants is an efficient way to recover the enzyme without reducing its activity, because the enzyme is not denatured by reagents.

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