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Liquid–liquid extraction of xylitol dehydrogenase from *Candida guilliermondii* homogenate by reversed micelles

Ely Vieira Cortez [a], Adalberto Pessoa Jr. [a,*], Maria das Graças de Almeida Felipe [b], Inês Conceição Roberto [b,1], Michele Vitolo [a]

[a] *Biochemical and Pharmaceutical Technology Department/FCF, University of São Paulo, P.O. Box 66083, CEP 05315-970 São Paulo, SP, Brazil*
[b] *Biotechnology Department/FAENQUIL, Rod. Itajuba-Lorena, Km 74.5, 12.600-000 Lorena, SP, Brazil*

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Abstract

The intracellular enzyme xylitol dehydrogenase (XD, EC 1.1.1.9) from *Candida guilliermondii*, grown in sugarcane bagasse hydrolysate, was separated by reversed micelles of BDBAC [*N*-benzyl-*N*-dodecyl-*N*-bis (2-hydroxyethyl) ammonium chloride] cationic surfactant. An experimental design was employed to evaluate the influence of the following factors on the enzyme separation: temperature, co-solvent concentration and surfactant concentration. The results showed that just the temperature did not show significant effect on XD recovery. A model was used to represent the activity recovery and fit the experimental data. Under optimized conditions, the recovery of total activity was about 121%, and the purity increased 2.3-fold.

Keywords: *Candida guilliermondii*; Xylitol dehydrogenase

1. Introduction

Liquid–liquid extraction by reversed micelles is a useful and very versatile tool for separating biomolecules. This process shows a close similarity to the traditional liquid–liquid extraction process, since both are biphasic and consist on partitioning a target solute between an aqueous phase and an organic phase, with subsequent back transfer to a second aqueous stripping phase [1]. Reversed micellar systems have great potential for industrial application, since they provide a favorable environment for protein solubilization in the organic phase with preservation of biological activity [2]. A number of recent studies on reversed micellar system clearly demonstrates the interest in this technique for the separation of biotechnological products. Both intra- and extracellular biomolecules can be extracted from various sources and at the same time purified and concentrated to the same extent by relatively simple means, using processes that are easy to scale up [1]. A reversed micellar system consists of aggregates of surfactant molecules containing an inner water core dispersed in an organic solvent medium. The polar microenvironment inside the reversed micelle allows the solubilization of hydrophilic proteins while maintaining its native structure. The overall liquid–liquid extraction process by reversed micelles involves two fundamental steps: a forward extraction, by which the proteins are transferred from an aqueous solution to a reversed micellar organic phase (MPI), and a back extraction, by which the proteins are released from the reversed micelles and transferred to a fresh aqueous phase (APII), so that, they can be subsequently recovered [3,4]. The separation of the target protein can also be achieved by its retention in the remaining aqueous phase of the forward extraction (API), whereas the contaminants are transferred into the reversed micelles. In this case, the purification process is more simpler and more economic because it is not necessary the back-extraction step of the target biomolecule. The extraction process is mainly governed by electrostatic interactions between the net charged protein and the charged surface of the reversed micelles. During the forward extraction, the protein is transferred to the aqueous core of the reversed micelles, since the pH of the aqueous phase is such that the net surface charge of the protein is electrically opposite to that of the surfactant head groups. Although not usual, biomolecules such as enzymes, can also be extracted by hydrophobic interactions between

* Corresponding author. Tel.: +55-1130913862; fax: +55-1138156386.
E-mail addresses: pessoajr@usp.br (A. Pessoa Jr.), ines@debiq.faenquil.br (I.C. Roberto).
[1] Fax: +55-125533165.

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the nonpolar regions of the molecule and the surfactant tail [3]. 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 should be increased by the addition of salts. Therefore, repulsion forces between the surfactant molecules and the protein will be present, and the diameter of the micelles will be lowered due to the screening of the electrostatic interactions, resulting in the release of the protein from the reversed micelles. Low ionic strength favors protein transfer to reversed micelles, and high values promote protein release [5].

A good perspective should be to utilize this technique to purify a target protein directly from a crude microbial homogenate, aiming to remove a specific protein. In this respect, two-phase reversed micellar systems could be used for the removal of xylitol dehydrogenase from a *Candida guilliermondii* cell homogenate. The cell homogenate could then be used for 'in vitro' conversion of xylose into xylitol catalyzed by xylose reductase, an enzyme also present in *C. guilliermondii* extract. The removal of xylitol dehydrogenase is important because this enzyme catalyzes the oxidation of xylitol to xylulose, leading to the decrease of the overall xylitol production yield [6]. Xylitol is a sweetener, presenting anti-cariogenic property, low caloric value, and negative dissolution heat. Because it can be used successfully in food and pharmaceutical formulations, its production is in great demand [7]. The enzymatic xylose/xylitol conversion could become an alternative to the conventional process of xylose reduction by inorganic catalysis (Ni or Pt). Moreover, the xylitol dehydrogenase removed could become commercially available as an analytical reagent.

In this paper, the utilization of liquid–liquid extraction by BDBAC [*N*-benzyl-*N*-dodecyl-*N*-bis (2-hydroxyethyl) ammonium chloride] reversed micelles to separate and purify the enzyme xylitol dehydrogenase from *C. guilliermondii*, grown in sugarcane bagasse hydrolysate, was studied. The extraction and recovery of XD was investigated particularly from the point of view of recovery of the enzymatic activity.

2. Experimental

2.1. Preparation of hemicellulosic hydrolysate

Sugarcane bagasse was hydrolyzed in a 250-l reactor at 121 °C for 20 min with H_2SO_4 (100 milligrams acid per gram dry matter and solid:liquid ratio of 1:10). A portion of the hydrolysate was further concentrated under vacuum at 70 °C to increase xylose concentration four-fold. The vacuum procedure was necessary to avoid sugar degradation. The hydrolysate was then treated as described by Alves et al. [8], to reduce the concentrations of toxic substances.

2.2. Inoculum preparation, medium, and fermentation conditions

The microorganism utilized was *C. guilliermondii* FTI 20037 [9]. A medium containing 30.0 g l^{-1} of xylose supplemented with 20.0 g l^{-1} of rice bran extract, 2.0 g l^{-1} of $(NH_4)_2SO_4$ and 0.1 g l^{-1} of $CaCl_2{\cdot}2H_2O$ was used to grow the inoculum. Erlenmeyer flasks (125 ml), each containing

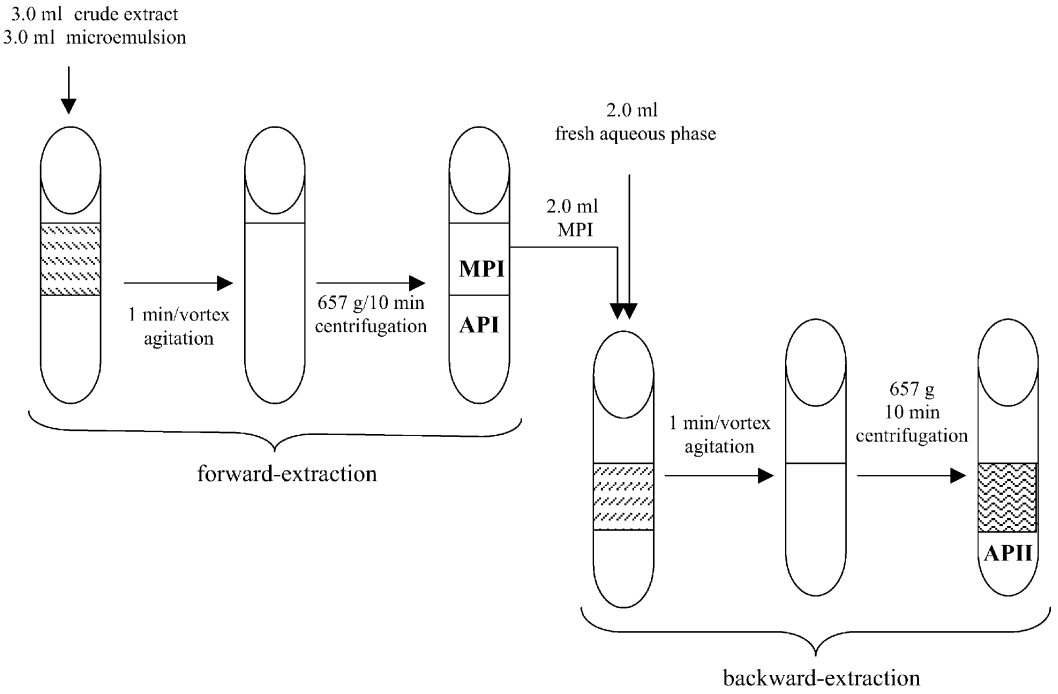

Fig. 1. Flow sheet of the partitioning process.

50 ml of medium with inoculum (initial pH 5.5), were incubated on a rotary shaker (200 rpm) at 30 °C for 24 h.

For the fermentation, concentrated bagasse hemicellulosic hydrolysate (containing 42 g l^{-1} of xylose, 3.1 g l^{-1} of glucose, 3.9 g l^{-1} of arabinose, 3.7 g l^{-1} of acetic acid, and 0.0420 g l^{-1} total phenols) was employed. The hydrolysate was supplemented with the same nutrients used for the inoculum preparation. The batch cultivation was carried out in a 1.25-l fermentor BIOFLO III (New Brunswick Scientific Co., Inc, Edison, New Jersey, USA), under agitation of 300 min^{-1} and aeration rate of 0.6 vvm ($K_L a$ 22.5 h^{-1}), at 30 °C, initial pH 5.5. The cells were maintained on malt-extract agar slants at 4 °C.

2.3. Preparation of cell-free extracts

Cells were harvested by centrifugation (800 × *g* during 15 min), washed with 0.1 M potassium phosphate buffer (pH 7.2), centrifuged and resuspended with the same buffer and stored at −18 °C. Cells were disrupted by sonication applying 1 s pulses with 1 s intervals for a period of 40 min using a disrupter (VC-100; Sonics & Materials, Newton, CT) at a frequency of 20 kHz. Cell homogenate was then centrifuged at 10,000 × *g* (Jouan, Model BR4i, St. Herblain, France) at 4 °C for 10 min, and the cell-free extract (crude extract) was analyzed.

2.4. Enzyme assays

Xylitol dehydrogenase activity was determined spectrophotometrically (Beckman DU 640, California, USA) at 340 nm at room temperature, as described by Alexander [10], using NAD^+ as cofactor. One enzyme unit was defined as μmol of NAD^+ reduced using an extinction coefficient of 6.22×10^{-3} M^{-1} cm^{-1}. Specific activity was expressed as units per milligram of total protein, with the total protein concentration being obtained according to Lowry et al. [11], using bovine serum albumin as the standard.

2.5. Liquid–liquid extraction

The enzyme was extracted, from the crude extracts (cell homogenate), in BDBAC-reversed-micelles in isooctane, by a two-step procedure (Fig. 1). In the first step (forward-extraction), 3.0 ml of the crude extract (containing XD) was mixed with an equal volume of reversed micellar microemulsion (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 (aqueous phase I: API, and micellar phase I: MPI) by centrifugation at 657 × *g* for 10 min (Jouan Centrifuge Mod. 1812, Saint-Herblain, France). Afterwards, 2 ml of XD-BDBAC-reversed micellar phase was mixed with 2.0 ml of fresh aqueous phase (acetate buffer 1.0 M at pH 5.5 containing 1.0 M NaCl), in order to transfer (backward-extraction) the enzyme from the micelles to the fresh aqueous phase, called the second aqueous phase II (APII), which was finally collected by centrifugation (657 × *g*; 10 min). Both aqueous phases (API and APII), and the crude extract were assayed to determine enzyme activity and total protein concentration. The extraction results are reported in terms of total activity recovered (%) in the first aqueous phase using the XD content of the crude extract as a reference. In this work, the forward extraction pH was maintained at 7.0 and electrical conductivity at 14 mS cm^{-1}, to avoid loss of activity. Employing these two values we attained good results, with no need to test other values.

2.6. Experimental design and statistical analysis

To verify the influence of temperature, co-solvent and surfactant concentrations on the activity recovery (*Y*) a 2^3 full factorial design with three repetitions at the center point was employed (Table 1). For each of the three factors, high (coded value: +1), center (coded value: 0), and low (coded value: −1) set points were selected. Extractions representing all the eight set point combinations (2^3) were performed, as

Table 1
Matrix and results of a 2^3 full factorial design with center point

Run no.	Actual values			Coded values			Recovery yield (%)	
	T (°C)	C_s (%)	*S* (M)	*T*	C_s	*S*	API[a]	APII[b]
1	5	6	0.10	−	−	−	4	46
2	30	6	0.10	+	−	−	0	0
3	5	9	0.10	−	+	−	92	0
4	30	9	0.10	+	+	−	78	0
5	5	6	0.20	−	−	+	0	0
6	30	6	0.20	+	−	+	71	0
7	5	9	0.20	−	+	+	5	11
8	30	9	0.20	+	+	+	1	3
9	17.5	7.5	0.15	0	0	0	0	2
10	17.5	7.5	0.15	0	0	0	5	2
11	17.5	7.5	0.15	0	0	0	7	3

T: temperature; C_s: co-solvent; *S*: surfactant. Conditions of the cell homogenate: pH = 7.0 and electrical conductivity = 14 mS cm^{-1}.

[a] API: aqueous phase I.

[b] APII: aqueous phase II.

Table 2
Analysis of variance (ANOVA) of the studied factors and interactions for XD extraction process by reversed micelles of BDBAC

Source of variation	Sum of squares	Degrees of freedom	Mean square	F value	P value
X_1: temperature	303.81	1	303.81	0.56	0.5045
X_2: co-solvent	1,303.05	1	1,303.05	2.39	0.1971
X_3: surfactant	1,178.55	1	1,178.55	2.16	0.2156
X_1X_2	905.25	1	905.25	1.66	0.2672
X_1X_3	884.10	1	884.10	1.62	0.2720
X_2X_3	6,699.03	1	6,699.03	12.28	0.0248
Total error	2,182.42	4	545.61		
Total (correlation)	13,456.22	10			

$R^2 = 0.84$.

well as the three extractions representing the center point (coded value: 0). Assays were conducted randomly.

2.7. Chemicals

The following pure reagents were used in this study: BDBAC, sulfuric acid, acetic acid, isooctane, and hexanol (Merck, Darmstadt, Germany); xylose, xylitol, nicotinamide adenine dinucleotide (NAD), bovine albumin, glucose, arabinose (Sigma, St. Louis, MO). All other reagents were of analytical grade.

3. Results and discussion

Table 1 shows the results of experiments based on a 2^3 full factorial matrix. As can be seen, under some extraction conditions (runs no. 2, 5, 7, 8, 9, 10, and 11), the recovery of activity in both phases was very low. Under these extraction conditions, the solvents or surfactants, probably, interacted with the enzyme resulting in denaturation. On the other hand, XD recoveries higher than 70% were attained in the experiments 3, 4, and 6, but the enzyme was not efficiently transferred into the reversed micelles, since in the fresh aqueous phase (APII) its activity was zero. The isoelectric point (pI) of XD, produced by *C. guilliermondii* in sugarcane bagasse hydrolysate is unknown. However, the XD described in this study should have a pI value around or higher than pH 7.0, since there was no attraction between the enzyme and the reversed micelles composed by the cationic surfactant BDBAC. The reversed micelles radius was calculated 4 nm. Since this micelle size allows the encapsulation of proteins with molecular mass of up to 100,000 relative molecular mass, it appears that the effect of size exclusion was not responsible for the low transfer of XD into the micelles, since the molecular mass of this enzyme is around 60,000 relative molecular mass. Moreover, the electrostatic or hydrophobic interactions, between the micelle and the enzyme, were not the extraction driving force. The electrostatic interaction is one of the most important factors in reversed micelle extraction, and this explains the high recovery of XD in aqueous phase I. This interaction can cause the enzyme migration to the micellar core, when the electrical net charge of the enzyme and the surfactant charge are opposite. Considering that there is no literature on XD extraction with reversed micelles, it can be concluded that the initial results were quite good, since the recovery of enzyme activity was around 92% in the remaining aqueous phase, and free of cell debris and some contaminants.

Table 2 gives the analysis of variance of the factors and interactions that were important for the XD separation by liquid–liquid extraction. As can be seen, only the interaction X_2X_3 (combination between surfactant and co-solvent) was significant at 95% confidence level. Therefore, it can be inferred that the temperature had no influence on XD recovery yield over the range evaluated. To verify the validity of the statistical model, considering only the significant factors (surfactant and co-solvent concentrations), another analysis of variance was performed, as shown in Table 3. The low value of determination coefficient ($R^2 = 0.68$) indicates that the linear model is inadequate to explain the results of XD recovery. Then, a new 2^2 full factorial matrix with centered face and three repetitions at the center point was designed, to evaluate if a second-order model could explain the extraction

Table 3
Analysis of variance (ANOVA) of the significant factors and interactions for XD extraction process by reversed micelles of BDBAC

Source of variation	Sum of squares	Degrees of freedom	Mean square	F value	P value
X_2: co-solvent	1,303.05	1	1,303.05	2.39	0.1971
X_3: surfactant	1,178.55	1	1,178.55	2.16	0.2156
X_2X_3	6,699.03	1	6,699.03	12.28	0.0248
Total error	4,275.58	7	610.79		
Total (correlation)	13,456.22	10			

$R^2 = 0.68$.

Table 4
Matrix and results of a 2^2 full factorial design with centered face and three repetitions at the center point

Run no.	Actual values		Coded values		Recovery (%)	
	X_2 (%)	X_3 (M)	X_2	X_3	API[a]	APII[b]
1	6	0.10	−	−	4	46
2	9	0.10	+	−	85	0
3	6	0.20	−	+	0	0
4	9	0.20	+	+	5	11
5	7.5	0.15	0	0	89	29
6	7.5	0.15	0	0	74	29
7	7.5	0.15	0	0	93	37
8	7.5	0.15	0	0	106	12
9	6	0.15	−	0	8	22
10	9	0.15	+	0	123	10
11	7.5	0.10	0	−	125	19
12	7.5	0.20	0	+	3	6

C_s: co-solvent; S: surfactant. Conditions of the cell homogenate: temperature = 5 °C, pH = 7.0, and electrical conductivity = 14 mS cm^{-1}.
[a] API: aqueous phase I.
[b] APII: aqueous phase II.

process. It was considered the factors co-solvent (X_2) and surfactant (X_3). The temperature (X_1) was maintained constant at 5 °C to prevent thermal denaturation of the enzyme. Table 4 gives the results of the experiments based on the 2^2 full factorial matrix. As can be seen, the recovery yield of the XD in the aqueous phase I strongly increased (up to 125%; run no. 11), and it was confirmed that the co-solvent and the surfactant concentrations influence significantly the enzyme partitioning. The XD recovery was above 100% since the extraction process reduced the concentration of several enzyme inhibitors, originally present in the cell homogenate derived from the acid hemicellulosic hydrolysate (mainly hydrophobic compounds such as furfural, hydroxymethylfurfural and phenols) used in the fermentation process by *C. guilliermondii* as a carbon source.

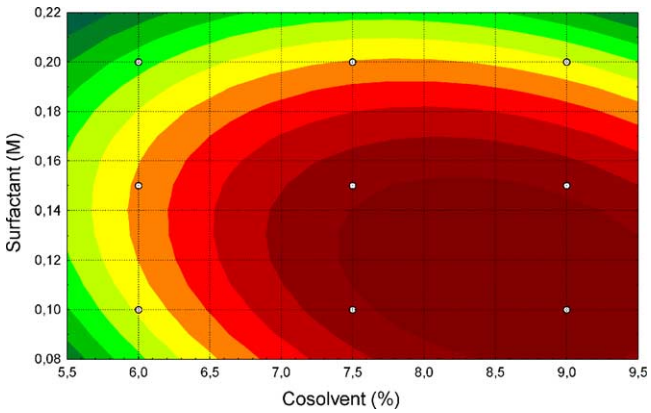

Fig. 2. Isoresponse contour plot of XD recovery showing the effect of co-solvent and surfactant concentrations (temperature and electrical conductivity were kept constant at 5 °C and 14 mS cm^{-1}, respectively).

By applying multiple regression analysis on the experimental data, the following second order polynomial equation (Eq. (1)) was obtained to explain the XD recovery, over the studied experimental region.

$$\hat{y} = 93.08 + 33.50X_2 - 34.33X_3 - 32.75X_2^2 - 34.25X_3^2 - 19.00X_2X_3 \quad (1)$$

The statistical significance of the second-order model was evaluated by the F-test analysis of variance (Table 5), which showed that the regression is statistically significant ($P = 0.0214$) at 5% confidence level and also presents a good determination coefficient ($R^2 = 0.84$). Fig. 2 represents the isoresponse contour plot for XD recovery ($\hat{y}$) as a function of the independent variables co-solvent concentration (X_2) and surfactant concentration (X_3). Over the values investigated, the optimum level of these variables for higher XD extraction can be attained at 8.5% (coded level =

Table 5
Analysis of variance (ANOVA) for the model regression representing XD extraction process by reversed micelles of BDBAC

Source	Sum of squares	Degrees of freedom	Mean square	F value	P value
Model	24,230.42	5	4,846.08	6.40	0.0214
Residual	4,542	6	757.08		
Lack of fit	4,021.50	3	1,340.50	7.72	0.0636
Pure error	521.00	3	173.67		
Total	28,772.92	11			

$R^2 = 0.84$.

Table 6
Purification of XD produced by *C. guilliermondii* using BDBAC reversed micelles

Purification steps	Total protein (mg ml^{-1})	Total activity (U ml^{-1})	Specific activity (U mg^{-1})	Purification factor	Recovery (%)
Cell homogenate	1.68	0.333	0.198	1.0	–
Aqueous phase I	0.77	0.353	0.458	2.3	121

Extraction conditions: T = 5 °C; co-solvent = 8.5%; surfactant = 0.12 M; pH = 7.0; and electrical conductivity = 14 mS cm^{-1}.

+0.70) co-solvent and 0.12 M (coded level = −0.60) of surfactant. Under these conditions, the model predicted a XD recovery of 117% (a variation of 94–140% being possible) in the confidence range of 95%. To confirm these promising results, an extraction assay at the optimum conditions obtained was performed, and the purification factor and recovery yield calculated (Table 6). As can be seen, the purification factor increased 2.3-fold and the recovery yield was 121%. These results showed that the model fitted well the experimental data, and thus described well the region studied.

4. Conclusions

This study demonstrated that liquid–liquid extraction by reversed micelles is a process able to separate, increase the enzymatic activity, and purify the XD produced by *C. guilliermondii* cultivated in sugarcane bagasse hydrolysate, since the recovery yield obtained in this work was around 121% and the enrichment factor was 2.3. The response surface methodology was a useful tool to qualitatively understand the mechanism of enzyme extraction, and the results indicated that the statistical model obtained was adequate for the process. This study presented promising results, since the high recovery yield and satisfactory enrichment factor were achieved in the remaining aqueous phase, meaning a simple and economic technique to purify XD.

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