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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

realized using a Shodex KC-811 column (8.0 × 300 mm, 6 μm) and a mobile phase 0.1% formic acid, with an Ultimate 3000 systems (Dionex, Germany) equipped with a refractive index detector. The column oven and the refractive index detector were set at 50 °C; the flow rate was 0.6 mL/min and the injection volume was 10 μL. The concentration of the xylose and arabinose were quantified using peak areas as compared with those for their respective standards.

2.8. Purification and characterization of xylo-oligosaccharides

The hydrolysis products obtained from sugarcane bagasse were purified by Preparative layer chromatography (PLC) using 10 × 20 cm silica gel 60 F_{254} plates (Merck, Germany) as was described for TLC. Two spots were scraped from the plate, extracted with methanol and evaporated with vacuum. After dissolving the samples in D_2O, proton nuclear magnetic resonance (^1H NMR) and total correlation spectroscopy nuclear magnetic resonance (TOCSY-NMR) spectra were performed on a 400 MHz NMR (Brucker Advance DPX 400 spectrometer).

3. Results and discussion

3.1. Selection of precipitating reagents

Agents commonly used in protein fractionation, such as solvents, ammonium sulfate and detergents, were assayed for the precipitation step in order to synthesize the Xyl-CLEA from the crude extract produced by *Cohnella* sp. AR92. The enzymatic activity was successfully recovered when the samples were insolubilized with isopropanol (80% v/v) and with ammonium sulfate (60% w/v), reaching maximum values of 160 IU/mg and of 76 IU/mg, respectively. These values corresponded to 318% and 180% of the specific activity of the starting crude supernatant (Fig. 1). On the other hand, detergents did not yield a significant xylanase activity as precipitating agent (data not shown).

Both, isopropanol and ammonium sulfate have been reported as useful reagents for CLEA preparation, although they produce different protein configurations [21,22]. Then, the influence of both treatments on the subsequent cross-linking assays was assessed by valuing the xylanase activity after the cross-linking assays as a percentage of the activities after the precipitation. The isopropanol-treated samples showed up to 60% of the activity with 25% GA, while near 100% of the enzyme activity was recovered using 1.0 and 2.5% GA when the ammonium sulfate was used (Fig. 2). The isopropanol precipitation allowed the recovery of a higher enzymatic activity than the salting out procedure.

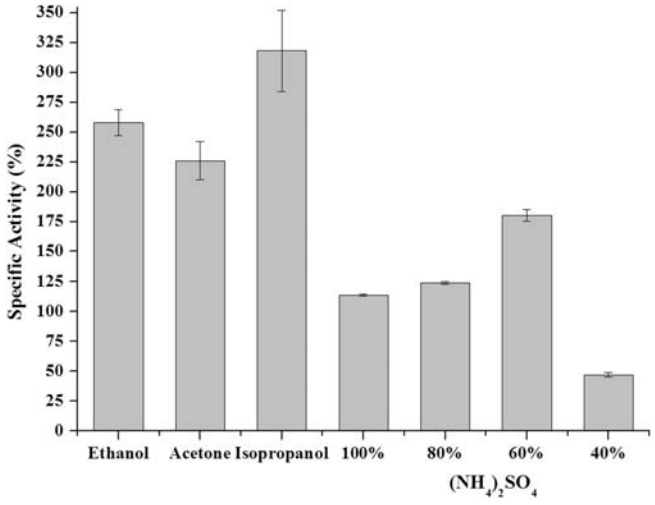

Fig. 1. Effect of solvent and ammonium sulfate, as precipitating agent. The initial protein concentration used was 46.28 IU/mg (100%). Specific activity (U/mg protein) was used to determine the aggregation yield (%).

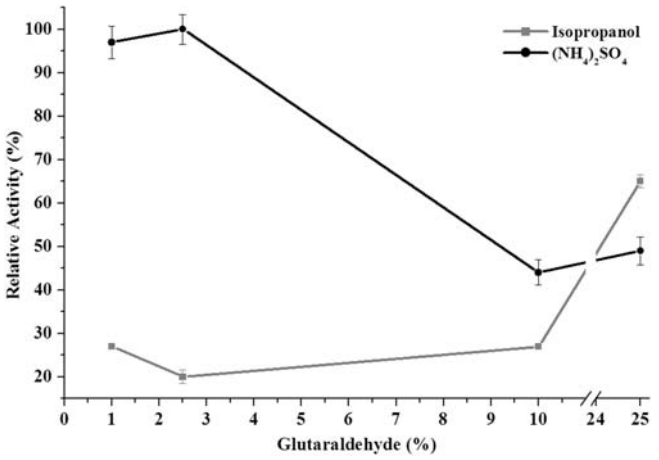

Fig. 2. Effect of glutaraldehyde (1–25%) as cross-linker solution for preparation of (•) proteins precipitate from $(NH4)_2SO_4$ (■) protein precipitate from Isopropanol was evaluated. The maximum activity was 1.34 IU/mL (100%).

However, the best overall yield was obtained after the salting-out precipitation and the cross-linking using the lowest GA concentration evaluated, 1.0 and 2.5% GA (Table 1).

The precipitating agents utilized promote structural protein changes thus affecting the following glutaraldehyde reaction. The organic solvents act by reducing the dielectric constant of the solution and displacing the water molecules around the protein surfaces, thus facilitating the protein aggregation via electrostatic interactions. Differently, the high salt concentrations pull the water away from proteins and their aggregation proceeds mainly via hydrophobic interactions [21,22]. Then, compatibility assays of the precipitating and the cross-linking agents are key to evaluate efficient CLEA generation from different enzymes and sources. Our results confirmed that the different solvent environments strongly influenced the cross-linking reaction, fact that endorsed the selection of 60% ammonium sulfate and 1.0%–2.5% GA for the Xyl-CLEA synthesis. These conditions were therefore settled upon the effectiveness in protein removal and the compatibility with the GA-mediated aggregation, as well as to avoid the use of organic solvents and high concentrations of GA.

3.2. Optimization of Xyl-CLEA synthesis

The selection of the optimal enzyme immobilization conditions is dependent on a delicate balance of factors that has often been determined by trial and error. Here, this was approached by means of a two-steps statistical approach comprising a Plackett-Burman design (PBD) followed by a response surface methodology (RSM). According to the PB results (Table S1 and Fig. S1), the variables that significantly affected the Xyl-CLEA synthesis were temperature, pH, GA concentration and ionic strength, all of which favored the Xyl-CLEA synthesis at their lower levels. On the other hand, the variables reaction time, reaction volume and $CaCl_2$, did not presented significant effect. The presence of Fe^{3+} was adverse in all the levels tested, probably due to the oxidizing nature of this metal ion in glycoside hydrolases [23]. A negative

Table 1
Specific activities and yield of: free enzymes after isopropanol and (NH4)2SO4 precipitation and cross-linking precipitates with glutaraldehyde for Xyl-CLEAs synthesis.

Condition	Activity (IU)	Specific Activity	Yield (%)
$(NH_4)_2SO_4$ precipitation	11.29	75.76	100.0
CLEAs from $(NH_4)_2SO_4$	0.57	3.82	5.0
Isopropanol precipitation	37.86	293.46	100.0
CLEAs from Isopropanol	0.49	3.80	1.3

effect on the activity of the xylanase from *Cohnella* sp.AR92 was previously observed [15].

Subsequently, the RSM based approach was assayed considering the factors glutaraldehyde concentration (A), ionic strength (B) and pH (C) as independent variables at fixed conditions of temperature, 4 °C, reaction volume, 1 mL, reaction time, 2 h, and absence of Ca^{2+} and Fe^{3+} (Table S2). The response xylanase activities obtained (XyAct, IU/mL) were then fitted into a second order polynomial equation by only considering the significant terms, as follows:

$$\textbf{\textit{XyAct}} = 1.9173 - 0.2896\,A - 0.1473\,B + 0.0936\,B^2 - 0.1088\,C^2 - 0.2014\,A*C + 0.1272\,B*C \quad (2)$$

The coefficients R^2 and R^2 adjusted (R^2adj) were 89.52% and 86.78%, respectively, meaning that 89.52% of the response variation is related to the variation of the independent variables; these values emphasized that the model was highly significant and suitable for the representation of the real relationship between these variables.

The RSM-based analysis indicated that the variables that influenced the most the Xyl-CLEA synthesis were GA concentration and ionic strength (IS) (Table 2). It also showed interactive effects between GA and pH (Table 2), as the Xyl-CLEA activity increased along with decrements of the GA concentration and pH increments (Fig. S2A, B and C). Besides, the pH influence was strongly affected by the ionic strength (Table 2), since IS values ≤10 mM improved the enzyme activities at 1% GA (Fig. S2D, E and F).

The GA concentration has shown to be a critical factor for a proficient CLEA synthesis since excessive amount may react with catalytically crucial residues. This results in rigid and cramped structures with limited diffusion of reactants, therefore affecting negatively the enzyme activity [24]. This potential effect was minimized by the use of the lowest GA concentrations tested, which showed to be the most convenient according to our results (Fig. 2) [10,14,25–27].

The pH showed a profound effect on the polymerization rate of glutaraldehyde molecules in solution since the high reactivity of its free aldehyde groups in aqueous media promotes a strong tendency for self-polymerization. In agreement, it was here observed that GA mediated cross-linking resulted effective at pH 4 (Table 2), conditions that favors the cross linking via Schiff bases formation with the amino groups of proteins [28].

As a result, the optimal conditions for the Xyl-CLEA synthesis consisted of a precipitation step with 60% ammonium sulfate, followed by the aggregation using 0.5% GA at pH 3.4, at 4 °C and an ionic strength of 10 mM, during 2 h. The xylanase activity of the immobilized enzyme was then improved 1.68 times with respect to the starting conditions. This was confirmed by the high correlation obtained between the experimental (2.51 ± 0.03 IU/mL) and predicted enzyme activities (2.46 ± 0.05 IU/mL).

The possible influence of the enzyme production medium on the Xyl-CLEA synthesis was also examined. Unexpectedly, the Xyl-CLEA produced from complex medium reached 2.81 IU/mL of xylanase activity, while the one obtained from a mineral medium made 1.23 IU/mL.

Table 2
Effect of variable and statistical analysis using an RSM approach for Xyl-CLEA synthesis.

Factors	ANOVA			Estimated Effect	
	Degree of freedom	*F*-Value	*p*-Value	Effect	T-Value
GA (A)	1	104.86	0.000	−0.5793	−10.24
IS (B)	1	27.13	0.000	−0.2946	−5.21
pH (C)	1	0.08	0.777	0.0162	0.29
GA * GA	1	0.82	0.375	0.0756	0.91
IS * IS	1	5.37	0.031	0.1930	2.32
pH * pH	1	6.47	0.019	−0.2118	−2.54
GA * IS	1	0.21	0.649	−0.0370	−0.46
GA * pH	1	25.36	0.000	−0.4029	−5.04
IS * pH	1	10.11	0.005	0.2544	3.18

This outcome was consistently reproduced regardless that the highest xylanase titles were obtained with a mineral-based medium [15], and may be due to the co-precipitation of xylanolytic enzymes with other proteins present in the culture supernatant. It has been reported that the presence of extra lysine residues during the CLEA synthesis, such as after the addition of BSA, would bind to glutaraldehyde producing a better stabilization and yield [8]. Likewise, the higher enzyme activity of the Xyl-CLEA obtained from complex medium supernatant in comparison to the mineral based-medium derivate, might be due to the presence of a diverse and abundant extracellular protein content (Fig. S3). These proteins may contribute with extra lysine residues for the Xyl-CLEA synthesis, thus exerting a stabilizing effect on the biocatalyst produced. Hence, the co-precipitation with other proteins from the same supernatant might work as the extra residues from BSA as was described.

3.3. Morphology and structure of the Xyl-CLEA

The shape, size and morphology are key properties of any aggregated-based enzyme immobilization as they define mass transfer limitations and stability against mechanical shear stress [22,29]. Those characters of were assessed by electron microscopy and FTIR analysis. By SEM observation, the free enzyme preparation showed a smooth surface whereas the Xyl-CLEAs appeared as a close-packed structure exposing several cavities (Fig. 3A and B-1). This increased surface area may facilitate the access of the substrate molecules to the catalytic site, as was described for aggregates of Type II [30]. In addition, spherical structures of 300–400 nm, or clusters, potentially containing a variable amount of aggregates were observed (Fig. 3B-2, B-3 and B-4). These clusters were similar to those defined as 'typical chemical aggregates' of Type I [31], which were also reported in Human Serum Albumin (HAS) that were successfully reduced after a treatment with L-dopa [32]. Interestingly, this molecule as well as others, such as ascorbic acid, were reported as inhibitors of protein aggregation. The Xyl-CLEA synthesis developed rendered the coexistence of both types of aggregates, as was confirmed after a sonication treatment of the clusters that rendered detached arrangements of 100–200 nm (Fig. 3C-1 and C-2). Then, such compounds could be helpful to control the aggregation step to improve the biocatalyst design to synthesize uniform and custom CLEAs [33–34].

A comparative FTIR spectra for the free enzyme and for the Xyl-CLEA showed differences at typical absorption bands of proteins and amino acids (Fig. 4). The peaks between 3500 cm^{-1} to 3200 cm^{-1} were assigned to the symmetric stretching N—H and peaks between 2900 cm^{-1} to 2850 cm^{-1} represented C—H vibrations [30]. Also, amide bands between 1600 and 1500 cm^{-1} and a smooth peak at 1210 cm^{-1}confirmed the presence of cross-linked residues in the Xyl-CLEA. The Amide *I* band (between 1600 and 1700 cm^{-1}) are primarily caused by stretching vibrations of C═O coupled weakly with C—N stretch and N—H bending [35]. The exact band position for the Xyl-CLEA corresponded to 1656 and 1546 cm^{-1}, while for the free enzyme a slight difference was detected as the peak was at 1650 and 1525 cm^{-1}. This change could be due to a conformational sensitivity of the Amide *I* band as consequence of the cross-linking. The Amide II band at 1500–1600 cm^{-1}, mainly derived from the C—N stretch along with N—H in-plane bending; the peak at 1547 cm^{-1} detected only for the Xyl-CLEA might correspond to this band. Lastly, the Amide III band was found at 1200–1300 cm^{-1}, which showed vibrations corresponding to a complex mix of N—H bending and C—N stretching along with deformation vibrations of C—H and N—H [35].

3.4. Characterization of the Xyl-CLEA

The thermal stability assessment of the Xyl-CLEA showed improvements of 41% and 25% with respect to the free enzyme after the treatments at 45 °C and 50 °C ($p < 0.05$), respectively (Fig. 5A), indicating

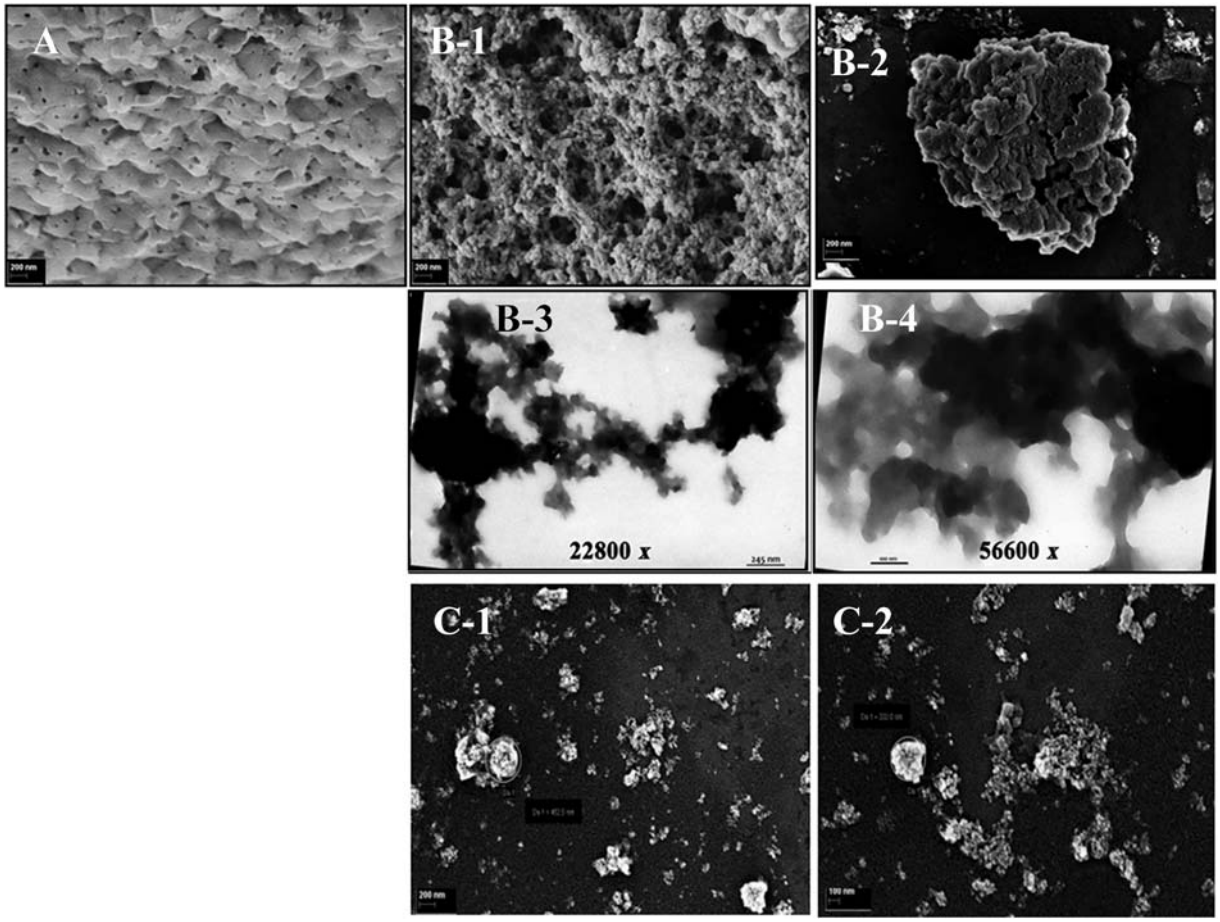

Fig. 3. SEM images of free enzyme and Xyl-CLEA. **A**: SEM image of protein precipitate (60.00 K X), **B-1**: SEM image of type II aggregates Xyl-CLEAs (60.00 K X), **B-2**: SEM image of type I spherical aggregates Xyl-CLEAs (60.00 K X); **B-3**, **B-4**: TEM image of Xyl-CLEA with electrodensity zones.**C-1**, **C-2**: SEM image of spherical aggregates arrangements sized between 100 and 200 nm after sonication were observed.

that the CLEA formation could outfit the immobilized enzymes against thermal denaturation. As was proposed, the configuration acquired after the cross-linking often confers to the enzymes a better thermal stability than conventional immobilization procedures, possibly due to the formation of stabilizing linkages that maintain the catalytically active conformation even at elevated temperatures [8,28,36]. Therefore, the procedure applied contributed to support the industrial properties of the xylanase preparation from *Cohnella* sp. AR92 [28,29].

Stability within an ethanol environment was assayed in order to evaluate the potential of the Xyl-CLEA for applications such as second-generation ethanol production or alkyl-glycosides synthesis [37]. The immobilized biocatalyst retained over 75% of activity in the presence of 15% (v/v) ethanol at pH 6.0, 37 °C during 72 h; whereas the free enzyme preparation lost 63% of its activity after 8 h in identical conditions (Fig. 5B). This effect could be related to the presence of nano-environment granted by surrounding protein molecules, which may reduce the concentration of organic solvents near to the enzyme thus preventing the dehydrating effect of the ethanol [36].

Recycling of the biocatalyst, a key property for a cost-effective suitability for industrial applications, showed that the Xyl-CLEA retained

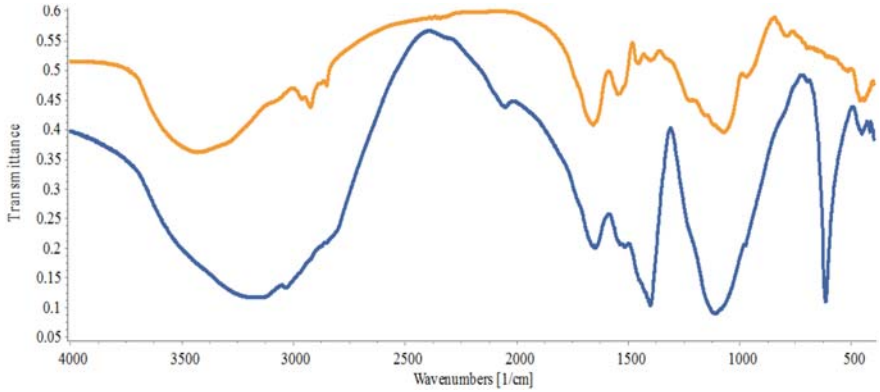

Fig. 4. FTIR spectroscopy in 4000–400 cm^{-1} region for free proteins (blue line) and Xyl-CLEA (red line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

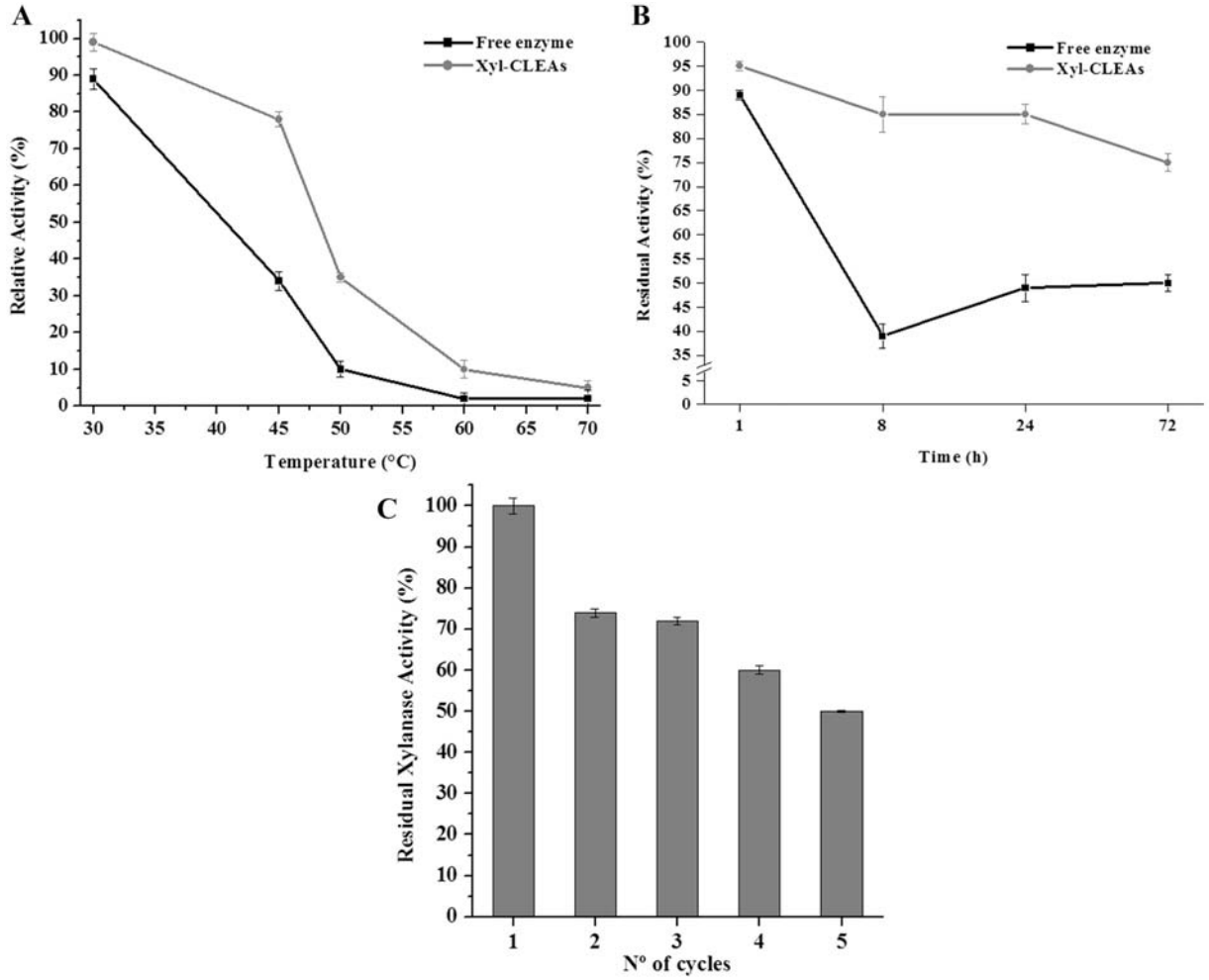

Fig. 5. Characterization of the Xyl-CLEAs. **A**: Thermal stability. **B**: Ethanol 15%- tolerance. **C**: Reuse cycle.

over 70% of activity after 3 cycles and 50% at the fifth cycle of reuse (Fig. 5C). The decay of the enzymatic activity observed after further cycles might be due to a partial leaching of the enzyme from the composite after multiple washing steps and/or to the clusters formation due to compression forces during centrifugation [31].

3.5. Bioconversion of agroindustrial waste biomass

Xylose (X1) was the main hydrolysis product of the free enzyme from an alkali pretreated sugarcane bagasse used as substrate (Table 3). The xilose concentration was observed to grow after 24; 48 and 72 h of reaction (Fig. 6A and B). Otherwise, the immobilized xylanase produced oligosaccharides as major product from identical substrate, as was observed by TLC analysis (Fig. 6B). The spots with a lower retention factor than ×1, Compounds 1 and 2, were purified by PLC in order to determine their nature by ^{1}H NMR and TOCSY NMR analysis. Table 4 indicates the chemical shifts of the 1H NMR spectra for both compounds, which were in the range reported for xylooligosaccharides [38]. For xylose residues, the signals at 5.11 and 4.50 ppm arise from the anomeric proton of the reducing Xyl*p* end groups, Xα and Xβ, respectively. The signals at 4.40 and 4.38 ppm corresponded to the anomeric protons of internal (X*i*) (only in Compound **1**) and non-reducing end (X*t*) xylosyl residues. The spin systems of X*i* and X*t* determined by TOCSY assays were assigned on the basis of published data [39,40]. The weak signal at 5.32 ppm was due to the α-L-arabinofuranosyl (α-L-Ara*f*) residue linked with the β-D-xylopyranose (β-D-Xyl*p*) residue [41–43].

The NMR analysis indicated that the two compounds were xylooligosaccharides. Whereas the Compound **1** was identified as a molecule containing three xylopiranose units linked by β-(1,4) bonds with an arabinofuranosyl residue, the Compound **2** contained two xylopiranose units linked by β-(1,4) bonds with an arabinofuranosyl residue as substituent. The arabinofuranosyl residue present in both

Table 3
HPLC analysis of hydrolysis products since sugarcane bagasse after the treatment with free enzyme and Xyl-CLEAs. Xylose was the main hydrolysis product for the free enzyme, increasing with extended reaction times. Xyl-CLEA produced a lower amount of sugars according to its retention time.

Treatment	Hydrolysis Time (h)	β-D-Xylose (mg/mL)[a,b]	α-L-Arabinose (mg/mL)[c,d]
Xyl-CLEA	24	0.05 ± 0.02	0.06 ± 0.02
	48	0.08 ± 0.02	0.09 ± 0.02
	72	0.07 ± 0.02	0.10 ± 0.02
Free Enzyme	24	2.20 ± 0.04	0.20 ± 0.02
	48	2.05 ± 0.04	0.10 ± 0.02
	72	2.30 ± 0.04	0.20 ± 0.02

[a] Limit of detection (LOD) β-D-xylose: 0.02 mg/mL.
[b] Limit of quantitation β-D-xylose: 0.04 mg/mL.
[c] LOD α-L-arabinose: 0.02 mg/mL.
[d] LOQ α-L-arabinose: 0.04 mg/mL.

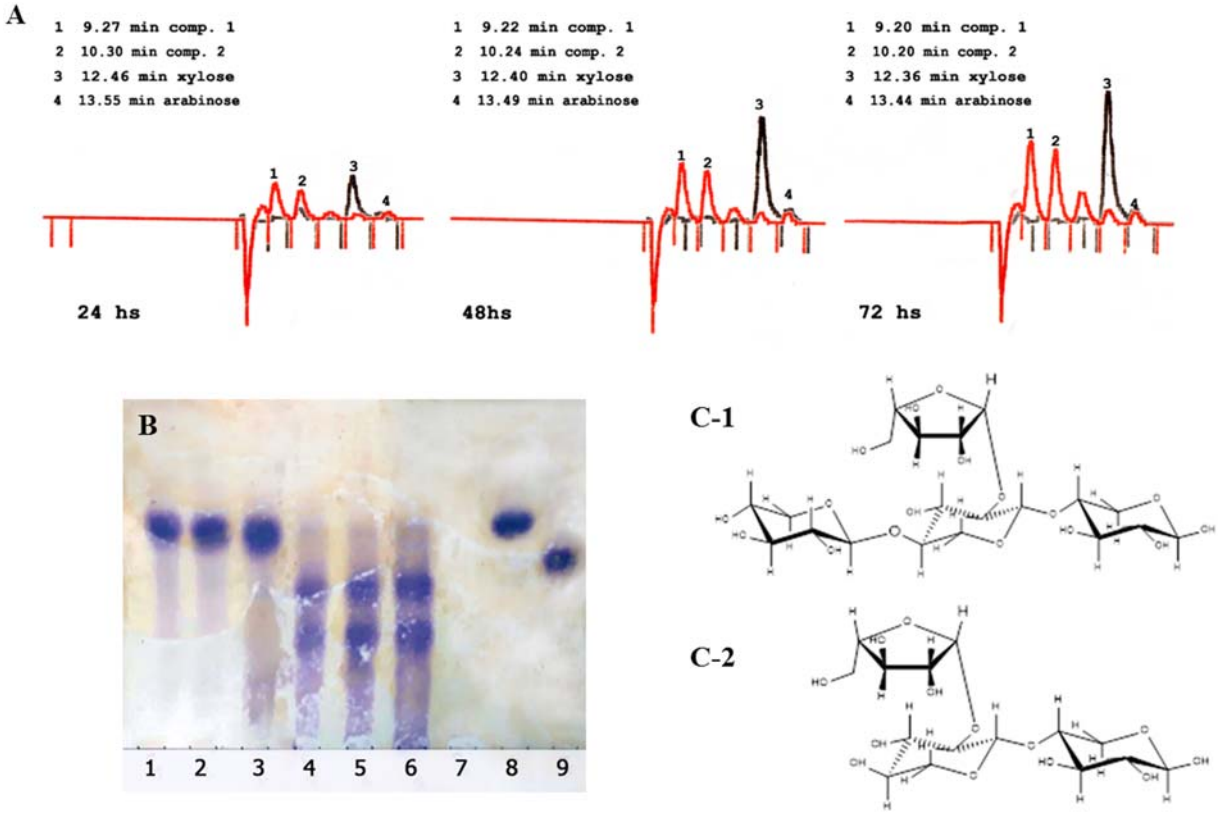

Fig. 6. Arabinoxylooligosaccharides (AXOS) production from sugarcane bagasse through Xyl-CLEA hydrolysis. **A**: HPLC quantification of AXOS produced from 1 g of sugarcane bagasse after 24, 48 and 72 h of hydrolysis by Xyl-CLEA (Red line) and free enzyme (Black line). **B**: Thin layer chromatography showing AXOS formation. Lane 1: Free enzyme 24 h. Line 2: Free enzyme 48 h. Line 3: Free enzyme 72 h. Line 4: Xyl-CLEA 24 h. Line 5: Xyl-CLEA 48 h. Line 6: Xyl-CLEA 72 h. Line 7: Sugarcane bagasse (control). Line 8: Xylose standard. Line 9: Arabinose standard. **C**: AXOS probable structures elucidated by NMR. **C-1**: Probable structure of Compound 1 (Rf 1), **C-2**: Compound 2 (Rf 2); where Rf 1 < Rf 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

compounds could be attached to the free OH groups of carbons C-2 and C-3 of any xylopyranose (Fig. 6C). Therefore, both compounds were identified as arabinoxylooligosaccharides (AXOS).

Table 4
Chemical shifts (1H, ppm) for compounds 1 and 2 obtained from 1H and TOCSY NMR spectra.

Element[a]	Chemical shift, ^{1}H (ppm)	Assignment
Compound 1		
Xα	5.11	H-1
Xβ	4.50	H-1
X*i*	4.40	H-1
	3.20	H-2
	3.48	H-3
	3.71	H-4
	3.32/4.03	H-5
X*t*	4.38	H-1
	n.d	H-2
	3.35	H-3
	3.54	H-4
	3.22/3.89	H-5
A*t*	5.32	H-1
Compound 2		
Xα	5.11	H-1
Xβ	4.51	H-1
X*t*	4.38	H-1
	n.d	H-2
	3.35	H-3
	3.54	H-4
	3.22/3.89	H-5
A*t*	5.32	H-1

[a] Xα and Xβ, β-D-xylopyranose (Xyl*p*) reducing ends; X*i* and X*t*, internal and non-reducing ends; A*t*, α-Ara*f* non-reducing end, n.d., not detected.

4. Conclusions

The cross-linked aggregate technology may have a significant impact on cost-effective biocatalysts production for lignocellulosic biomass bioconversion. Here, a systematic approach based on statistical tools was used to develop and depict the CLEA synthesis with a reduced number of assays. As a start material for enzyme immobilization, a crude xylanase from *Conhella* sp. AR92 produced with a nutritive medium was used. This preparation contained proteins that possibly served as source of amino groups to improve the cross-linking of the enzyme of interest, thus contributing to the simplification and to the practicability of the process. As a result, the stability and recycling performance of the biocatalysts obtained, contributed to improve the industrial properties of the xylanase from the strain AR92. The Xyl-CLEA synthesized displayed a different specificity of the enzymes (xylanases) that constitute the extracellular extract from the strain AR92; whereas the free enzymes produced xylose as a majority product from pretreated SCB, the CLEAs obtained from such extract generated xylooligosaccharides from the same substrate.

Finally, further knowledge needs to be applied in order to upgrade the carrier-free immobilized biocatalysts from crude enzymatic preparations. Our results provided valuable data to approach studies using proteomic tools and additives during the synthesis for better understand and control the aggregation step to improve the CLEAs designing protocols.

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Appendix A. Supplementary data

The complete methods are given in Table S1 and S2. The complete optimization design is given in Fig. S1 and S2. The Native-PAGE of different enzymes production is given in Fig. S3. Supplementary data associated with this article can be found in the online version, at https://doi.org/10.1016/j.ijbiomac.2017.12.166.

References

[1] M. Coma, E. Martínez-Hernández, F. Abeln, S. Raikova, J. Donnelly, M.J. Allen, D.D. Hong, C.J. Chuck, Organic waste as a sustainable feedstock for platform chemicals, Faraday Discuss. 202 (2017) 175–195.

[2] B. Kamm, M. Kamm, Principles of biorefineries, Appl. Microbiol. Biotechnol. 64 (2004) 137–145.

[3] H. Chakdar, M. Kumar, K. Pandiyan, A. Singh, K. Nanjappan, P.L. Kashyap, A.K. Srivastava, Bacterial xylanases: biology to biotechnology, Biotech 3 (6) (2016) 150.

[4] A. Bhattacharya, B. Pletschke, Strategic optimization of xylanase–mannanase combi-CLEAs for synergistic and efficient hydrolysis of complex lignocellulosic substrates, J. Mol. Catal. B Enzym. 11 (2015) 140–150.

[5] S. Kumar, I. Haq, A. Yadav, J. Prakash, A. Raj, Immobilization and biochemical properties of purified xylanase from *Bacillus amyloliquefaciens* SK-3 and its application in Kraft pulp biobleaching, J. Clin. Microbiol. Biochem. Technol. 2 (2016) 026–034.

[6] S. Velasco-Lozano, F. López-Gallego, J.C. Mateos-Díaz, E. Favela-Torres, Cross-linked enzyme aggregates (CLEA) in enzyme improvement – a review, Biocatalysis 1 (2016) 166–177.

[7] R.A. Sheldon, Cross-linked enzyme aggregates (CLEAs): stable and recyclable biocatalysts, Biochem. Soc. Trans. 35 (2007) 1583–1587.

[8] I. Roy, J. Mukherjee, M.N. Gupta, Cross-linked enzyme aggregates for applications in aqueous and nonaqueous media, Methods Mol. Biol. 1504 (2017) 109–123.

[9] A. Bhattacharya, B. Pletschke, Magnetic cross-linked enzyme aggregates (CLEAs): a novel concept towards carrier free immobilization of lignocellulolytic enzymes, Enzym. Microb. Technol. 61–62 (2014) 17–27.

[10] L. Li, G. Li, L.C. Cao, G.H. Ren, W. Kong, S.D. Wang, G.S. Guo, Y.H. Liu, Characterization of the cross-linked enzyme aggregates of a novel β-galactosidase, a potential catalyst for the synthesis of galacto-oligosaccharides, J. Agric. Food Chem. 63 (2015) 894–901.

[11] R.A. Sheldon, Engineering a more sustainable world through catalysis and green chemistry, J. R. Soc. Interface 13 (2016) 20160087.

[12] Q. Zhen, M. Wang, W. Qi, R. Su, Z. He, Preparation of β-mannanase CLEAs using macromolecular cross-linkers, Cat. Sci. Technol. 3 (2013) 1937–1941.

[13] F. Secundo, Conformational changes of enzymes upon immobilisation, Chem. Soc. Rev. 42 (2013) 6250–6261.

[14] M. Bilal, M. Asgher, H.M.N. Iqbal, H. Hu, X. Zhang, Bio-based degradation of emerging endocrine-disrupting and dye-based pollutants using cross-linked enzyme aggregates, Environ. Sci. Pollut. Res. Int. 24 (2017) 7035–7041.

[15] J.H. Pisa, A.P. Manfredi, N.I. Perotti, H.G. Romero, J.D. Breccia, M.A. Martínez, Agro-wastes as feedstock for the production of endo-β-xylanase from *Cohnella* sp. strain AR92, J. Mol. Microbiol. Biotechnol. 27 (2017) 277–288.

[16] A.P. Manfredi, N.I. Perotti, M.A. Martínez, Cellulose degrading bacteria isolated from industrial samples and the gut of native insects from northwest of Argentina, J. Basic Microbiol. 55 (2015) 1384–1393.

[17] E. Di Marco, P.M. Soraire, C.M. Romero, L.B. Villegas, M.A. Martínez, Raw sugarcane bagasse as carbon source for xylanase production by *Paenibacillus* species: a potential degrader of agricultural wastes, Environ. Sci. Pollut. Res. Int. 24 (2017) 19057–19067.

[18] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem. 31 (1959) 426–428.

[19] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[20] A. Cruz-Izquierdo, E.A. Picó, C. López, J.L. Serra, M.J. Llama, Magnetic cross-linked enzyme aggregates (mCLEAs) of *Candida antarctica* lipase: an efficient and stable biocatalyst for biodiesel synthesis, PLoS One 9 (2014), e115202.

[21] S. Rehman, H.N. Bhatti, M. Bilal, M. Asgher, Cross-linked enzyme aggregates (CLEAs) of *Pencilluim notatum* lipase enzyme with improved activity, stability and reusability characteristics, Int. J. Biol. Macromol. 91 (2016) 1161–1169.

[22] S. Talekar, A. Joshi, G. Joshi, P. Kamat, R. Haripurkar, S. Kambale, Parameters in preparation and characterization of cross linked enzyme aggregates (CLEAs), RSC Adv. 3 (2013) 12485–12511.

[23] C. Vieille, G.J. Zeikus, Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability, Microbiol. Mol. Biol. Rev. 65 (2001) 1–43.

[24] J. Mukherjee, M.N. Gupta, Protein aggregates: forms, functions and applications, Int. J. Biol. Macromol. 97 (2017) 778–789.

[25] S. Dalal, A. Sharma, M.N. Gupta, A multipurpose immobilized biocatalyst with pectinase, xylanase and cellulase activities, Chem. Cent. J. 8 (2007) 1–16.

[26] S. Hernández-García, M.I. García-García, F. García-Carmona, Improving the production, activity, and stability of CLEAs with diepoxides, Biotechnol. Prog. (2017)https://doi.org/10.1002/btpr.2505.

[27] X. Hu, L. Liu, D. Chen, Y. Wang, J. Zhang, L. Shao, Co-expression of the recombined alcohol dehydrogenase and glucose dehydrogenase and cross-linked enzyme aggregates stabilization, Bioresour. Technol. 224 (2017) 531–535.

[28] I. Migneault, C. Dartiguenave, M.J. Bertrand, K.C. Waldron, Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking, BioTechniques 37 (2004) 798–802.

[29] H. Chen, J. Ullah, J. Jia, Progress in *Bacillus subtilis* spore surface display technology towards environment, vaccine development, and biocatalysis, J. Mol. Microbiol. Biotechnol. 27 (2017) 159–167.

[30] S.A. Chaudhari, R.S. Singhal, A strategic approach for direct recovery and stabilization of *Fusarium* sp. ICT SAC1 cutinase from solid state fermented broth by carrier free cross-linked enzyme aggregates, Int. J. Biol. Macromol. 98 (2017) 610–621.

[31] R. Schoevaart, M.W. Wolbers, M. Golubovic, M. Ottens, A.P. Kieboom, F. van Rantwijk, L.A. van der Wielen, R.A. Sheldon, Preparation, optimization, and structures of cross-linked enzyme aggregates (CLEAs), Biotechnol. Bioeng. 87 (2004) 754–762.

[32] M.R. Ajmal, T.I. Chandel, P. Alam, N. Zaidi, M. Zaman, S. Nusrat, et al., Fibrillogenesis of human serum albumin in the presence of levodopa - spectroscopic, calorimetric and microscopic studies, Int. J. Biol. Macromol. 94 (2017) 301–308.

[33] P. Alam, A.Z. Beg, M.K. Siddiqi, S.K. Chaturvedi, R.K. Rajpoot, M.R. Ajmal, et al., Ascorbic acid inhibits human insulin aggregation and protects against amyloid induced cytotoxicity, Arch. Biochem. Biophys. 621 (2017) 54–62.

[34] S. Nusrat, N. Zaidi, M.K. Siddiqi, M. Zaman, I.A. Siddique, M.R. Ajmal, et al., Anti-Parkinsonian L-dopa can also act as anti-systemic amyloidosis - a mechanistic exploration, Int. J. Biol. Macromol. 99 (2017) 630–640.

[35] S.E. Glassford, B. Byrne, S.G. Kazarian, Recent applications of ATR FTIR spectroscopy and imaging to proteins, Biochim. Biophys. Acta 1834 (2013) 2849–2858.

[36] L. Wilson, A. Illanes, O. Abián, B.C.C. Pessela, R. Fernández-Lafuente, J.M. Guisán, Co-aggregation of penicillin G acylase and polyionic polymers: an easy methodology to prepare enzyme biocatalysts stable in organic media, Biomacromolecules 5 (2004) 852–857.

[37] M.Y. Rather, S. Mishra, β-Glycosidases: an alternative enzyme based method for synthesis of alkyl-glycosides, Sustainable Chem. Processes (2013)https://doi.org/10.1186/2043-7129-1-7.

[38] S.S. Reddy, C. Krishnan, Production of high-pure xylooligosaccharides from sugarcane bagasse using crude β-xylosidase-free xylanase of *Bacillus subtilis* KCX006 and their bifidogenic function, LWT Food Sci. Technol. 65 (2016) 237–245.

[39] J. Bian, F. Peng, X.P. Peng, P. Peng, F. Xu, R.C. Sun, Structural features and antioxidant activity of xylooligosaccharides enzymatically produced from sugarcane bagasse, Bioresour. Technol. 127 (2013) 236–241.

[40] P. Gullón, M.J. González-Muñoz, M.P. van Gool, H.A. Schols, J. Hirsch, A. Ebringerová, J.C. Parajó, Production, refining, structural characterization and fermentability of rice husk xylooligosaccharides, J. Agric. Food Chem. 58 (2010) 3632–3641.

[41] S.S. Reddy, C. Krishnan, Characterization of enzyme released antioxidant phenolic acids and xylooligosaccharides from different *Graminaceae* or *Poaceae* members, Food Biotechnol. 27 (2013) 357–370.

[42] J.X. Sun, X.F. Sun, R.C. Sun, Y.Q. Su, Fractional extraction and structural characterization of sugarcane bagasse hemicelluloses, Carbohydr. Polym. 56 (2004) 195–204.

[43] Y. Sun, S.W. Cui, X. Gu, J. Zhang, Isolation and structural characterization of water unextractable arabinoxylans from Chinese black-grained wheat bran, Carbohydr. Polym. 85 (2011) 615–621.