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Screening of Variables in Xylanase Recovery Using BDBAC Reversed Micelles

E. M. G. Rodrigues, A. Pessoa, Jr., and A. M. F. Milagres*

Department of Biotechnology, Faculty of Chemical Engineering of Lorena, 12.600-000, Lorena/SP-Brazil, E-mail: adriane@debiq.faenquil.br

Abstract

Xylanase recovery by reversed micelles using cationic surfactant (N-benzyl-N-dodecyl-N-bis(2-hydroxyethhyl)ammonium chloride) BDBAC was evaluated under different experimental conditions. A full factorial design with center point was employed to verify the influence of different factors on the recovery. A mathematical model was found to represent the xylanase yield (Y) as a function of BDBAC and hexanol: $Y = 4.32 + 5.1B + 2.64D + 0.83B^2 + 1.46D^2$, where B = BDBAC and D = hexanol. The highest xylanase recovery (27%), indicated by the model was attained at pH = 8.1, BDBAC = 0.38 *M* and hexanol = 8.6%. Under these conditions, and to test the model, a new xylanase extraction was performed in laboratory, giving 29.4% recovery yield, this value was similar to that predicted by the model.

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

Introduction

Plants are the source of renewable natural fibers used in the paper and textile industries. Normally, the cellulosic fibers are bleached by chemicals processes, which are harmful to the environment. Environmental problems could be avoided by replacing the chemical bleaching processes by biologically oriented processes, such as the use of xylanase *(1–5)*. In fact, using xylanases to facilitate pulp bleaching lowers chlorine consumption and reduces toxic discharges *(6–10)*. Besides, xylanases can also be employed in baking processes increasing loaf volume *(11)*, clarification of beer and juice *(12)*, and partial xylan hydrolysis in animal feed *(13)*. *Penicillium janthinellum* strain produces xylanase with high xylanolytic activity, but no detectable cellulolytic activity *(2)*. At present, xylanases are produced from

*Author to whom all correspondence and reprint requests should be addressed.

processed or refined substrates such as sugars, cellulose, and xylan. However, these are expensive substrates that account for the high costs of industrial-scale production *(14)*. Such costs would lower if cheaper substrates were employed. Sugar cane bagasse hemicellulosic hydrolysate, which is mainly composed of xylose oligomers, is certainly a cheap substrate. Cultivation conditions for producing xylanase using this hydrolysate were tested by *(15)*, but downstream processing still needs to be studied.

Reverse micellar systems have been extensively studied as a technique for the extraction and purification of proteins *(16–19)*. This technique allows the recovery and concentration of protein from a dilute aqueous solution containing other bioproducts *(17)*. 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 micelles permits the solubilization of protein while maintaining its native structure. The overall liquid-liquid extraction process by reverse micelles consists of 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 reverse micelles and transferred to an aqueous phase to be subsequently recovered *(16,17)*. Because the extraction process is governed by the electrostatic interaction between the charged protein and the micellar wall, protein transfer can only take place during the forward extraction when the pH of the aqueous phase is such that the net surface charge of the protein is electrically opposite to that of the surfactant headgroups. In the back extraction, however, the pH value must be such that the protein has the same charge as the surfactant molecules; in this way repulsion forces are created, causing the release of protein from the reverse micelles. Ionic strength impedes protein transfer to reverse micelles but seems to promote protein release *(17,20,21)*. This technique is therefore particularly interesting for the recovery of extracellular enzymes, which are presently purified by conventional processes *(22)*.

This work describes the transfer of extracellular xylanase from *P. janthinellum* to a reversed micellar phase of the cationic surfactant BDBAC *(23,24)* and the influence of the following factors: pH, concentration of salts (ionic strength), temperature, solvent/co-solvent volume ratio, surfactant concentration, agitation time, and buffer concentration *(16,21,25)*. The extraction and recovery of xylanase enzymic protein has been investigated with a particular reference to the yield of the extraction process. 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 1. requiring a large number of experiments and 2. missing the interactions among the variables. An alternative is the use of fractional factorial designs, which require fewer experiments *(26)*. This methodology makes it possible to study the effects of the variables individually and their interactions.

Materials and Methods

Microorganism and Growth Conditions

The isolation of *P. janthinellum* from decaying wood was described by Milagres *(27)*. This microorganism was identified by the Biosystematic Research Center of Canada and deposited in their collection under the designation of CRC 87M-115. The strain was initially maintained in silica stocks and then transfered to agar slants. The fungi were 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 *(28)*, and 2% agar-agar. The medium was autoclaved at 112°C for 15 min. In order to obtain the spore inocula, 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.

Preparation of Sugar Cane Bagasse Acid Hydrolysate

In order to prepare the hydrolysate for cultivation, 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.

Enzyme Production

The cultivation medium consisted of the aforementioned sugar cane bagasse hemicellulosic hydrolysate supplemented with 2% (v/v) concentrated salt solution based on Vogel's medium and 0.1% yeast extract *(29)*. The medium was autoclaved for 15 min at 112°C and poured into 125 mL Erlenmeyer flasks for cultivation. Inocula were prepared by inoculating 10^5 spores per ml into Erlenmeyer flasks containin 25 mL of pre-sterilized media. Standard cultivation conditions were: temperature 30°C; initial pH 5.5 (uncontrolled); agitation 60 rpm, cultivation time of 96 h.

Liquid-Liquid Extraction

The liquid-liquid extraction was performed using an experimental design. The enzyme was extracted from the whole clarified fermentation broth by BDBAC-reversed-micelles in isooctan by a two-step procedure. In the first step (forward-extraction), 4.0 mL of the aqueous xylanase solution was mixed with an equal volume of micellar microemulsion (BDBAC in isooctan/hexanol), the pH of the aqueous phase solution being adjusted by adding phosphate or acetate buffer with an appropriate pH, to a desirable final concentration. The equilibrium phase was obtained by intense agitation on a vortex. The phases were separated by centrifugation at 2800*g* for 5 min. Two mL of xylanase-BDBAC-micellar phase was mixed with 2.0 mL of fresh aqueous phase (acetate buffer 1.0 M at pH 5.5 with 0.5 M NaCl), in order to transfer the enzyme from the micelles to this aqueous phase (backward-extraction), which was finally collected by centrifugation

(2800*g*; 5 min). Both aqueous phases were assayed to determine enzyme activity. The extraction results are reported in terms of total activity recovered (%) in the stripping phase using the xylanase content of the initial aqueous phase for reference. Usually, about 10% of the total activity was lost in the balance, which could be attributed to denaturation.

Determination of Enzyme Activities

Xylanase activities were determined according to Bailey et al. *(30)*. The released reducing-equivalents were measured by a colorimetric assay *(31)* using xylose solution as a standard reference. Activity units were expressed as micromoles of reducing equivalents released/min at 50°C. The enzymatic activity during the extraction experiments varied between 40 and 60 U/mL.

Water Determination

Water content (Wo: water in oil) is defined as the ratio of water molecules over surfactant molecules per reverse micelle ($Wo = [H_2O]/[BDBAC]$). It is proportional to the radius of the aqueous core of the reverse micelle (Rm), i.e., $Rm = (3.Wo.M_{aqu})/(a_{surf}.N_{av}.\rho_{aqu})$, where: M_{aqu} is the water molecular weight, ρ_{aqu} the density of water, and a_{surf}. denotes the area per surfactant molecule in the interface, which depends on the properties of the surfactant and of the aqueous and organic phases. For ionic surfactants at room temperature, a_{surf}. is in the range of 0.5–0.7 nm^2 *(11)* and N_{av} is the Avogadro's number. Water content values are expressed as % by weight using Karl-Fischer-Moisture Titrator (Mettler DL18).

Experimental Designs

After selecting the most significant factors(indicated by the 2^{7-3} and 2^{5-1} fractional factorial designs) another statistical design was made to determine the optimized factors and the statistical model. A 2^4 full factorial central composite design with eight star points and four replicates at the center point and sixteen points was employed to fit a second-order polynomial model, which indicated that 28 experiments would be required for this procedure. BDBAC and buffer concentrations, pH and co-solvent/solvent ratio were selected as experimental factors. The range and levels of the variables investigated in this study are shown in Table 1.

Chemicals

Birchwood 4-O-methyl-β-D-glucuroxylan (90% xylose) was obtained from Sigma (St. Louis, MO). The cationic surfactant BDBAC [N-Benzyl-N-bis(2-hydroxyethyl)-ammonium chloride] (purity > 99%) was purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Table 1
Factors and Levels Used in the Assays
of Reversed Micelle Liquid-Liquid Extraction

	Range and levels				
Variables	–2	–1	0	+1	+2
pH	5.1	5.9	6.6	7.4	8.1
BDBAC (M)	0.18	0.23	0.28	0.33	0.38
Buffer (M)	0	0.01	0.05	0.08	0.12
Hexanol (%)	3.6	4.8	6.1	7.3	8.6

Results and Discussion

Seven variables at two levels (pH, 5.5 and 7.0, temperature, 10 and 30°C; agitation time, 1 and 3 min; percentage of hexanol, 7.5 and 10%; and concentrations of BDBAC, 0.1 and 0.2 *M*; buffer, 0.03; and 0.1 *M* and NaCl, 0 and 0.1 *M*, were selected and their effects on the extraction by reversed micelles was studied. In order to reduce the number of assays, a fractional factorial design (2^{7-3}) was performed. The statistical analysis showed that 5 variables (pH, NaCl, BDBAC, buffer, and hexanol concentration) presented significant effects. Another fractional factorial design (2^{5-1}) was employed. The analysis of variance of the results showed a significant curvature ($p < 0.05$ and $R^2 = 0.99$). This fact indicates that the region where the experiments were performed is located around the surface curvature. Therefore, this region is better represented by a model of order higher than 1. A new experimental design with ascending points of the four significant variables (pH, concentrations of BDBAC, buffer, and hexanol) was performed in order to adjust a second-order model.

The experiments corresponding to the ascending points were conducted to determine the region of best recovery. The 2^4 full rotational experimental design with center face required 28 experiments. As can be seen in Table 2, the best enzyme recovery was ~20%. The statistical analysis of the results showed significant effects only for the main variables A and B, and no evidence of any interactions involving these variables were obtained. The interactions BC and CD and the quadractic interactions B^2 and D^2 were significant at 95% confidence level for xylanase recovery (Table 3). The other variables were not significants and therefore were not included in the equation described by the model.

The adjustment of the second-order model by statistical criteria showed a significant level of 99%. However, the model adjusted to the data presented a correlation coefficient of 71% (Tables 4 and 5). The mathematical model to represent the extraction process can be expressed by Eq. 1.

$$Y = 8.32 - 2.00A + 2.36B - 1.37BC - 1.32CD + 0.83B^2 + 1.46D^2 \quad (1)$$

Table 2
Factors and Yields of the 2^4 Full Factorial Design Rotational with Center Face

	Factors[a]				
Assay	A	B	C	D	Yield (%)
1	–1	–1	–1	–1	10.45
2	+1	–1	–1	–1	6.70
3	–1	+1	–1	–1	14.25
4	+1	+1	–1	–1	14.65
5	–1	–1	+1	–1	11.70
6	+1	–1	+1	–1	7.45
7	–1	+1	+1	–1	16.95
8	+1	+1	+1	–1	10.65
9	–1	–1	–1	+1	11.30
10	+1	–1	–1	+1	6.45
11	–1	+1	–1	+1	20.10
12	+1	+1	–1	+1	17.60
13	–1	–1	+1	+1	10.05
14	+1	–1	+1	+1	6.80
15	–1	+1	+1	+1	11.90
16	+1	+1	+1	+1	6.30
17	–2	0	0	0	10.40
18	+2	0	0	0	1.35
19	0	–2	0	0	6.15
20	0	+2	0	0	13.75
21	0	0	–2	0	2.60
22	0	0	+2	0	9.70
23	0	0	0	–2	14.90
24	0	0	0	+2	10.00
25	0	0	0	0	9.00
26	0	0	0	0	8.00
27	0	0	0	0	10.70
28	0	0	0	0	7.98

[a]A, pH (–2 = 5.1; –1 = 5.9; 0 = 6.6; +1 = 7.4; +2 = 8.1). B, BDBAC (M) (–2 = 0.18; –1 = 0.23; 0 = 0.28; +1 = 0.33; +2 = 0.38). C, buffer (M) (–2 = 0; –1 = 0.01; 0 = 0.05; +1 = 0.08; +2 = 0.12). D, Hexanol (%) (–2 = 3.6; –1 = 4.8; 0 = 6.1; +1 = 7.3; +2 = 8.6).
Average of two repetitions.

Where:

Y = Yield (%)
A = pH
B = BDBAC (M)
C = buffer (M)
D = hexanol (%)

This model (Eq. 1) is composed of 4 variables and its graphical representation (contour lines and surface response) is not possible. Then coded values were given to pH (A) and buffer concentration (C) and Eqs. 2, 3, 4,

Table 3
Analysis of the Effects and Interactions
of the Full Factorial Design 2^4 Rotational with Center Face

Effects[a]	Estimates	Standard error	*t* Value
Average	8.920	+/–0.639	—
A	–4.017	+/–0.522	7.695[b]
B	4.725	+/–0.522	9.052[b]
C	–0.458	+/–0.522	0.877
D	–1.008	+/–0.522	1.931
AB	0.263	+/–0.639	0.412
AC	–1.088	+/–0.639	1.703
AD	–0.288	+/–0.639	0.451
BC	–2.738	+/–0.639	4.285[b]
BD	0.138	+/–0.639	0.216
CD	–2.638	+/–0.639	4.128[b]
AA	–0.573	+/–0.522	1.098
BB	1.465	+/–0.522	2.807[b]
CC	–0.435	+/–0.522	0.833
DD	2.715	+/–0.522	5.201[b]

[a]A, pH; B, BDBAC (M); C, Buffer (M); D, Hexanol (%).
[b]Significant ($t_{3,0,95} = 3.182$)

Table 4
Factors, Regression Coefficients, Standard Error, *t* Value,
and Significance Level to the Model Represents the Extraction Process
by Reversed Micelles Using a 2^4 Rotational Full Factorial Design with Center Face

Factors	Coefficients	Standard error	*t* Value	*p* Value
Average	8.316	+/–0.833	9.98	0.000
A	–2.008	+/–0.538	3.73	0.001
B	2.362	+/–0.538	4.39	0.000
BC	–1.369	+/–0.659	2.08	0.050
CD	–1.319	+/–0.659	2.00	0.058
B^2	0.833	+/–0.510	1.63	0.117
D^2	1.458	+/–0.510	2.86	0.009

Table 5
Analysis of Variance of the Model Regression the Extraction Process
by Reversed Micelles Using a 2^4 Rotational Full Factorial Design with Center Face

Source of variations	Degrees of freedom	Sum of squares	Mean square	*F* Value	*p* Value[a]
Model	6	354.818	59.134	8.5181	0.0001
Residue	21	145.791	6.942		
Total	27	500,608			

[a]$p < 0.05$.

Table 6
Observed Responses and Predicted Yields

Observation number[a]	Factors A	B	C	D	Predicted yields by the model (%)	Actual[b] yield(%)
1	–2	–2	2	–2	26.6	24.9
2	2	2	–2	2	27.0	29.4
3	2	2	2	–2	18.6	19.2
4	–2	2	–2	2	35.0	20.3

[a]The observation numbers refer to the following equations 1, $Y = 12.32 – 0.38B – 2.64D + 0.83B^2 + 1.46D^2$ (A = –2, C = 2). 2, $Y = 4.32 + 5.10B + 2.64D + 0.83B^2 + 1.46D^2$ (A = 2, C = –2). 3, $Y = 4.32 – 0.38B – 2.64D + 0.83B^2 + 1.46D^2$ (A = 2, C = 2). 4, $Y = 12.32 + 5.1B + 2.64D + 0.83B^2 + 1.46D^2$ (A = –2, C = –2).

[b]Average of two repetitions.

A, pH (–2 = 5.1; 2 = 8.1). B, BDBAC(mol/L) (–2 = 0.18; 2 = 0.38). C, Buffer(mol/L) (–2 = 0; 2 = 1.2). D, Hexanol (%) (–2 = 3.6; 2 = 8.6).

and 5 were obtained from Eq. 1 by replacing the coded values of A and C. They are:

$$Y = 12.32 – 0.38B – 2.64D + 0.83B^2 + 1.46D^2 \quad (A = –2, C = 2) \qquad (2)$$

$$Y = 4.32 + 5.10B + 2.64D + 0.83B^2 + 1.46D^2 \quad (A = 2, C = –2) \qquad (3)$$

$$Y = 4.32 – 0.38B – 2.64D + 0.83B^2 + 1.46D^2 \quad (A = 2, C = 2) \qquad (4)$$

$$Y = 12.32 + 5.10B + 2.64D + 0.83B^2 + 1.46D^2 \quad (A = –2, C = –2) \qquad (5)$$

where: Y = yield (%); B = BDBAC (M); D = hexanol (%).

New extractions under theoretically optimal conditions were performed using the values predicted by each model (Table 6). As can be seen the yield predicted by Eq. 4 was 40% higher than the actual value, however the other assay were good reproducible (<10%). Among these assays the highest yield was 29.4%. This experimental finding is in close agreement with the model prediction (27%). The water content of these micelles (Wo) was 45.8 (micellar radius = 6.8 nm), which showed that the micelle size was not a limiting variable during the extraction.

The recovery and purification xylanase by reversed micelles was low if compaired with those obtained by precipitation with acetone *(32)*, ammonium sulfate *(33)*, and ethanol *(34)*; however, this technique enables other industrial applications if the enzymes are utilized entrapped within reversed micelles *(35,36)*. The xylanase recovery by reversed micelles using BDBAC surfactant provided an enrichment factor of 2.3-fold and is better represented by Eq. 3. The surface response and the contour lines corresponding to this model are represented by Fig. 1.

Conclusions

The extraction process by reverse micelles is a new purification method in downstream processing of xylanase. However, its efficiency depends on

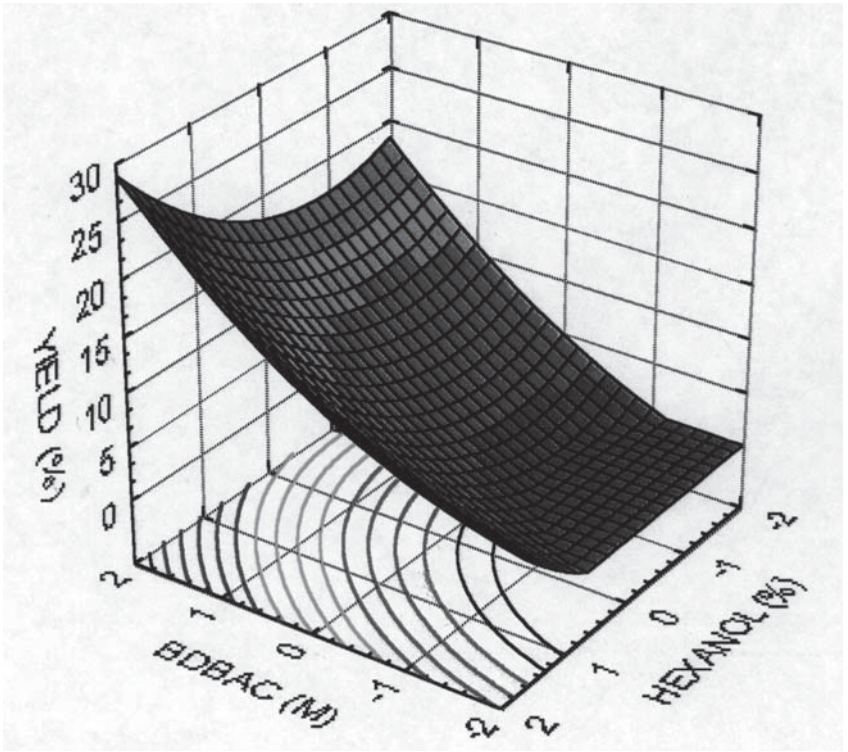

Fig. 1. Surface response and contour lines described by Eq. 3.

the choice of the extraction parameters. In our study, the highest xylanase recovery value attained was ~30%, under the following conditions: pH 8.1, 0.38 *M* BDBAC cationic surfactant and 8.6% hexanol.

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