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Designing cross-linked xylanase aggregates for bioconversion of agroindustrial waste biomass towards potential production of nutraceuticals

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ABSTRACT

Immobilized biocatalysts design has the potential to efficiently produce valuable bioproducts from lignocellulosic biomass. Among them, the carrier-free immobilization through the cross-linked enzyme aggregates technology is a simple and low-cost alternative. A two steps statistical approach was utilized to evaluate the synthesis of a cross-linked enzyme aggregate from a xylanolytic preparation, which was produced by *Cohnella* sp. AR92 grown in a peptone-based culture medium.

The resulting immobilized biocatalyst, Xyl-CLEA, was significate more stable (25 to 45%) towards temperatures up to 50 °C with respect to the free enzyme, and retained over 50% of its initial activity after 5 consecutive cycles of reuse. By means of infrared spectroscopy and electron microscopy, the Xyl-CLEA showed architectural features described as signature of type I and type II of protein aggregates. These, were the result of the simultaneous aggregation of a multiplicity of proteins from the crude enzymatic extract.

The enzymatic activity was assessed using alkali pretreated sugar cane bagasse as substrate. Whereas the free enzymatic preparation released xylose as the main product, the immobilized xylanase produced xylooligosaccharides, thus showing that the immobilization procedure modified the potential application of the extracellular xylanase from *Conhella* sp. AR92.

1. Introduction

The emerging biorefinery concept is aimed to integrate an optimized sequence for biomass and agro-waste utilization towards a maximization of the productivity in the generation of marketable intermediates and products [1]. Therefore, a wide range of technologies are focused to develop feasible alternatives to the bioconversions aimed to biofuels and other value-added products. Such integrated approach is intended to improve the bioprocesses economics as well as its environmental sustainability. In this context, the expansion of agro-waste valorization and the biocatalysts design are key subjects [2,3].

Within the strategies for enzyme immobilization to meet the biotechnological requirements of stability and recycling capability of biocatalysts, the cross-linked enzyme aggregates (CLEA) technology has emerged as an alternative that presents both technical and economic advantages [4,5]. This carrier-free immobilization approach offers simplicity of preparation, low cost of production and prompt optimization. One of the clear advantages is that the CLEA allows to feed reactors with a higher amount of enzyme with no volume consumed by the immobilization support [6]. Furthermore, it involves a simultaneous purification and immobilization, which additionally streamlines the co-immobilization of multiple enzymes [7,8].

The CLEA technology has been described as an attractive candidate for the bioconversion of lignocellulosic biomass [4,9–12]. However, additional efforts are still necessary to better understand the phenomena governing the behavior and performance of the structural chance of the enzymes in the immobilized aggregated form [13]. In order to produce an efficient biocatalyst, particular protocols must be established for each enzyme, bearing in mind that the aggregation conditions to obtain CLEA can alter the selectivity and/or activity of biocatalysts [6,14].

In the present work, we address the selection of insolubilization and cross-linking conditions for the preparation of a xylanolytic biocatalyst (Xyl-CLEA) from a crude enzymatic preparation produced by *Conhella* sp. AR92 [15]. Our study also delivers a structural analysis of the biocatalysts produced, as well as a comparative evaluation of the free

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enzyme and the CLEAs for the hydrolysis of pre-treated sugarcane bagasse.

2. Material and methods

2.1. Growth and enzyme production

The xylanolytic *Cohnella* sp. AR92 [16] was cultivated in diluted (1:2) commercial Tryptic Soya Broth (DTSB, Britania), amended with 5.00 g/L of hemicellulose from sugarcane bagasse (HC-SCB) fractioned from local samples [17].

Cells recovered from DTSB after 24 h of cultivation were washed twice with saline solution (0.9%, w/v NaCl), suspended to *c.a.* 10^6 CFU/mL and used as inocula (10%). The enzyme production assays were performed in triplicate at 30 °C using 125 mL flasks containing 20 mL of HC-SCB-DTSB with orbital shaking at 200 rpm. Assays were run for 144 h sampling every 24 h. The culture supernatants obtained after centrifugation at 8000 rpm for 10 min were used as enzyme source.

2.2. Endo-xylanase assays

Endo-xylanase activity was measured using 0.05 mL of suitably diluted culture supernatants and 0.45 mL of 1.0% (w/v) birchwood xylan (Sigma) in 100 mM sodium phosphate buffer (pH 6.0). After 30 min at 50 °C, reducing sugars released were quantified by the dinitrosalicylic acid method [18]. One international unit (IU) of enzyme activity was defined as the amount of enzyme that releases 1 μmol of reducing sugars (measured as xylose) per milliliter and per minute. All samples were analyzed in triplicate and mean values and standard deviations were calculated. Protein determination was assayed according to Lowry et al. [19] and specific activity was determined as IU per mg of total protein content.

2.3. Insolubilization and aggregation of the xylanolytic crude extract

Protein precipitation was assayed by triplicate from 1 mL samples of culture broth as follows: (i) different concentrations of ammonium sulfate (40%; 60%; 80% and 100%, w/v), stepwise added at regular intervals and stirring the samples at 4.0 ± 0.5 °C, were then kept undisturbed for 1 h; (ii) 80% (v/v) stock solutions of ethanol, acetone and 2-propanol were added in a 1:9 proportion (sample:solvent) and maintained at −20 °C for 1 h; and (iii) Tween 20 and Tween 80 were added to 10 mM as final concentration [20].

The protein pellets obtained after centrifugation at 10,000 rpm for 15 min were used for cross-linking assays performed during 15, 40 and 120 min at room temperature with glutaraldehyde solutions (25%; 10%; 2.5% and 1% v/v in 100 mM sodium phosphate buffer, pH 6.0). The samples were washed three or four times with phosphate buffer (pH 6.0) to remove any excess of glutaraldehyde, and then stored at 4.0 ± 0.5 °C. The efficiency of all steps involved in protein insolubilization and aggregation (*i.e.* CLEAs preparation) were assessed by xylanase activity quantification.

2.4. Screening and optimization of the factors affecting enzyme aggregation

Eight independent variables at two levels were analyzed by means of a Plackett-Burman statistical design that included 12 mixtures with their respective random triplicates (Table S1). The *residual xylanase activity* of the immobilized biocatalyst was the response evaluated (Y). Enzyme reactions were performed at 50 °C with gentle agitation (60 rpm), and the reducing sugars released from birchwood xylan were estimated with the DNS reagent. The Minitab® 17 software (PA, USA, Minitab Inc.) was used to analyze the effects of each independent variable, according to the *t*-test and *p*-value statistical parameters obtained.

The levels of glutaraldehyde (A), ionic strength (B) and pH (C) and their interactions headed to a maximization of the residual xylanase activity of CLEAs (Y) were assessed through a response surface methodology with a Box-Behnken design (Minitab® 17). A total of 30 treatment combinations were generated and randomized, considering the factors at 3 different levels (−1; 0; +1), plus six replicates at the center point that were used for the estimation of pure error and sum of squares. Experiments were performed in duplicate (Table S2).

2.5. Statistical analysis

The following model equation was used to analyze the design based on 3 factors:

$$\boldsymbol{Y} = \beta_0 + \beta^1 x^1 + \beta^2 x^2 + \beta^3 x^3 + \beta^{11} x^{1\,2} + \beta^{22} x^{2\,2} + \beta^{33} x^{3\,2} + \beta^{12} x^1 x^2 + \beta^{13} x^1 x^3 + \beta^{23} x^2 x^3 + \varepsilon \quad (1)$$

where, Y is the predicted response for Xyl-CLEA production; β_0 is the value of the fitted response at the center point of the design; β^1, β^2 and β^3 are the linear coefficients; β^{11}, β^{22} and β^{33} are the quadratic coefficients; β^{12}, β^{13} and β^{23} are the interaction coefficients; and ε is the random error. The MINITAB® 17 software was used to obtain the coefficients of the equation. The responses under different combinations as defined by the design were analyzed using analysis of variance (ANOVA) to estimate the statistical parameters.

2.6. Structural and biochemical analysis of the Xyl-CLEAs

Samples obtained after the precipitation and aggregation steps were vacuum dried and coated with gold particles using a sputter coater for scanning electron microscopy (SEM) observation using a ZEISS Supra 40VP (CIME, Argentina). Replica samples were included into potassium bromide tablets and subjected to Fourier transform infrared spectroscopy (FTIR, Perkin Elmer 1600), performing the measurements at room temperature and at a pressure of 7 atm.

The thermal stability of the Xyl-CLEAs produced and suspended in 100 mM phosphate buffer (pH 6.0) was assessed after a 4 h preincubation period at 30; 45; 50; 60 and 70 °C. The stability in a water-miscible solvent was assayed incubating diluted samples 1:1 with ethanol (30% v/v), for 1; 8; 24 and 72 h at 37 °C. Controls without treatments were simultaneously processed and considered as 100% of enzymatic activity.

Recycling assays of the Xyl-CLEAs were performed using 10 g/l of filtered xylan (0.22 μm) as substrate. Cycles of 30 min at 37 °C–40 °C were followed by separation of the Xyl-CLEAs by centrifugation (10,000 rpm; 5 min). After 3 thorough washes with 100 mM phosphate buffer (pH 6.0), the biocatalysts were re-used with freshly charged buffer and reactant for a subsequent cycle. The residual enzymatic activity of the CLEAs after each cycle was estimated considering the enzyme activity of the first cycle as 100%.

2.7. Activity of immobilized xylanase on pretreated sugarcane bagasse

Samples of milled sugarcane bagasse (20 mesh) were pretreated with NaOH according to Manfredi et al. [16] to be used as substrate. Reaction mixtures containing 2% (w/v) of total solids in 100 mM phosphate buffer (pH 6.0) and 15 IU of the immobilized xylanolytic preparation were incubated at 37 °C with gentle agitation (60 rpm). Aliquots were sampled at 24 h; 48 h and 72 h for enzyme activity evaluation.

The products of enzymatic hydrolysis were analyzed using thin layer chromatography (TLC) in 10 × 20 cm silica gel 60 F_{254} plates (Merck, Germany) with methanol-dichloromethane-acetone-concentrated ammonium hydroxide (42:17:25:17 v/v/v/v). The detection reagent was 0.2% (w/v) orcinol dissolved in a mixture of ethanol-concentrated sulfuric acid (90:10 v/v). High pressure liquid chromatography (HPLC) was