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SHORT COMMUNICATION

Optimization of β-xylosidase recovery by reversed micelles using response surface methodology

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Abbreviations: ANOVA: analysis of variance;
CTAB: Cetyl Trimethyl Ammonium Bromide;
Da: Dalton;
pI: isoelectric point;
R_m: micellar radius;
SDS: sodiumdodecyl sulfate;
W_o: water in oil.

β-Xylosidase recovery by reversed micelles using CTAB cationic surfactant was performed under different experimental conditions. A 22-full orthogonal design with center points was employed to optimize the recovery of this enzyme and to evaluate the influence of the factors CTAB (A) and butanol concentration (B) on the enzyme extraction. A mathematical model to represent the enzyme recovery as a function of A and B was proposed. According to the model, the CTAB and butanol concentrations necessary to attain the maximum β-xylosidase recovery (43%) were 0.26 M and 29.4%, respectively. The results showed that the recovery value predicted by the model was similar to that obtained experimentally (44.3%).

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Improvements in downstream processing are necessary for biotechnological researches to achieve their goals. A number of recent studies on reversed micellar methodology clearly demonstrate the scientific interest in reversed micelles for the separation of biotechnological products (Kilikian et al. 2000). Recovery of proteins from an aqueous solution using reversed micellar extraction is, in principle, a promising method (Rodrigues et al. 1999; Hasmann et al. 2000). Reversed micelles are a spontaneous and reversible formation of spherical aggregates of amphiphilic molecules in non-polar liquids. Their inner core is able to solubilize enzymes and other polar substances, improving their purity (Rodrigues et al. 1999).

The recovery of enzymes by ionic surfactant systems depends on the choice of appropriate set of experimental factors such as: pH (by controlling the aqueous-phase pH), temperature, ionic strength, surfactant and organic solvent (Kilikian et al. 2000). β-Xylosidase (EC 3.2.1.37), the enzyme employed in this study, catalyses the hydrolysis of xylooligosaccharides and xylobiose from the non-reducing end to release xylose. According to Pan et al. 2001 one feature of the fungal β-Xylosidase is its transxylosylation activity, which is particularly useful for preparation of β-Xylosides.

In our experiments, β-Xylosidase produced and secreted by *Penicillium janthinellum* was extracted by a reversed micellar system of the cationic surfactant CTAB [Cetyl Trimethyl Ammonium Bromide]. The influence of two operational factors (surfactant and co-solvent concentrations) on the recovery efficiency was verified by means of an experimental design, and the extraction process (recovered β-Xylosidase activity) was optimised.

The results achieved in this work complete the findings reported in earlier publications on the extraction of the same enzyme using another reversed micellar system (cationic surfactant BADBAC/Isooctane/hexanol) and different experimental conditions (Hasmann et al. 1999; Hasmann et al. 2000; Hasmann et al. 2001).

MATERIALS AND METHODS

Preparation of acid sugar cane bagasse hydrolysate

To preparate the hydrolysate for cultivation, 800g of dry milled bagasse (from Usina Nova América, Tarumã, Brazil) was mixed with 8L of H_2SO_4 solution (0.25% v/v) and autoclaved at 121ºC for 45 min. The liquid fraction was separated by filtration and the pH was adjusted to 5.5 with 1.0 N NaOH.

Cultivation medium and enzyme production

Penicillium janthinellum fungus (CRC 87M-115), isolated from decayed wood by Milagres et al. 1993 and maintained in silica stocks, was transferred to agar slants and cultivated at 30ºC for 5d in medium containing 2% glucose, 0.25% yeast extract, 2% (v/v) concentrated salt solution based on Vogel's minimal medium (Vogel, 1956), and 2% agar-agar. The medium was autoclaved at 121ºC for 15 min. In order to obtain the inoculum, the spores were suspended in water and the suspension was filtered through gauze placed on Erlenmeyer flasks. The final spore concentration was 10^5/mL.

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 minimal medium (Vogel, 1956) and 0.1% yeast extract. The medium was autoclaved for 15 min at 121ºC. The cultivation was carried out in Erlenmeyers flasks (125 mL) containing 25 mL of medium at initial pH 5.5 (uncontrolled). The flasks were agitated for 96 hrs (60 rpm) at 30ºC. The enzyme activity in the fermented medium was 0,2 U/mL".

β-Xylosidase precipitation

The enzyme was precipitated with ethanol at 20 and 60% (v/v) according to the method described by Cortez and Pessoa Jr., 1999. The pH value of the precipitation medium was adjusted to 4.5 by adding 1 M acetate buffer (pH 4.0) in the ratio of 9:1 (v/v). The ethanol was slowly mixed with the medium in a refrigerated bath at -4ºC and the mixture was centrifuged (2,000 g for 15 min) at 2ºC. To prepare the aqueous phase, samples of the precipitate formed were solubilized separately in phosphate buffer (0.03 M) to obtain pH 8.0. The electrical conductivity of the solubilized samples was adjusted to 4.0 mS/cm by adding NaCl.

Reversed micellar liquid-liquid extraction

The enzyme was extracted from solubilized precipitate by CTAB-reversed-micelles in isooctane, hexanol (in ratio of 9:1) and butanol by a two-step procedure. Micellar solutions with different CTAB and butanol concentrations were employed; the extraction was performed using an experimental design.

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 (CTAB in isooctan/hexanol/butanol). This mixture was agitated on a

vortex for 1 min, to obtain the equilibrium phase, and again separated into two phases by centrifugation at 1,677 g for 10 min (Jouan Centrifuge Mod. 1812, Saint-Herblain, France). Two milliliters of the micellar phase (containing β-xylosidase) was mixed with 2.0 mL of fresh aqueous phase (acetate buffer 1.0 M at pH 5.5 with 1.0 M NaCl), in order to transfer the enzyme from the micelles to this fresh aqueous, called the second aqueous phase (backward-extraction), which was finally collected by centrifugation (1677 g; 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.

Analyses

β-Xylosidase activity. β-Xylosidase activity was measured using the method described by Kumar and Ramón, 1996. Specific activity was expressed as Units/milligram of protein based on protein determination, according to the method of Lowry, using bovine serum albumin as the standard.

***W_o* and *R_m*.** The size of a reversed micelle is expressed by the surfactant-to-water ratio (Equation 1), hence experiments were performed involving changing the concentration of water (Luisi et al. 1988, Lye et al. 1995). The water content in the CTAB reversed micelles was checked by means of a Karl-Fischer titrator.

$$W_o=[H_2O]/[CTAB] \quad \textbf{[1]}$$

Where:

$[H_2O]$ = water concentration
$[CTAB]$ = CTAB concentration
W_o = "water in oil" parameter, water content

The micellar radius is given by Equation 2:

$$R_m=1.64W_o \quad \textbf{[2]}$$

Where:
R_m = micellar radius (Angstroms)

Experimental design and statistical analysis. To verify the influence of the butanol and CTAB concentrations on the enzyme recovery by reversed micelles, a 2^2-full ortogonal design with 3 repetitions at the centre point was employed (Table 1). High, centre and low set points, coded into +1, 0 and -1 respectively, were selected for each of the two factors. Extractions representing all the 9 set point combinations were made, as well as three extractions representing the centre point. Assays were conducted randomly. To analyse the results the STATGRAPHIC® software version 6.0 was employed.

Electrophoretic analysis. The molecular weight the proteins were estimated by SDS-polyacrilamide gel electrophoresis, as described by Weber et al. 1972.

RESULTS AND DISCUSSION

Table 1 shows the recovery values of β-Xylosidase obtained from the CTAB reversed micelle extractions performed according to the 2^2-full orthogonal design with 3 repetitions at the center point. All the assays were carried in triplicate. The highest β-Xylosidase recovery (45%) was observed during run number 9, when the factors CTAB and butanol concentration were used at their central levels. This recovery value was 2.1 and 2.6-fold higher than those observed for runs number 1 and 4, respectively, when the related factors were used at the lowest and the highest levels. These results suggest that the maximum point of recovery can be found in the experimental range studied.

When cationic surfactants are used, the transport of enzymes to the interior of the reversed micelles is favored by their negative electric charge, which is reached with the use of pH values higher than the pI (isoelectric point) of the enzyme. In the case of β-Xylosidase, pI is between 4.9 and 6.0. Therefore, in extractions performed with cationic surfactants, the enzyme is expected to migrate to the interior of the reversed micelles when the pH of the aqueous phase is higher than the pI of the enzyme. In this work, pH 8.0 (higher than pI of β-Xylosidase) was used, and so the transfer of the enzyme to the micellar phase was governed by electrostatic interactions. The use of high concentrations of CTAB is consequently necessary to obtain larger micelles capable of including β-Xylosidase (100 kDa), due to the fact that micellar size can be assumed to be approximately proportional to the surfactant concentration (Ihara et al. 1995). On the other hand, changing the amount of one or more elements can displace the equilibrium, since the reversed micellar phase ceases to exist. This explains the non-recovery of β-Xylosidase when higher CTAB and butanol concentrations were used.

Regression analysis was carried out to fit the response (β-Xylosidase recovery) with the experimental data (Table 2).

The second-order equation was evaluated by the *F-test* analysis of variance (ANOVA), which showed statistically significant ($F_{model}>F_{Tab}=5.05$). The model did not show lack-of-fit and the determination coefficient (R^2 = 0.93) indicated that the model can explain 93% of the variability in the recovery (Table 3).

A mathematical model to represent the recovery of β-Xylosidase for reversed micelles of CTAB (in iso-octano

and hexanol) can, therefore, be expressed by Equation 3:

$$\hat{Y} = 42.35 - 3.07A + 5.79B + 5.67AB - 18.72A^2 - 13.32B^2 \quad [3]$$

Where:

$\hat{Y}$ = Enzyme recovery (%);
A = CTAB concentration;
B = Butanol concentration.

The maximum recovery value of β-Xylosidase (43%) corresponded to the point defined by a CTAB concentration of 0.26 M (A = - 0.05) and a butanol concentration of 29.4% (B = 0.2). The optimum value predicted from the results using the response surface model is shown in Figure1.

To validate the mathematical model, β-Xylosidase was extracted under different experimental conditions (Table 4).

The differences between the values experimentally obtained and the values predicted by the model are small and can be explained by experimental errors. Under the optimum conditions predicted by the model, a purification factor of 5.34 times was attained for the enzyme reextracted in the aqueous phase (Table 5).

Table 6 shows the micelle water content (W_o) and the radius of the reversed micelles (R_m) measured by the model under the optimized conditions (pH 8.0, hexanol concentration 10%, electric conductivity 4 mS/cm, temperature 26ºC, CTAB concentration 0.26 M and butanol concentration 29.4%). It can be seen that both parameters were higher in the forward extraction phase when compared with the backward extraction phase. A strong increase in the water content (W_o) or size of the reversed micelles was also reported by Regalado et al. 1994 for AOT/iso-octane microemulsion, and by Pessoa and Vitolo, 1998 for BDBAC/iso-octane-hexanol microemulsion. Bearing in mind that β-Xylosidase has a high molecular weight (~110 kDa), thus requiring reversed micelles with large diameters for its encapsulation. According to Krei and Hustedt, 1992, the reversed micelles should have a radius larger than 5 nm to absorbe proteins with molecular weight of around 100 kDa. As the process was conducted at pH 8.0 and this value was higher than the enzyme pI value, the extraction depended only on electrostatic interactions.

This work was concluded with an electrophoretic analysis of the enzyme extracted under the optimal conditions defined by the experimental design (pH 8.0, hexanol concentration 10%, electric conductivity 4 mS/cm, temperature 26ºC, CTAB concentration 0.26M and butanol concentration 29.4%). Figure 2 shows the electrophoretic separation of the proteins by SDS-PAGE. No proteins were detected in the fraction of the solubilized precipitate (lanes 3 and 4). Lane 5 and 6 (reextracted aqueous phase at the optimum point of β-Xylosidase recovery) shows the presence of two bands with molecular weight between 100 and 110 kDa. These results are in accordance to Cortez and Pessoa Jr., 1999 . These authors purified β-Xylosidase from *Penicillium janthinellum* and concluded that the enzyme has molecular weight in the range between 100 and 110 kDa. By cultivating *Aspergillus niger* in arabinoxilana, VanPeij-NNME et al. 1997 also purified and identified by SDS-PAGE that β-Xylosidase, and verified a molecular weight of 110 kDa.

CONCLUDING REMARKS

This study demonstrates that liquid-liquid extraction by micelles reversed is a process able to recover and increase the purity of β-Xylosidase produced by *Penicillium janthinellum* fungus. Under optimized conditions, 43% of β-Xylosidase was recovered and an enrichment factor of 5.34-fold in the reextracted aqueous phase was obtained.

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APPENDIX

Figures

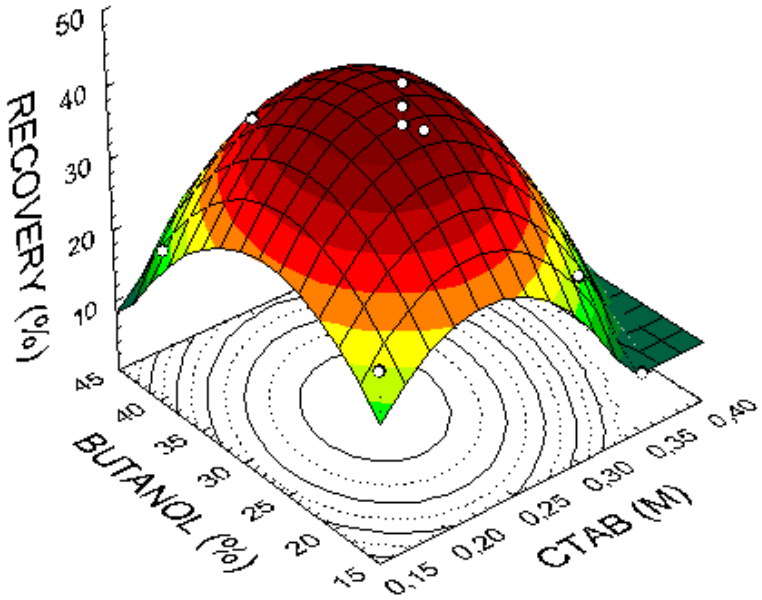

Figure 1. Response surface described by the model, which represents β-Xylosidase recovery as a function of CTAB and butanol concentration. Extraction conditions: pH 8.0, hexanol concentration 10%, electrical conductivity 4 mS/cm, temperature 26ºC, CTAB concentration 0.26M and butanol concentration 29.4%.

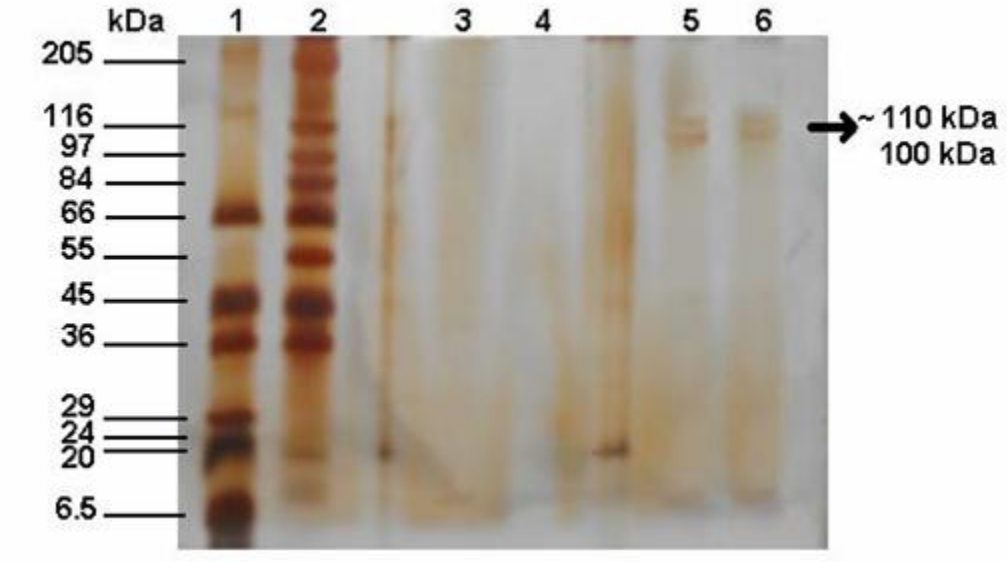

Figure 2. Electrophoresis (SDS-PAGE) of the optimized conditions of liquid-liquid extraction of β-Xylosidase in reversed micellar system.
Lanes 1 and 2: molecular weight markers;
Lanes 3 and 4: solubilized precipitated;
Lanes 5 and 6: reextracted aqueous phase.

Tables

Table 1. Experimental design and results of the 2^2-full orthogonal design with 3 repetitions at the center point.

Run number	Coded levels		Actual values		β-xylosidase activity* (U/mL)	β-xylosidase recovery (%)
	CTAB (M)	Butanol (%)	CTAB (M)	Butanol (%)		
1	-1	-1	0.15	15	0,73	21.8
2	+1	-1	0.35	15	0,63	0.0
3	-1	+1	0.15	39	0,81	16.5
4	+1	+1	0.35	39	0,76	17.4
5	0	$+2^{1/2}$	0.25	44	0,55	2.6
6	0	$-2^{1/2}$	0.25	10	0,65	0.0
7	$2^{1/2}$	0	0.39	27	0,6	0.0
8	$-2^{1/2}$	0	0.11	27	0,5	24.2
9	0	0	0.25	27	0,69	45.74
10	0	0	0.25	27	0,68	42.10
11	0	0	0.25	27	0,69	39.2

* solubilized precipitate

Table 2. Results of the regression analysis using the 2^2-full orthogonal design with 3 repetitions at the center point.

Term	Coefficient	*t*-statistic	*p*-value
Intercept	42.35	10.97	<0.005
CTAB (A)	-3.07	-0.65	0.12
Butanol (B)	5.79	1.22	0.03
AB	5.67	0.84	0.07
AA	-18.72	-3.32	0.005
BB	-13.32	-2.36	0.01

Table 3. Analysis of variance (ANOVA) for the model regression representing β-xylosidase recovery.

Source of variations	Sum of squares	Degrees of freedom	Mean squares	*F*-value
Model	3452.35	5	690.47	15.40
Residue	224.15	5	44.83	
Total (corr)	3676.50	10		

$R^2 = 0.93$

Table 4. Predicted and obtained values of β-Xylosidase recovery under different experimental conditions using the mathematical model (equation 3).

Experimental conditions		Recovery (%)	
CTAB (M)	Butanol (%)	Predicted by the model	Experimentally obtained
0.26	29.4	43	44.3*
0.10	20.0	21.5	18.5

Note: The runs were performed twice (each one with three replicates).
Optimum conditions predicted by the model

Table 5. Purification factor of β-Xylosidase extracted by reversed micelles.

Sample	Specific Activity (U/mg)
Reextracted aqueous phase	21.58
Solubilized precipitate	4.04
Purification factor	5.34

Note: In the culture both: β-Xylosidase activity: 0.98 U
Total protein: 0.78 mg

Table 6. Micelle water content (W_o) and micellar radius (R_m) of CTAB reversed micelles, after forward and backward extractions.

Phase	W_o	R_m (nm)
Forward extraction	85.13	13.95
Backward extraction	11.00	1.80