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# Extraction by Reversed Micelles of the Intracellular Enzyme Xylose Reductase

ELY V. CORTEZ,[1] MARIA DAS GRAÇAS DE ALMEIDA FELIPE,[2] INÊS C. ROBERTO,[2] ADALBERTO PESSOA, JR,[1] AND MICHELE VITOLO*,[1]

*[1]Faculdade de Ciências Farmacêuticas/USP, PO Box 66083, São Paulo, SP, Brazil, E-mail: ines@debiq.faenquil.br; and [2]Faculdade de Engenharia Química de Lorena, CEP 12.600-970, PO Box 116, Lorena, SP, Brazil*

## Abstract

Xylose reductase enzyme (EC 1.1.1.21) produced by *Candida guilliermondii* in sugarcane bagasse was extracted by reversed micelles of *N*-benzyl-*N*-dodecyl-*N*-*bis* (2-hydroxyethyl) ammonium chloride cationic surfactant. An experimental design was employed to evaluate the influences of the following factors on the enzyme extraction: temperature, cosolvent, and surfactant concentration. A model was used to represent the enzyme recovery and fit of the experimental data. The extraction yielded a total recovery of 130%, and the purity increased 4.8-fold. This study demonstrates that liquid-liquid extraction by reversed micelles is a process able to recover and increase the enzymatic activity and purity of XR produced by *C. guilliermondii*.

**Index Entries:** Reversed micelles; xylose reductase; liquid-liquid extraction.

## 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 extraction process, because both are biphasic and consist in 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)*.

Reversed micellar systems have great potential for industrial application, because they provide a favorable environment for protein solubilization in the organic phase with preservation of biologic activity *(2)*. A number of recent studies on reversed micellar methodology clearly demonstrate

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

the interest in reversed micelles for the separation of biotechnologic 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)*.

Xylitol is a sweetener with important properties such as anticariogenicity, low caloric value, and negative dissolution heat. Because it can be used successfully in food formulations and in the pharmaceutical industry, its production is in great demand *(3)*. Xylitol can be obtained by microbiologic processes, because many yeasts and filamentous fungi synthesize the xylose reductase (XR) enzyme, which catalyzes the xylose reduction into xylitol as the first step in xylose metabolism. Xylitol production by biotechnologic means has several economic advantages in comparison to the conventional process based on the chemical reduction of xylose. The efficiency and productivity of this fermentation chiefly depend on the microorganism and the process conditions employed *(3)*. The present study evaluates the effectiveness of reversed micelles in extracting XR enzyme produced by *Candida guilliermondii* grown in sugarcane bagasse hydrolysate.

## Materials and Methods

### Microorganism

The cells were obtained from fermentations conducted with *C. guilliermondii* FTI 20037 as described by Barbosa et al. *(4)*. The yeast was maintained on malt-extract agar slants at 4°C.

### Preparation of Hemicellulosic Hydrolysate

Sugarcane bagasse was hydrolyzed in a 250-L reactor at 121°C for 20 min with $H_2SO_4$ (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 used to avoid sugar degradation. The hydrolysate was then treated as described by Alves et al. *(5)*, to reduce the concentrations of toxic substances.

### Inoculum Preparation, Medium, and Fermentation Conditions

A medium containing 3.0 g/L of xylose supplemented with 20.0 g/L of rice bran extract, 2.0 g/L of $(NH_4)_2SO_4$, and 0.1 g/L 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 (200 rpm) at 30°C for 24 h.

For the fermentation, concentrated bagasse hemicellulosic hydrolysate containing 42 g/L of xylose was employed. The hydrolysate was supplemented with the same nutrients as used for the inoculum preparation. Cultivation was done by a batch process in a 1.25-L fermentor (BIOFLO III; New Brunswick, Edison, NJ) under agitation of 300 rpm and aeration rate of 0.6 vvm ($K_La$ = 22.5 $h^{-1}$) at 30°C and an initial pH of 5.5.

### *Preparation of Cell-Free Extracts*

Cells were harvested by centrifuging at 800*g* and washed in phosphate buffer (50 m*M*, pH 7.2). Cell pellets were stored in a freezer. For enzymatic analysis, cell extracts were thawed and disrupted by a sonic disruption technique using a Sonics & Materials disrupter. The cell homogenate was then centrifuged at 10,000*g* (Jouan MR 1812) at 4°C for 10 min, and the supernatant solution was used for enzymatic assays.

### *Enzyme Assays*

XR activity was determined spectrophotometrically at 340 nm at room temperature *(6)*. One enzyme unit was defined as 1 µmol of NADPH oxidized per minute using an extinction coefficient of $6.22 \times 10^{-3}$ $M^{-1}/cm^{-1}$. Specific activity was expressed as units/milligram of protein based on protein determination, according to the method of Lowry et al. *(7)*, using bovine serum albumin as the standard.

### *Liquid-Liquid Extraction*

Liquid-liquid extraction was performed using an experimental design. The enzyme, from the crude extracts, was extracted by *N*-benzyl-*N*-dodecyl-*N*-*bis* (2-hydroxyethyl) ammonium chloride (BDBAC) reversed micelles in isooctane, by a two-step procedure. In the first step (forward extraction), 3.0 mL of the crude extract (containing XR) was mixed with an equal volume of 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 by centrifuging at 657*g* for 10 min (Jouan Centrifuge Model 1812; Saint-Herblain, France). Next, 2 mL of XR-BDBAC-micellar phase was mixed with 2.0 mL of fresh aqueous phase (1.0 *M* acetate buffer at pH 5.5 with 1.0 *M* NaCl), to transfer the enzyme from the micelles to this fresh aqueous, called the second aqueous phase (backward extraction), which was finally collected by centrifugation (657*g* for 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 (percent) in the second aqueous phase using the XR content of the crude extract as a reference.

### *Experimental Design and Statistical Analysis*

To verify the influence of temperature, cosolvent, and surfactant concentrations on the enzyme recovery ($Y$), a $2^3$ full factorial design with centered face and 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 14 set point combinations ($2^3$ + centered face) were performed, as well as three extractions representing the center point (coded value: 0). Assays were conducted randomly.

Table 1
Matrix for a $2^3$ Full Factorial Design with Centered Face

| Run no. | Coded values | | | Actual values | | |
|---|---|---|---|---|---|---|
| | Temp. (°C) | Cosolvent (%) | Surfactant (%) | Temp. (°C) | Cosolvent (%) | Surfactant (%) |
| 1 | –1 | –1 | –1 | 5 | 6 | 0.1 |
| 2 | +1 | –1 | –1 | 30 | 6 | 0.1 |
| 3 | –1 | +1 | –1 | 5 | 9 | 0.1 |
| 4 | +1 | +1 | –1 | 30 | 9 | 0.1 |
| 5 | –1 | –1 | +1 | 5 | 6 | 0.2 |
| 6 | +1 | –1 | +1 | 30 | 6 | 0.2 |
| 7 | –1 | +1 | +1 | 5 | 9 | 0.2 |
| 8 | +1 | +1 | +1 | 30 | 9 | 0.2 |
| 9 | 0 | 0 | 0 | 17.5 | 7.5 | 0.15 |
| 10 | 0 | 0 | 0 | 17.5 | 7.5 | 0.15 |
| 11 | 0 | 0 | 0 | 17.5 | 7.5 | 0.15 |
| 12 | –1 | 0 | 0 | 5 | 7.5 | 0.15 |
| 13 | +1 | 0 | 0 | 30 | 7.5 | 0.15 |
| 14 | 0 | –1 | 0 | 17.5 | 6 | 0.15 |
| 15 | 0 | +1 | 0 | 17.5 | 9 | 0.15 |
| 16 | 0 | 0 | –1 | 17.5 | 7.5 | 0.1 |
| 17 | 0 | 0 | +1 | 17.5 | 7.5 | 0.2 |

## Results and Discussion

Table 2 gives the results of experiments based on a $2^3$ full factorial matrix. A high recovery of XR activity could be observed in many experiments.

The isoelectric point (p*I*) of XR produced by *C. guilliermondii* in sugarcane bagasse hydrolysate is unknown. However, XR produced by *Pachysolen tannophilus* NRRL Y-2460 has a p*I* equal to 4.9 *(8)* and XR produced by *Candida tropicalis* has a p*I* between 4.1 and 4.15 *(9)*. Therefore, the XR described in the present study could have the same p*I* range and a negative global charge at pH 7.0. This would improve the electrostatic interaction between enzyme and cationic surfactant BDBAC *(1)*. 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 (Table 2). This interaction can cause the enzyme migration to the micellar core, when it is opposite to the electrical charge between enzyme and surfactant. 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 such as furfural, hydroxymethyl furfural, phenols) present in the crude extract. Using BDBAC reversed micelles, Hasmann et al. *(10)* recovered about 50% of β-xylosidase from fermented medium and Pessoa and Vitolo *(2)* recovered 90% of inulinase.

Table 2
Results of a $2^3$ Full Factorial Design with Centered Face

| Experiment no. | Initial activity (U/mL) | Backward extraction activity (U/mL) | Recovery (%) |
|---|---|---|---|
| 1 | 0.23 | 0.36 | 140 |
| 2 | 0.52 | 0.42 | 81 |
| 3 | 0.35 | 0.56 | 145 |
| 4 | 0.41 | 0.49 | 118 |
| 5 | 0.45 | 0.21 | 47 |
| 6 | 0.52 | 0.0 | 0 |
| 7 | 0.54 | 0.63 | 116 |
| 8 | 0.38 | 0.38 | 94 |
| 9 | 0.55 | 0.66 | 122 |
| 10 | 0.55 | 0.68 | 123 |
| 11 | 0.55 | 0.72 | 131 |
| 12 | 0.31 | 0.37 | 122 |
| 13 | 0.39 | 0.43 | 110 |
| 14 | 0.28 | 0.40 | 142 |
| 15 | 0.35 | 0.41 | 120 |
| 16 | 0.35 | 0.44 | 126 |
| 17 | 0.39 | 0.41 | 104 |

Table 3
Variance Analysis of Significant Factors and Interactions for XR Extraction Process by Reversed Micelles of BDBAC[a]

| Source of variation | Sum of squares | Degrees of freedom | Mean square | F Value | *p* Value |
|---|---|---|---|---|---|
| $X_1$: temperature | 2798.9 | 1 | 2798.9 | 6.28 | 0.0407 |
| $X_2$: cosolvent | 3319.7 | 1 | 3319.7 | 7.45 | 0.0294 |
| $X_3$: surfactant | 6170.2 | 1 | 6170.2 | 13.84 | 0.0075 |
| $X_2X_3$ | 1830.1 | 1 | 1830.1 | 4.10 | 0.0824 |
| $X_1X_1$ | 2924.4 | 1 | 2924.4 | 8.20 | 0.0169 |
| $X_3X_3$ | 1020.7 | 1 | 1020.7 | 2.86 | 0.1216 |
| Residue | 3567.2 | 10 | 356.7 | | |
| Total (corr.) | 21,631.37 | 16 | | | |

[a] $R^2 = 0.84$.

Considering that there is no literature on XR extraction with reversed micelles, we can say that our results were quite good.

In our work the pH was maintained at 7.0 and electrical conductivity at 14 mS/cm. Our objective was to avoid loss of activity, and with these two values we attained good results, with no need to test other values.

Table 3 gives the variance analysis of the factors and interactions that were important for the XR extraction process by reversed micelles. All the factors ($X_1$, $X_2$, and $X_3$) and the interaction $X_1X_1$ were significant

Table 4
Regression Coefficients

| | |
|---|---|
| Average | 129.24 |
| Temperature—$X_1$ | –16.73 |
| Cosolvent—$X_2$ | 18.22 |
| Surfactant—$X_3$ | –24.84 |
| $X_1X_1$ | –17.21 |
| $X_2X_3$ | 15.12 |
| $X_3X_3$ | –18.36 |

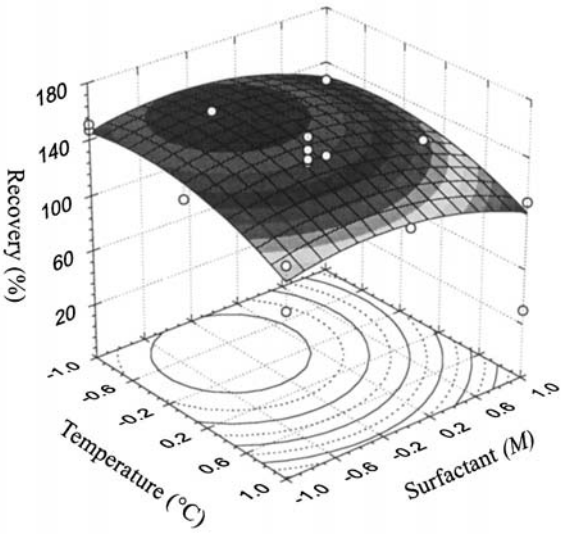


Fig. 1. Surface response described by Eq. 1 for temperature ($X_1$) and surfactant ($X_3$). Cosolvent concentration was maintained at superior level ($X_2$ = 1).

at a 95% confidence level. The interactions $X_2X_3$ and $X_3X_3$, although less significant (Table 4), were also taken into account for establishing the model.

Table 4 gives the regression coefficients of the second-order model (quadratic), which was used to estimate the percentage of enzyme recovery as a function of temperature, cosolvent, and surfactant concentration.

The statistical significance of the quadratic model was evaluated by the F test, which revealed that this regression is statistically significant at the 5% probability level. The model did not show lack of fit, and the determination coefficient ($R^2$ = 0.84) indicates that 84% of the variability in the recovery can be explained by the model. Thus, the mathematical model representing the XR extraction process in the experimental region studied can be expressed by Eq. 1 and by Fig. 1:

$$Y = 129.2 - 16.7X_1 + 18.2X_2 - 24.8X_3 + 15.1X_2X_3 - 17.2X_1^2 - 18.4X_3^2 \quad (1)$$

in which $Y$ is the enzyme recovery (%); and $X_1$, $X_2$, and $X_3$ are coded values for temperature, cosolvent, and surfactant concentrations, respectively.

Table 5
Purification of XR Produced by *C. guilliermondii* Using Reversed Micelles

| Purification step | Total protein (mg/mL) | Total activity (U/mL) | Specific activity (U/mg) | Purification factor |
|---|---|---|---|---|
| Crude extract | 1.38 | 0.41 | 0.30 | 1.0 |
| Aqueous phase after back-extraction | 0.39 | 0.55 | 1.42 | 4.8 |

The maximum recovery value of XR (152%) corresponded to the point defined by the temperature of 11.4°C ($X_1 = -0.49$), cosolvent concentration of 9% ($X_2 = 1$), and surfactant concentration of 0.14 $M$ ($X_3 = -0.26$). Triplicate XR extraction runs were performed at these optimum conditions predicted by the model, and the average recovery value was about 135 ± 3%, which is close to that predicted by the model (152 ± 23%).

Table 5 gives the results of total protein analysis regarding a point of maximal extraction. The purification factor increased 4.8-fold.

## Conclusion

This study demonstrates that liquid-liquid extraction by reversed micelles is a process able to recover and increase the enzymatic activity and purity of XR produced by *C. guilliermondii* cultivated in sugarcane bagasse hydrolysate. It was possible to obtain a representative model for the extraction of XR.

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