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Optimized extraction by cetyl trimethyl ammonium bromide reversed micelles of xylose reductase and xylitol dehydrogenase from *Candida guilliermondii* homogenate

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Abstract

The intracellular enzymes xylose reductase (XR, EC 1.1.1.21) and xylitol dehydrogenase (XD, EC 1.1.1.9) from *Candida guilliermondii*, grown in sugar cane bagasse hydrolysate, were separated by reversed micelles of cetyl trimethyl ammonium bromide (CTAB) cationic surfactant. An experimental design was employed to optimize the extraction conditions of both enzymes. Under these conditions (temperature = 5 °C, hexanol: isooctane proportion = 5% (v/v), butanol concentation = 22%, surfactant concentration = 0.15 M, pH = 7.0 and electrical conductivity = 14 mS cm^{-1}) recovery values of about 100 and 80% were achieved for the enzymes XR and XD, respectively. The purity of XR and XD increased 5.6- and 1.8-fold, respectively. The extraction process caused some structural modifications in the enzymes molecules, as evidenced by the alteration of K_M values determined before and after extraction, either in regard to the substrate (up 35% for XR and down 48% for XD) or cofactor (down 29% for XR and up 11% for XD). However, the average variation of V_{max} values for both enzymes was not higher than 7%, indicating that the modified affinity of enzymes for their respective substrates and cofactors, as consequence of structural modifications suffered by them during the extraction, are compensated in some extension. This study demonstrated that liquid–liquid extraction by CTAB reversed micelles is an efficient process to separate the enzymes XR and XD present in the cell extract, and simultaneously increase the enzymatic activity and the purity of both enzymes produced by *C. guilliermondii*.

Keywords: Reversed micelles; *Candida guilliermondii*; Xylitol dehydrogenase; Xylose reductase; Enzymes

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 liquid–liquid traditional extraction process, because both are biphasic and consist of partitioning a targeted solute between an aqueous feed phase and an organic phase, with a subsequent back transfer to a second aqueous stripping phase [1,2]. 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. A number of recent studies on reversed micellar methodology clearly demonstrate the interest in reversed micelles 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 reverse micellar system consists of aggregates of surfactant molecules containing an inner water core dispersed in an organic solvent medium. The micro-environment of the micelles may contribute to altered kinetic properties. The overall liquid–liquid extraction process by reverse micelles is conducted in two fundamental steps: a forward extraction, by which the target protein or the contaminants are transferred from an aqueous solution to a reverse micellar organic phase, and a back extraction, by which the biomolecules are released from the

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reversed micelles and transferred to a fresh aqueous phase, so that it can be subsequently recovered [3,4]. The separation of the target protein also can be achieved if, during the extration, it is retained in the remaining aqueous phase (API), and the contaminants are transferred into micelles, or vice versa.

In the first situation, the purification process is more simple and economic, because it is not necessary the back-extraction step of the target biomolecule. 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 to that of the surfactant head groups. Although is not usual, biomolecules, as enzymes, can also be extracted by hydrophobic interaction between the apolar 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 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 [5].

A good perspective should be to apply this technique directly in a crude microbial homogenate, aiming to remove a specific protein or enzyme. An example should be the removal of xylitol dehydrogenase (XD) from a *Candida guilliermondii* homogenate, which, after removing the cell debris, could be used in the "in vitro" conversion of xylose into xylitol catalyzed by xylose reductase (XR), an enzyme also present in the extract. If XD was not removed from the reaction medium, the xylitol formed, by the action of XR on xylose, would certainly be oxidized to xylulose, leading to the decrease of the overall production yield [6]. Nowadays, xylitol is a product in great demand because it can be used in food industry (for its sweetening power and insulin-independent metabolism), dentistry formulations (for its anticariogenicity, tooth rehardening and remineralization properties) and pharmaceutical formulations (for its capability of preventing otitis and its possibility of being used as a sweetener or excipient in syrups, tonics and vitamin formulations) [7].The enzymatic xylose/xylitol conversion could become an alternative to the conventional process based on the reduction of xylose with inorganic catalyst (Ni or Pt). Besides, the xylitol dehydrogenase attained could become commercially available as an analytical reagent.

This study evaluates the effectiveness of liquid–liquid extraction by CTAB reversed micelles in purifying XD and XR enzymes, in two different phases of the micellar system, from *C. guilliermondii*, grown in sugar cane bagasse hydrolysate. The extraction and recovery of XD and XR have been investigated with particular attention to the recovery of the enzymatic activities.

2. Materials and methods

2.1. Chemicals

The following pure reagents were used in this study: cetyl trimethylammonium bromide (CTAB) (Carlo Erba, Milan, Italy); acetic acid, Karl-Fischer solution, isooctane, butanol and hexanol (Merck, Darmstadt, Germany); xylose, NADPH, xylitol, NADP, bovine albumin, glucose, arabinose (Sigma, St. Louis, MO). All other reagents are analytical grade.

2.2. Preparation of hemicellulosic hydrolysate

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

2.3. Inoculum preparation, medium and fermentation conditions

The fermentations were conducted with *C. guilliermondii* FTI 20037 [9]. A medium containing 3.0 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 for growing the inoculum. Erlenmeyer flasks (125 ml), each containing 50 ml of medium with inoculum (initial pH 5.5), were incubated on a rotary shaker ((New Brunswich, model G-25, Edison, NJ) (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 cultivation was done by a batch process in a 1.25 l fermentor BIOFLO III (New Brunswick Scientific Co. Inc., Edison, NJ, 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.

2.4. Preparation of cell-free extracts

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

at 10,000 × *g* at 4 °C for 10 min, and the cell-free extract (crude extract) was analyzed.

2.5. Enzyme assays

The constituents of the reaction media for measuring the xylose reductase activity were 100 μl of 5 mM xylose, 50 μl of 3.0 μM NADPH, 250 μl of enzyme extract and 600 μl of phosphate buffer (pH 6.2), whereas for xylitol dehydrogenase were 300 μl of 5 mM xylitol, 50 μl of 2.5 μM NADP, 150 μl of enzyme extract and 500 μl of 0.5 M Tris–HCl buffer (pH 8.2). The reactions were carried out in 1 ml spectrophotometer cell at room temperature and the NADPH (for XR) or NADP (for XD) consumption followed by the variation of absorbance during 30 s at $\lambda = 340$ nm [10].

One XR or XD unit (U) was defined as the amount of enzyme catalyzing, respectively, the formation of 1 μmol of NADP per min or 1 μmol of NADPH per min. The specific activity was expressed as U mg^{-1} of protein. The protein concentration was measured by the conventional Lowry's method [11], using bovine albumin as standard protein (Sigma®, 99% purity). Each activity determination was made in triplicate, being the variation coefficient not higher than 5%.

2.6. Kinetic parameters determination

The kinetic constants K_M (Michaelis constant) and V_{max} (maximal enzyme activity) for both enzymes were determined through the conventional Lineweaver–Burk's method, by taking the reciprocal of both sides of the Michaelis–Menten equation. So that, the concentrations of xylose and NADPH for XR were varied from 0.001 to 0.23 M and from 0.02 to 0.20 M, respectively, meanwhile the concentrations of xylitol and NADP for XD were varied from 0.01 to 0.35 M and from 0.07 to 0.7 mM, respectively.

2.7. Liquid–liquid extraction

The liquid–liquid extraction was performed using an experimental design. The enzymes, from the crude extracts were separated by CTAB-reversed-micelles in isooctane, hexanol and butanol, by a two-step procedure. In the first step (forward-extraction), 3.0 ml of the crude extract (containing XD and XR) was mixed with an equal volume of micellar microemulsion (CTAB in isooctane/hexanol/butanol/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 model 1812, Saint-Herblain, France). Afterwards, 2 ml of CTAB-micellar phase (MPI) 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 enzymes from the micelles to this fresh aqueous, called the aqueous phase II (APII) (backward-extraction), which was finally collected by centrifugation (657 × *g*; 10 min). Both aqueous phases (first and second), and the crude extract were assayed to determine enzyme activity and protein concentration. The extraction results are reported in terms of total activity recovered (%) in the aqueous phases using the XD and XR content of the crude extract as a reference [12]. In this work the forward extraction pH was maintained at 7.0, electrical conductivity at 14 mS cm^{-1} and temperature at 5 °C. The objective was to avoid loss of activities, and with these three fixed conditions we attained good results, with no need to test other values.

2.8. Experimental design and optimization

2.8.1. Factorial design

To verify the influence of hexanol:isooctane ratio, butanol and surfactant concentrations on the XR and XD recovery yields (*Y*), a 2^3-full factorial design and three repetitions at the center point was employed (Table 1). In this experimental design, the main effects and interactions of different factors, each at two different levels, can be simultaneous investigated. 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 well as three extractions representing the center point (coded value: 0). For statistical calculation, the variables were coded according to Eq. (1):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

where x_i is the independent variable coded value, X_i the independent variable real value, X_0 the independent variable real value on the center point and ΔX_i is the step change value. Assays were conducted randomly. The range of values are in agreement with those reported by several authors [2–5].

2.8.2. Central composite design

Once the relevant variables were selected by factorial design, a central composite design with $\alpha = 1.414$ and three replicates at the center point was used, resulting in 11 experimental runs. The XR and XD recovery yield were taken as the dependent variables or responses of the design experiments. The quadratic model for predicting the optimal point was expressed according to Eq. (2):

$$\hat{y}_i = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2 \quad (2)$$

where $\hat{y}_i$ represents the response variable, b_0 the interception coefficient, b_1 and b_2 the linear terms, b_{11} and b_{22} the quadratic terms, and X_1 and X_2 represent the variables studied. Where possible, the model was simplified by the elimination of statistically insignificant terms.

The Design Expert version 5.0 (Stat-Ease Inc., Minneapolis, USA) and STATISTICA version 5.0 (Statsoft, USA)

Table 1
Experimental design, and results of the 2^3 factorial design

Run no.	Actual values			Coded values			Recovery yield (%)			
	X_1	X_2	X_3	X_1	X_2	X_3	API[a]		APII[b]	
							XR	XD	XR	XD
1	0.10	5	10	−	−	−	0	45.84	109.55	8.93
2	0.20	5	10	+	−	−	0	0	0	0
3	0.10	10	10	−	+	−	0	64.70	135.12	8.97
4	0.20	10	10	+	+	−	0	0	0	0
5	0.10	5	20	−	−	+	0	85.69	103.41	4.52
6	0.20	5	20	+	−	+	0	74.98	110.59	5.11
7	0.10	10	20	−	+	+	0	74.47	102.30	1.18
8	0.20	10	20	+	+	+	0	72.87	115.54	3.13
9	0.15	7.5	15	0	0	0	0	65.12	102.77	3.19
10	0.15	7.5	15	0	0	0	0	64.68	101.69	5.84
11	0.15	7.5	15	0	0	0	0	64.61	94.44	2.40

X_1 = CTAB concentration (M), X_2 = H:I (hexanol:isooctane) ratio (%) and X_3 = butanol concentration (%, v/v). Conditions of the cell homogenate: pH = 7.0 and electrical conductivity = 14 mS cm^{-1}, temperature = 5 °C.
[a] Aqueous phase I.
[b] Aqueous phase II.

softwares were used for regression and graphical analysis of the data obtained. The statistical significance of the regression coefficients was determined by Student's test, the second-order model equation was determined by Fischer's test and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2. The optimum extraction conditions were obtained by the graphical and numerical analysis using "design expert" program.

2.9. Water determination and micellar radii

Water content (W_o: water in oil) is defined as the ratio of water molecules over surfactant molecules per reverse micelle (Eq. (3)). It is proportional to the radius of the aqueous core of the reverse micelle (R_m) (Eq. (4)), 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 [13]. For ionic surfactants at room temperature, a_{surf} is in the range of 0.5–0.7 nm^2 [3], and N_{av} is the Avogadro's number. Water content values are expressed as wt.% using Karl–Fisher moisture titrator (Mettler DL31, Greifensee, Switzerland). The results were expressed as wt.%.

$$W_o = \frac{[H_2O]}{[BDBAC]} \quad (3)$$

$$R_m = \frac{3W_o M_{aqu}}{a_{surf} N_{av} \rho_{aqu}} \quad (4)$$

3. Results and discussion

Table 1 gives the extraction results of experiments based on a 2^3 full factorial design. Recovery yields higher than 100% for XR in aqueous phase II, and up to around 86% for XD in aqueous phase I were obtained. On the other hand, no XR recovery was obtained in aqueous phase I, and lower than 9% of XD recovery was extracted to aqueous phase II.

The XR extraction to the aqueous phase II can be explained by the electrostatic interaction between enzyme and cationic surfactant CTAB [1]. The isoelectric point of XR produced by *C. guilliermondii* in sugar cane bagasse hydrolysate is unknown. However, XR produced by *Pachysolen tannophilus* NRRL Y-2460 has the p*I* equal to 4.9 [14] and XR produced by *Candida tropicalis* has the pI between 4.1 and 4.15 [15]. Therefore, the XR described in this study could have the same pI range and a negative global charge at pH 7.0. This would improve the driving force of the extraction system. On the other hand, the XD enzyme was not efficiently transferred into reversed micelles, since the highest recovery in the fresh aqueous phase (APII) was ~9.0%. However, it could be observed that in the experiments 5, 6, 7 and 8, the XD recoveries, in aqueous phase I, were higher than 70%. The isoelectric point of XD, produced by *C. guilliermondii* in sugar cane bagasse hydrolysate, is also unknown. However, the XD described in this study should have pI around or higher than pH 7.0, since there was no attraction between the enzyme and the reversed micelles composed by the cationic surfactant CTAB.

The electrostatic interaction is one of the most predominant factors in the reversed micelle extraction, and this explains the high recovery of XR in our experiments. This interaction can cause the enzyme migration to the micellar core, since the enzyme and the surfactant have opposite electrical charge. The XR recovery was above 100% since all enzyme activity present in the crude extract was transferred to the fresh aqueous phase after backward-extraction, and the process reduced the concentration of several enzyme inhibitors (mainly hydrophobic compounds as furfural, hydroxymethyl furfural, phenols, etc.) present in the

Table 2
Estimated effects, standard errors, and Student's *t*-test results for the factorial design (2^3) with three center points

Factors	Effect estimates		Standard error		*t*-values	
	XR[a]	XD[b]	XR	XD	XR	XD
Mean	88.67*	55.72*	3.88	3.15	22.87*	17.66*
X_1 (CTAB)	−56.06*	−30.71*	9.09	7.40	−6.16*	−4.15*
X_2 (H:I ratio)	7.35	1.38	9.09	7.40	0.81	0.18
X_3 (Butanol)	46.79*	49.37*	9.09	7.40	5.14*	6.67*
$X_1 \times X_2$	−4.88	−2.44	9.09	7.40	−0.53	−0.33
$X_1 \times X_3$	−66.27*	24.56*	9.09	7.40	7.28*	3.31*
$X_2 \times X_3$	−5.43	−8.05	9.09	7.40	−0.59	−1.08

(*) Significant for a 95% confidence level; XR: xylose reductase; XD: xylitol dehydrogenase.
[a] Aqueous phase II (APII).
[b] Aqueous phase I (API).

crude extract. Considering that there is no literature on XD or XR extractions with reversed micelles, it can be concluded that the initial results were quite good, since the enzyme XD was ∼80% recovered in the remaining aqueous phase, the enzyme XR was totally recovered in the aqueous phase II, and there was no XR remaining in aqueous phase I. It is borne out that both phases were free of cell debris.

All of the three factors studied, surfactant concentration (X_1), H:I (hexanol:isooctane) ratio (X_2) and butanol concentration (X_3), seem to have played a critical role in the liquid–liquid extraction. The statistical analyses for each of the response variables evaluated, namely XD recovery in the aqueous phase I (Y_1), and XR recovery in the aqueous phase II (Y_2), are summarized in Table 2. As can be seen, the effect of the H:I ratio did not exert statistically significant effects for either XR or XD extraction yield, at 95% confidence level. On the other hand, the factors CTAB and butanol concentrations, and their interactions were significant at this level. According to Kilikian et al. [1], the main function of the hexanol in the formation of CTAB reversed micelles is to increase their radii. Therefore, the increase in hexanol concentration, from 5 to 10%, was not the main driving force responsible for the high XR recovery yield. Similar results were achieved by Krei and Hustedt [12] in reversed micellar extraction studies performed with the enzyme α-amylase.

From the analysis of the data, a linear model was tested and showed to be inadequate to represent the experimental results. Thus, new experiments were performed employing just the significant variables selected in the factorial design (surfactant and butanol concentration) aiming to optimize XR and XD extraction by CTAB micelles reversed. The design and experimental results of these experiments are given in Table 3. As can be seen, very good results were obtained.

Data from Table 3 were analyzed by non-linear multiple regression, and the second-order polynomial coefficients were calculated to estimate the responses of the dependent variables. The models expressed by equations [5,6] were generated, representing the XR recovery yield (Y_1) and XD recovery yield (Y_2) as a function of the more significant

Table 3
Experimental design, and results for extraction optimization

Run no.	Actual values		Coded values		Recovery yield (%)			
	X_1	X_3	X_1	X_3	API[a]		APII[b]	
					XR	XD	XR	XD
1	0.10	10	−	−	0	46	110	9
2	0.20	10	+	−	0	0	0	0
3	0.10	20	−	+	0	86	103	5
4	0.20	20	+	+	0	75	111	5
5	0.08	15	−1.41	0	0	88	103	18
6	0.22	15	+1.41	0	0	56	85	19
7	0.15	8	0	−1.41	0	0	0	0
8	0.15	22	0	+1.41	0	97	106	6
9	0.15	15	0	0	0	46	127	20
10	0.15	15	0	0	0	44	134	15
11	0.15	15	0	0	0	48	128	19

X_1 = CTAB concentration (M), X_3 = butanol concentration (%, v/v). Conditions of the cell homogenate: pH = 7.0 and electrical conductivity = 14 mS cm^{-1}, temperature = 5 °C, H:I = 5%.
[a] Aqueous phase I.
[b] Aqueous phase II.

Table 4
Analysis of variance (ANOVA) for the model regression representing XD extraction in the API

Source	SS	d.f.	MS	*F*-value	*P*-value
Model	10328.63	4	2582.16	65.77	<0.0001
Residual	235.55	6	39.26		
Lack of fit	227.55	4	56.83	14.22	0.0668
Pure error	8.00	2	4.00		
Total	10564.18	10			

$R^2 = 0.98$; CV = 11.76%; SS: sum of squares; d.f.: degrees of freedom; MS: mean square.

variables (X_1 and X_3 coded values):

$$\hat{y}_1 = 129.67 - 15.93x_1 + 31.74x_3 - 15.92x_1^2 - 36.46x_3^2 + 29.50x_1x_3 \quad (5)$$

$$\hat{y}_2 = 46.00 - 12.78x_1 + 31.52x_3 + 1.88x_1^2 + 8.75x_1x_3 \quad (6)$$

The analysis of variance (ANOVA) of the second-order model—for XD (Table 4) and XR (Table 5)—demonstrates, according to *F*-test and probability values, that the models are highly significant. The goodness of the models was checked by the analysis of the determination coefficient (R^2). Only 5.1 and 2.8% of total variations for XR, and XD, respectively, cannot be explained by the model.

The recovery yield values (XR and XD) for different concentrations of the variables can be predicted from the respective contour plots (Figs. 1 and 2). Based on the two models obtained, a graphical optimization was conducted using "design-expert" program. The method basically consists of overlaying the curves of the models according to the criteria imposed. The optimal working conditions were defined to attain high XR and XD recovery yield in the APII and API, respectively. The criteria adopted were: (a) XR extraction yield as a minimum of 100% and (b) XD extraction yield no less than 80%. The overlaying plot attained (Fig. 3) shows a shaded area where all the criteria imposed were satisfied. Thus, a point was chosen on the graph, which was assigned as optimum point corresponding to 0.15 M CTAB (coded value = 0) and 22% butanol concentration (coded value = +1.41). Under these conditions, the model

Table 5
Analysis of variance (ANOVA) for the model regression representing XR extraction in the APII

Source	SS	d.f.	MS	*F*-value	*P*-value
Model	21245.76	5	4249.15	18.69	<0.0001
Residual	1136.97	5	227.39		
Lack of fit	1108.30	3	369.43	25.77	0.0376
Pure error	28.67	2	14.33		
Total	0.400	10			

$R^2 = 0.95$; CV = 16.4%; SS: sum of squares; d.f.: degrees of freedom; MS: mean square.

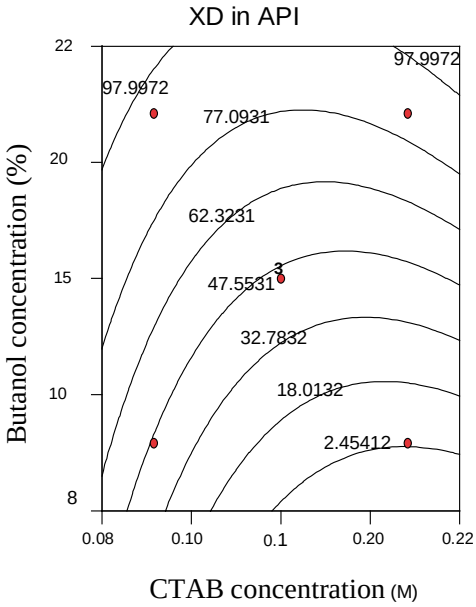

Fig. 1. Contour plot described by the model $\hat{y}_1$ which represents XD extraction in the API by CTAB reversed micellar system.

predicted a XR extraction in APII of 101.9% (a variation of 71.4–132% being possible) and XD extraction in API of 88.7% (a variation of 74.9–102.5% being possible) in the confidence range of 95%. These results are very interesting from an economic point of view, since both phases of the system (with identical composition) can be utilized to separate, and purify, two different enzymes with high recovery yields.

Extractions under optimized conditions were carried out and the radii of the micelles, present in the micellar phase I

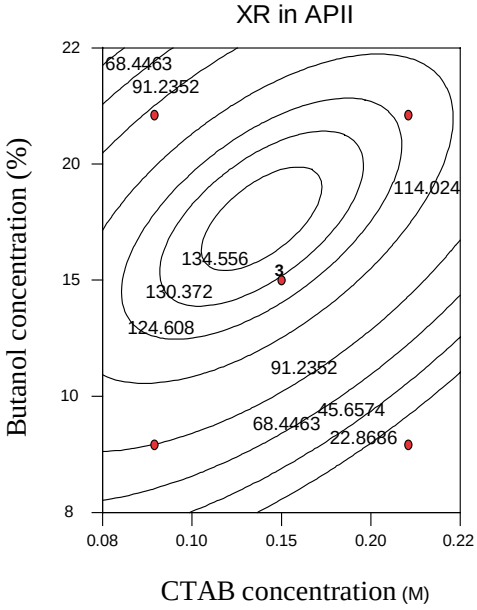

Fig. 2. Contour plot described by the model $\hat{y}_1$ which represents XR extraction in the APII by CTAB reversed micellar system.

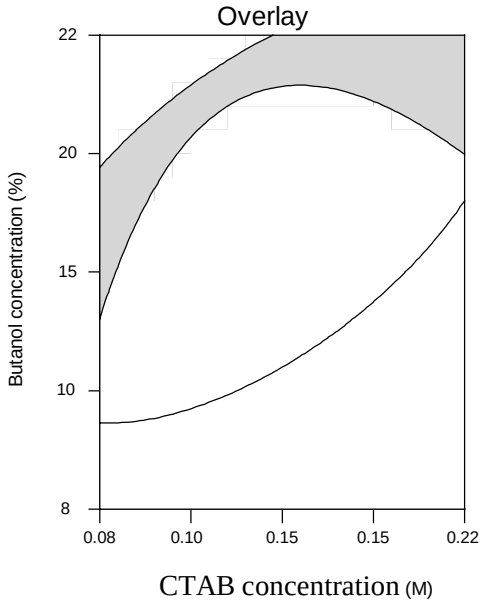

Fig. 3. The optimum region by overlay plots of the two responses evaluated (extraction of XD in API and XR in APII) as a function of surfactant and butanol concentration.

Table 6
Radii attained in micellar phase I (MPI) and in micellar phase II (MPII) after liquid–liquid extraction by reversed micelles

System phase	W_o (wt.%)	Micellar radius (nm)
MPI	38.07	6.24
MPII	26.70	4.40

pH = 7.0; electrical conductivity = 14 mS cm^{-1}; tempertaure = 5 °C; hexanol = 5%; butanol = 22%; CTAB = 0.15 M; H:I = 5%.

(after forward extraction—with encapsulated proteins), and in the micellar phase II (after backward-extraction—reduced amount of proteins) were determined (Table 6). The water content of the micellar phase I (W_o) was 38.07 (micelle radius = 6.24 nm), which showed that the micelle size was not a limiting variable during the extraction. According to Krei and Hustedt [12], this micelle can solubilize proteins with a molecular weight of 100 kDa, and this includes XR and XD. This shows that the micelles size do not limit the encapsulation of both enzymes. Therefore, the low XD recovery in aqueous phase II could not be ascribed to the micelle size. Besides, the electrostatic or hydrophobic interactions, between the micelle and the enzyme, were not the extraction driving force. However, the favorable micelle size associated to the ionic interactions provided the high XR recovery yields in the aqueous phase II.

Table 7
Purification factors of XD and XR produced by *C. guiliiermondii* using CTAB reversed micelles

Enzyme	Phase of the system	Enrichment factor (fold)
XR	APII	5.6
XD	API	1.8

Extraction conditions: pH = 7.0; electrical conductivity = 14 mS cm^{-1}; temperature = 5 °C; hexanol = 5%; butanol = 22%; CTAB = 0.15 M; H:I = 5%.

The XR and XD recovery by reversed micelles using CTAB surfactant provided satisfactory enrichment factors of 5.6 and 1.8, respectively (Table 7). These enrichment factor values are in accordance with the literature [3,16] after enzyme purification by reverse micelles. Similar results were reported by Zhang et al. [17] that purified (four-fold) the enzyme alcohol dehydrogenase by CTAB reversed micelles and the recovery yield was 99%.

Considering that the enzymes were separated and purified in different phases of the system with high recovery yields, the enzymes are intracellular, the homogenate contains a large variety of contaminants (soluble and insoluble components), and this is a very simple purification technique, it can be concluded that the overall procedure is quite good.

To evaluate the influence of the extraction conditions on XR and XD, the kinetic constants values for both enzymes were calculated and compared before (in the cell homogenate) and after the extraction. From Table 8 it can be seen that the values of V_{max} in regard to the substrates (xylose and xylitol) and cofactors (NADPH and NAD) for each enzyme did not vary more than 7%, indicating that this kinetic constant was significantly not affected by the extraction procedure. The same did not occur with the K_M values for both enzymes, because variations up to 48% (XD in regard to xylitol) were observed. As K_M relates in some extension

Table 8
K_M and V_{max} of the enzymes XR and XD in the cell homogenate, in aqueous phase I and aqueous phase II, and for different substrates

Enzyme	Substrate/cofactor	Extraction steps					
		Cell homogenate		API[a]		APII[b]	
		V_{max} (U ml^{-1})	K_M	V_{max} (U ml^{-1})	K_M (M)	V_{max} (U ml^{-1})	K_M (M)
XR	Xylose	0.76	0.011 M	–	–	0.81	0.017
	NADPH	0.87	0.119 mM	–	–	0.81	0.085
XD	Xylitol	0.93	0.023 M	0.98	0.012	–	–
	NAD^+	1.02	0.287 mM	1.09	0.323	–	–

[a] Aqueous phase I.
[b] Aqueous phase II.

to the three-dimensional protein structure, so its pronounced variation before and after extraction with reversed micelles indicates that the XR and XD structures were affected in some extension. The presence of hexanol, butanol and isooctane in the extraction medium were probably responsible for the reversible and/or irreversible modifications in the XR and XD structures. Moreover, for xylose reductase K_M increased 35% and decreased 29% in relation to xylose and NADPH, respectively, and for XD, K_M decreased 48% and increased 11%, respectively, in regard to xylitol and NADP. The small V_{max} and the high K_M variations could occur as consequence of the particular catalytic mechanism presented by both enzymes, which is an ordered sequential bi–bi type. In this case, both the substrate and the cofactor attach simultaneously to the active site of the enzyme [18]. Thereby, if the enzyme requirement is simultaneously up for the substrate (for instance, XR requires 35% more xylose after extraction) and down for the cofactor (for instance, XR requires 29% less NADPH after extraction) the V_{max} of the overall reaction could practically result unchanged. Though the reversed micelles extraction process promotes some structural modifications in the XR and XD molecules, its advantages continue remarkable, because the technique is simple and reliable, presenting quite good recovery yield (100%) and purification factor (5.6- and 1.8-fold, respectively, for XR and XD). Moreover, the process does not cause net loss in the overall catalytic reaction rate of both enzymes. Particularly in the case of XR, the enzyme could be used in the "in vitro" xylose/xylitol conversion. The simultaneous increasing and decreasing of K_M in regard to xylose and NADPH, respectively, could present advantage in the xylose/xylitol conversion in terms of overall cost of the process, resulting from the huge price difference between NADPH and xylose.

4. Conclusions

This study demonstrated that liquid–liquid extraction by reversed micelles is a process able to separate, to increase the enzymatic activity, and to purify the XD and XR produced by *C. guilliermondii* cultivated in sugar-cane-bagasse hydrolysate, since the recovery yields were around 100% for both enzymes and the enrichment factor was 1.8 for XD and 5.6 for XR. Using the methodology of experimental factorial design, and response surface analysis it was possible to determine optimal extraction conditions to obtain high recovery yield and satisfactory enrichment factor in the aqueous phase I (remaining aqueous phase) and in the aqueous phase II, simultaneously, meaning a simple and economic technique to purify the enzymes and separate XR from XD. Finally, in the case of xylose reductase, which could be used in the in vitro xylose/xylitol conversion, the simultaneous increasing and decreasing of K_M in relation, respectively, to xylose and NADPH, could advantageously be explored, due to the significant price difference between NADPH, much more expensive, and xylose.

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