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Research Article

Laccase-Based CLEAs: Chitosan as a Novel Cross-Linking Agent

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Laccase from *Coriolopsis Polyzona* was insolubilized as cross-linked enzyme aggregates (CLEAs) for the first time with chitosan as the cross-linking agent. Concentrations between 0.01 and 1.867 g/L of chitosan were used and between 0.05 and 600 mM of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. The laccase was precipitated using ammonium sulphate and cross-linked simultaneously. Specific activity and thermal stability of these biocatalysts were measured. Activities of up to 737 U/g were obtained when 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was used as a substrate. Moreover, the stability of these biocatalysts was improved with regards to thermal degradation compared to free laccase when exposed to denaturing conditions of high temperature and low pH. The CLEAs stability against chemical denaturants was also tested but no significant improvement was detected. The total amount of ABTS to be oxidized during thermal degradation by CLEAs and free laccase was calculated and the insolubilized enzymes were reported to oxidize more substrate than free laccase. The formation conditions were analyzed by response surface methodology in order to determine an optimal environment for the production of efficient laccase-based CLEAs using chitosan as the cross-linking agent. After 24 hours of formation at pH 3 and at 4°C without agitation, the CLEAs exhibit the best specific activity.

1. Introduction

There is growing interest in the use of enzymes in industrial bioprocesses dedicated to bioremediation purposes [1, 2]. Over the last years, laccases (polyphenoloxidase, EC 1.10.3.2) have gained attention due to their ability to convert a wide range of pollutants present in different environmental matrices [2–6]. Laccases are produced by fungi, higher plants, bacteria, and insects. These multicopper oxidases catalyze the oxidation of various phenol-like compounds, aromatic amines, and some inorganic compounds. They have received a growing attention due to their intrinsic properties such as relatively low substrate specificity, stability, and the simple and inexpensive culture media that could be used to produce them [7].

However, two major obstacles hamper the use of laccases in industrial bioprocesses: (1) their sensitivity to various environmental denaturants such as salts, solvents, and proteolytic enzymes [8] and (2) the difficulty of retaining the enzyme in a continuous flow bioreactor. These obstacles make the use of laccases a costly alternative to conventional environmental remediation alternatives.

In the interest of enhancing the industrial applicability of laccase, including the improvement of its stability and its repeated utilization, substantial efforts have been made to immobilize this enzyme with or without a solid support [9]. A well-known strategy to immobilize enzyme is to bind them covalently or through ionic interactions to a solid support or by trapping them in a matrix made of (bio)polymer [10]. These methods produce stable and reusable biocatalysts but can reduce considerably their specific activity [11]. The formation of cross-linked enzyme aggregates (CLEAs) can overcome this drawback. Insolubilization of enzyme as CLEAs is a simple technique to produce a biocatalyst with

high enzyme activity per unit volume. Since it does not use a support to insolubilize the enzyme, it increases the specific activity of the biocatalyst formed [10]. An industrial process using CLEAs can make use of them in smaller reactors than the enzymes immobilized on a solid support.

CLEAs of laccase secreted by the white rot fungus (WRF) *Coriolopsis polyzona* have been prepared by Cabana et al. [5] using glutaraldehyde (GLU) as the cross-linking agent. These CLEAs have shown high enzyme activity and higher stability than free laccase against physical, chemical, and biological denaturants and good kinetics of reaction. These biocatalysts have been successfully used for the continuous treatment of water contaminated by the endocrine disrupting chemicals bisphenol A, nonylphenol, and triclosan [11]. In addition, Matijošyte et al. [12] have produced CLEAs with laccases from the WRF *Trametes versicolor*, *Trametes villosa*, and *Agaricus bisporus*. Their laccase CLEAs have also shown higher stability than the free enzymes and have been used in a laccase/mediator system for the successful oxidation of C_5–C_{10} aliphatic alcohols.

The formation of CLEAs requires the use of a cross-linking agent. Generally, GLU is chosen for this purpose due to its low cost, ease of manipulation, and its ability to generate covalent bonds with most enzymes [10]. Even if this chemical is used in several applications, it presents adverse effects on the aquatic environment and on the health of the workers [13, 14]. GLU is suspected of reducing the hatching rate of some aquatic species eggs [14, 15]. It also has many effects on human health: it can provoke asthma, eczema, and respiratory tract and skin irritation [13]. The utilization of GLU for the formation of CLEAs dedicated to environmental applications can pose a problem because this cross-linking agent can potentially leach from the biocatalysts to the receiving environment where it can cause adverse effects to the aquatic ecosystems. To overcome this potential issue, an alternative must be found to cross-link the aggregated enzymes destined for environmental processes.

The renewable biopolymer chitosan represents an attractive candidate for the cross-linking of the enzyme aggregates. Chitosan is obtained from the deacetylation of the naturally occurring polymer chitin. The use of this biopolymer is favored by: (1) its high amino group content which favors link formation with enzymes, (2) its good mechanical strength and its resistance to chemical degradation, and (3) its biocompatibility and biodegradability [16]. Furthermore, its production is of low cost and ecologically interesting. The amino groups present on its chain can react with activated carboxylic group present in nonessential amino acid of the enzyme and form amide bonds [17]. Moreover, one molecule of chitosan can react with more than one carboxylic group and therefore more than one enzyme since it has many amino groups on its chain. This way, laccase aggregates can be covalently attached and permanently insolubilized. The activation of the carboxylic group is done through carbodiimide chemistry. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) is perhaps one of the most popular chemicals used for the binding of an enzyme to chitosan. Some investigations have used this strategy for the conjugation of free laccases to chitosan [18, 19]. However, the objective of this study is to use chitosan and EDAC as a cross-linking complex rather than a binding support.

The first objective of this study was to produce CLEAs of laccase from the WRF *C. polyzona* by using chitosan as the cross-linking agent and characterize them. The second objective was to determine the effects of the conditions of formation (pH, temperature, reaction time, and shaking speed) on the characteristics of the CLEAs produced by this new approach.

2. Materials and Methods

2.1. Materials. The WRF strain *C. polyzona* (MUCL 38443) was provided by the Belgian Coordinated Collections of Microorganisms (BCCM/MUCL). Cellulose membranes for dialysis came from Fisher Scientific (Pittsburgh, PA). All other reactants used came from Sigma-Aldrich (St. Louis, MO) and were of analytical grade or the highest grade available.

2.2. Laccase Production. The inoculum was grown in a rotary shaker at 150 rpm and 27°C in 250-mL Erlenmeyers containing 100 mL of standard medium: 10 g/L glucose, 2 g/L NH_4NO_3, 0.8 g/L KH_2PO_4, 0.4 g/L Na_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 2 g/L yeast extract. The medium was adjusted to pH 6.0 with 2 M NaOH prior to autoclaving. After 10 days of cultivation or after reaching a laccase activity over 2000 U/L in the broth, the biomass was filtered and the supernatant was conserved. Enzymes were precipitated using 600 g/L ammonium sulphate. The resulting solution was then centrifuged at 10000 ×g for 5 minutes, and the supernatant was removed. The precipitation steps were repeated until no laccase activity was detected in the supernatant. The precipitates were solubilized in deionized water. The resulting preparation was dialyzed against distilled water using a regenerated cellulose membrane with a molecular cut-off of 13 kDa and then used as the source of laccase.

2.3. Enzyme Assay. Laccase activity was determined by monitoring the oxidation of 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) to its radical cation ($ABTS^{\bullet+}$) [20]. The assay mixture contained 0.5 mM ABTS. The pH was adjusted to 3 using 60 mM citric acid/disodium hydrogen phosphate buffer at room temperature. One unit of activity was defined as the amount of enzyme forming 1 mmol of $ABTS^{\bullet+}$ per min.

2.4. Chitosan Solution Preparation. Chitosan (mean molecular weight of 750 kDa and 64% deacetylated) was solubilized in HCl (0.1 M) solution to a final concentration of 5 g/L. The solution was shaken in a sonification bath during one hour.

2.5. CLEAs Production. CLEAs were prepared by simultaneously aggregating and cross-linking the laccases [5]. In 10 mL of solution, 550 g/L of ammonium sulphate, 10 units of laccase, chitosan and EDAC were added to a 500 mM phosphate buffer at pH 5. This reaction solution was stored

Table 1: Chitosan and EDAC concentrations tested for the optimization of the CLEAs formation at 4°C during 48 hours.

Experimental design type	Sample names	Chitosan concentration (g/L)	EDAC concentration (mM)
3^2	CLEA-0.2-200	0.2	200
	CLEA-0.2-400	0.2	400
	CLEA-0.2-600	0.2	600
	CLEA-0.6-200	0.6	200
	CLEA-0.6-400	0.6	400
	CLEA-0.6-600	0.6	600
	CLEA-1.0-200	1.0	200
	CLEA-1.0-400	1.0	400
	CLEA-1.0-600	1.0	600
Modified Central Composite	CLEA-0.5-1	0.5	1
	CLEA-0.5-100	0.5	100
	CLEA-1.5-1	1.5	1
	CLEA-1.5-100	1.5	100
	CLEA-1.0-0.05	1.0	0.05
	CLEA-1.0-50.5	1.0	50.5
	CLEA-1.0-136	1.0	136
	CLEA-0.134-50.5	0.134	50.5
	CLEA-1.867-50.5	1.867	50.5

at 4°C for 48 h. These formation conditions were used unless other conditions are cited. Subsequently, the solution was centrifuged at 10000 ×g for 5 minutes. The supernatant was discarded, and the precipitate was washed with 3 mL of 50 mM acetate buffer at pH 6.5 and centrifuged at 10000 ×g for 5 minutes. The washing and centrifugation steps were then repeated with deionised water until no laccase activity was detected in the supernatant. The washed precipitate was then suspended in 5 mL of deionised water. The CLEAs samples were identified as follows: CLEA-concentration of chitosan (in g/L), concentration of EDAC (in mM) (e.g., CLEA-1.0-200 for CLEA prepared with 1,0 g/L of chitosan and 200 mM of EDAC).

2.5.1. Concentrations Optimization. The impact of chitosan and EDAC concentrations on the performances of CLEAs was tested in two experimental designs. The concentrations used in these experiments are shown in Table 1. The center composite design was modified to have positive values of EDAC concentrations. The specific activity, the thermal stability, the half-life under thermal degradation, and the total amount of ABTS oxidized by the prepared CLEAs were evaluated. Design-Expert 6.0 (Stat-Ease, Minneapolis, MN) software was used to evaluate the influence of chitosan and EDAC concentrations on these parameters.

2.5.2. Optimization of CLEAs Preparation Conditions. The physical conditions of CLEAs formation were also optimized to reach better specific activity and thermal stability. The different conditions tested are shown in Table 2. The concentrations of chitosan and EDAC were fixed at 1.87 g/L and 50.5 mM, respectively. Each condition was tested twice for a total of 72 samples. The specific activity and thermal stability of each sample were measured twice each, and an analysis of variance was conducted on the results with the Stat-Ease software Design-Expert 6.0 (Minneapolis, MN).

Table 2: Conditions tested for the optimization of CLEAs characteristics.

Condition	Value
pH	3 and 5
Temperature	4, 20 and 30°C
Agitation	0 and 150 RPM
Reaction time	8, 16 and 24 hours

2.6. CLEAs and Free Laccase Thermal Stability. The thermal stability was determined by monitoring free laccase and CLEAs activity through time when exposed to a temperature of 40°C and a pH of 3. CLEAs or laccase solution (400 μL) was incubated in 400 μL of 50 mM citric acid/sodium phosphate buffer. Activity was measured twice and at different moments during the degradation. The inactivation of free laccase and CLEAs was modeled using the 3-parameter phenomenological model proposed by Aymard and Belarbi [21]. This biexponential model is expressed by

$$\frac{(A)_t}{(A)_0} = C * e^{-\alpha * t} + (1 - C) * e^{-\beta * t}. \quad (1)$$

The ratio $(A)_t/(A)_0$ represents the enzyme activity remaining after a time t (A_t) compared to initial activity (A_0). The physical meaning of the parameters and their expressions as a function of individual rate constants differs according to the mechanism considered. This expression can be used irrespective of the thermal inactivation mechanism involved [21].The values of the different parameters of this model (C, *alpha*, and *beta*) were obtained by curve fitting of the plot of the residual enzyme activity versus time using the Sigma Plot 7.0 software (SPSS Inc., Chicago, IL).

2.7. Total Amount of ABTS Oxidized under Denaturing Conditions. To consider both the initial activity of the biocatalysts formed and their thermal stability, the total amount of ABTS oxidized by the different biocatalysts under the thermal denaturing conditions was calculated. To do so the Aymard-Belarbi model was integrated to determine the theoretical total amount of ABTS oxidized under the thermal degradation conditions used:

$$Q_t = (A)_0 * \int \left(C * e^{-\alpha * t} + (1 - C) * e^{-\beta * t}\right) dt. \quad (2)$$

The amount of ABTS oxidized (Q_t (mmol)) was calculated on a 24-hour interval using the parameters C, alpha, and beta obtained from the thermal degradation tests (see Section 2.7) and the initial activity of the biocatalyst (A_0).

2.8. Enzyme Kinetics. Michaelis-Menten parameters of CLEAs and free laccase were determined using ABTS as a substrate at various concentrations (0.05, 0.1, 0.2, 0.4, 0.5, 0.75, 1.0, 1.5, and 2.0 mM). The activity with each substrate concentration was determined twice. The parameter values were obtained by curve fitting the plot of reaction rate versus substrate concentrations using the Sigma Plot 7.0 software.

2.9. CLEAs and Free Laccase Stability to Chemicals Denaturants. The CLEAs and free laccase were also exposed to chemicals to determine their stability against denaturing environments. Solutions of $CaCl_2$ (10 μM), $ZnCl_2$ (10 μM), ethylenediaminetetraacetic acid (EDTA) (10 μM), NaN_3 (30 μM), acetone (25% (v/v)), and methanol (25% (v/v)) were prepared separately by dissolving the powders or diluting the solvents in a 50 mM citric acid/phosphate buffer at pH 3. CLEAs and free laccase duplicates (100 μL each) were exposed to each of these chemicals (400 μL) separately for 4 hours.

The stability of CLEAs and free laccase has also been tested after an exposition of 24 hours to an effluent of a wastewater treatment plant (WWTP). The effluent was taken from the Mont St-Grégoire (Québec, Canada) WWTP. As for the stability against chemical denaturants test, 100 μL of CLEAs or free laccase solution was mixed with 400 μL of denaturing medium. The methods used to characterize the wastewater samples were ICP-MS for total phosphorus content [22], infrared spectroscopy for COD [22], gravimetric for suspended particles [22], spectrophotometry for nitrogen-NH_3 [22], HPLC for nitrites and nitrates [22], and hexane extraction and gravimetric method for oils and grease [22].

2.10. Scanning Electron Microscopy of CLEAs. Scanning electron micrographs (SEMs) of CLEAs were obtained on Hitachi S-4700 FESEM and S-3000N VPSEM (Tokyo, Japan) electron microscopes. The samples were previously dried at ambient temperature and then coated with platinum using an Emitech K550 (Ashford, UK) sputter coater.

2.11. Particle Size of CLEAs. The size of the CLEAs was measured by photon correlation spectroscopy (PCS) with a ZetaPlus zeta potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA). CLEAs suspension was diluted in a 1 : 1 ratio with deionised water in 2 mL cuvettes at 20°C. Each CLEAs particle size was measured twice.

3. Results

3.1. Preliminary Screening. Table 3 shows the specific activities, the thermal stability, the half-life under thermal degradation, and the total amount of ABTS oxidized by CLEAs and free laccase. The specific activity of the CLEAs prepared was between 16 and 737 U/g while the free laccase used had a specific activity of 284 U/g.

Free laccase has a half-life of 19 hours while CLEA-1.867-50.5 and CLEA-1.5-100 have half-life of 81 and 78 hours, respectively. CLEA-0.5-1, CLEA1.0-136, and CLEA-0.6-400 all have a half-life under 2 hours. These half-lifes were calculated by using the kinetics of thermal degradation model proposed by Aymard and Belarbi [21].

In order to have a good basis to compare biocatalysts with different initial activities and stability, the total amount of ABTS theoretically oxidized during the thermal denaturation of the biocatalysts for 24 hours was calculated. After a 24-hour period of thermal denaturation, CLEA-1.5-100 and CLEA-1.867-50.5 both oxidized more ABTS than free laccase. CLEA-1.5-100 and CLEA-1.867-50.5, respectively, oxidize 1.103 mmol/U and 1.094 mmol/U of ABTS while free laccase oxidizes only 0.787 mmol/U. CLEA-1.0-136 and CLEA-1.0-50.5 have catalyzed a comparable number of reactions than free laccase after 24 hours (resp., 0.539 and 0.565 mmol/U).

From this point, only the four samples that oxidized the highest amount of ABTS in 24 h have been used to characterize the CLEAs. These samples are CLEA-1.0-136, CLEA-1.5-100, CLEA-1.867-50.5, and CLEA-1.0-50.5. They were the most promising samples for the preparation of efficient and stable biocatalysts dedicated to bioremediation purposes.

3.2. Optimization of CLEAs Preparation Conditions. Table 4 presents the results of the analysis of variance (ANOVA) performed on the results of specific activity for each of the prepared samples.

The ANOVA performed show a significant effect of the temperature and reaction time on the specific activity of the CLEAs. Figures 1(a) and 1(b) show how these parameters influence the specific activity of the CLEAs while Figure 1(c) illustrates the interaction between the agitation and reaction time.

The specific activity of CLEAs prepared at 30°C is lower than those prepared at 20°C and 4°C. The longer the cross-linking reaction is, the more active the CLEAs will be. The reaction has to last more than 16 hours though according to Figure 1(b).

3.3. Michaelis-Menten Kinetic Parameters. Table 5 shows the enzyme kinetic parameters for the oxidation of ABTS by CLEAs and free laccase. They all follow Michaelis-Menten kinetics according to the correlation factor obtained by the curve-fitting analysis (results not shown).

CLEA-1.0-136 and CLEA-1.0-50.5 have a comparable affinity for ABTS to free laccase according to the Michaelis-Menten constant (K_m). Free laccase has a K_m of 0.082 mM, and CLEA-1.0-136 and CLEA-1.0-50.5 K_m are, respectively, 0.083 and 0.101 mM. The same CLEAs have a higher maximum rate of ABTS transformation than free laccase. CLEA-1.0-136 and CLEA-1.0-50.5 have k_{cat} of 30.760 and 5.295 μmol/s/mg, respectively, while free laccase has a k_{cat} of 2.694 μmol/s/mg. The biocatalytic efficiencies (k_{cat}/K_m) of CLEAs are equivalent to free laccase except for CLEA-1.0-136 that has a k_{cat}/K_m 10 times higher than free laccase.

3.4. Stability against Chemical Denaturants. Figure 2 shows the resistance to chemical degradation of free laccase and

Table 3: Specific activity, thermal stability, and total amount of ABTS oxidized by all the prepared samples.

Sample	Specific activity* (U/g)	Thermal stability*,[a] (%)	Aymard-Belarbi parameters			Half-life (h)	Amount of ABTS oxidized in 24 h under denaturing conditions (mmol/U)
			C	α	β		
CLEA-0.2-200	298 ± 44	7.9 ± 1.2	0,71	0,15	1,78	2.36	0.305
CLEA-0.2-400	65.3 ± 1.9	13.5 ± 0.4	0,12	−0,01	0,19	5.79	0.135
CLEA-0.2-600	42.9 ± 3.5	17.7 ± 1.4	0,38	0,04	0,77	2.79	0.101
CLEA-0.6-200	18.6 ± 3.5	81.5 ± 15.3	0,79	0,006	183166	44.2	0.178
CLEA-0.6-400	103 ± 21	31.4 ± 6.4	0,74	0,025	49,72	0.84	0.094
CLEA-0.6-600	133 ± 29	34.4 ± 7.5	1,04	0,04	7,65	18.7	0.172
CLEA-1.0-200	77.1 ± 0.3	47.6 ± 0.2	19,3	0,34	0,34	20.8	0.159
CLEA-1.0-400	14.7 ± 3.2	68.6 ± 14.9	1,32	0,13	0,125	62.3	0.301
CLEA-1.0-600	24.4 ± 0.2	22.3 ± 0.2	0,76	0,027	41,2	16.5	0.123
CLEA-0.5-1	35.6 ± 4.1	13.6 ± 7.9	0,32	0,03	1,27	1.51	0.379
CLEA-0.5-100	156 ± 6	38.5 ± 4.6	0,76	0,026	56,8	5.84	0.334
CLEA-1.5-1	69 ± 14	82.9 ± 17.3	0,80	0,006	34,8	7.00	0.259
CLEA-1.5-100	39.5 ± 1.9	118.5 ± 1.8	1,58	0,03	0,03	78.48	1.103
CLEA1.0-0.05	21.2 ± 6.6	85.5 ± 28.6	13,4	0,025	0,025	2.77	ND
CLEA-1.0-50.5	78.8 ± 9.9	60.0 ± 4.8	0,51	0,027	15048	7.54	0.565
CLEA-1.0-136	737 ± 24	68.8 ± 2.7	0,465	0,024	2,62	1.29	0.539
CLEA-0.134-50.5	186 ± 10	37.2 ± 3.2	0,413	0,005	0,117	11.9	ND
CLEA-1.867-50.5	16.0 ± 2.9	91.8 ± 2.5	1,58	0,0264	0,0259	81.1	1.094
Free laccase	284 ± 33	21.3 ± 2.5	0,433	0,0189	0,0997	19.7	0.787

* The values represent means of duplicate experiments for the specific activity and thermal stability ± standard deviation.
[a]Residual activity after 24 hours at 40°C and pH 3.
ND: Not determined.

Table 4: Results of the analysis of variance (ANOVA) performed on the specific activities of the CLEA-1.867-50.5.

Source	Sum of squares	Degree of freedom	Mean squares	*F* Value	*P*-value
Model	101.15	8	12.64	5.04	<0.0001
Agitation	5.21	1	5.21	2.07	0.1547
Temperature	27.36	2	13.68	5.45	0.0066
pH	7.09	1	7.09	2.82	0.0978
Reaction time	36.98	2	18.49	7.37	0.0013
Interaction Agitation/Reaction time	24.01	2	12.00	4.78	0.0116
Residues	158.07	63	2.51		
Lack of Fit	86.03	27	3.19	1.59	0.0955
Pure error	72.03	36	2.00		
Total	259.22	71			

Table 5: Michaelis-Menten kinetic constants of laccase CLEAs for the oxidation of ABTS[a].

Sample	K_m (mM)	K_{cat} (μmol/s/mg)	K_{cat}/K_m (L·mg/s)
CLEA-1.0-136	0.083 ± 0,015	30.760 ± 0.900	0.369
CLEA-1.5-100	0.156 ± 0,026	2.524 ± 0.091	0.016
CLEA-1.867-50.5	0.259 ± 0,044	0.986 ± 0.046	0.004
CLEA-1.0-50.5	0.101 ± 0,027	5.295 ± 0.250	0.053
Free laccase	0.082 ± 0,010	2.694 ± 0.060	0.033

[a]Results are mean of triplicate measures ± standard deviation.

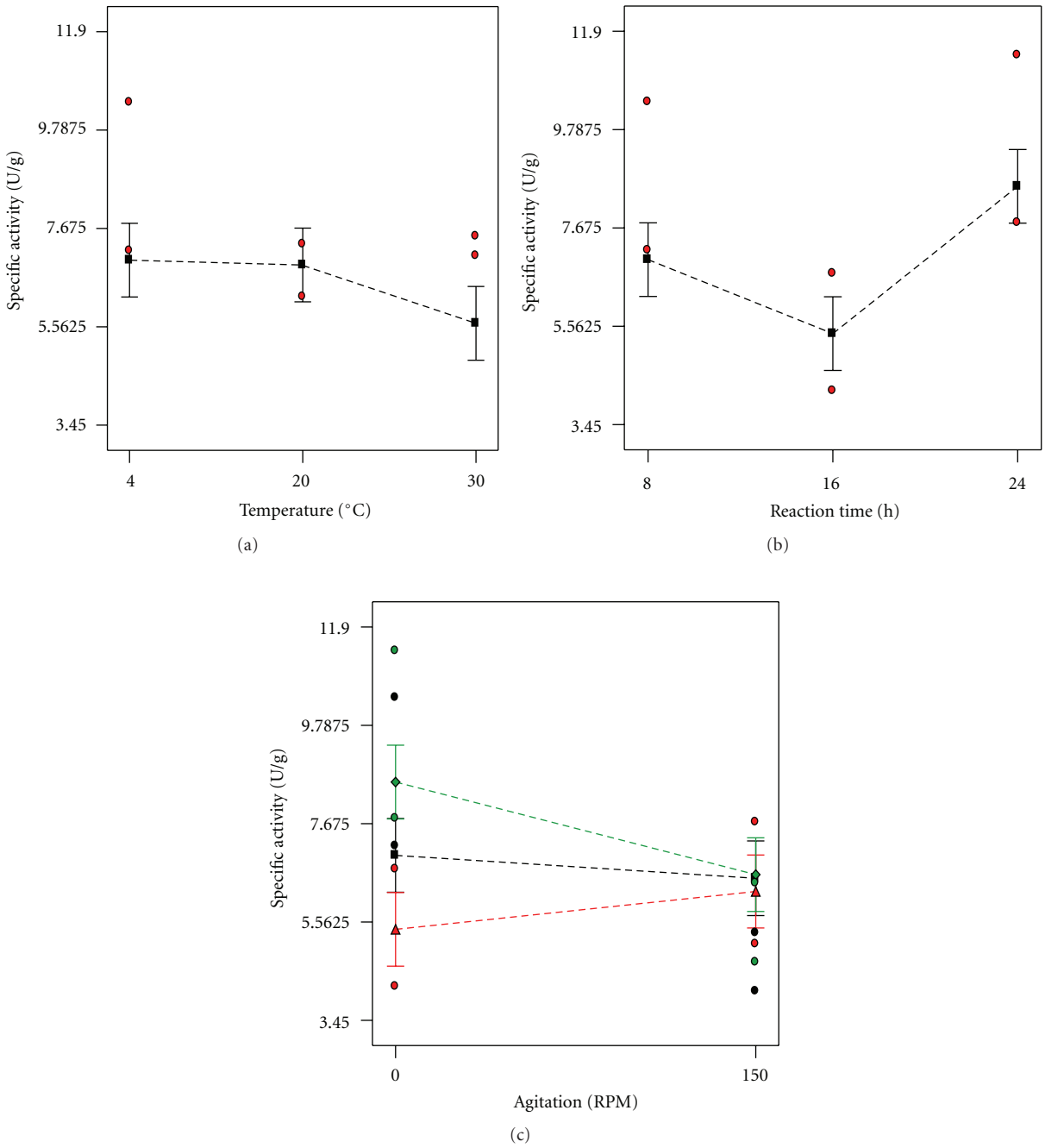


Figure 1: Influence of the temperature (a), the reaction time (b), and the interaction between agitation and reaction time (c) on the specific activity of the CLEA-1.867-50.5. On graph (c): Reaction time of 8 hours (black square), 16 hours (red triangle), and 24 hours (green diamond).

CLEAs to various chemical denaturants. The resistance of CLEAs and free laccase to the chemical denaturants was similar except for the sodium azide and the organic solvents.

When exposed to sodium azide, the residual activity of CLEAs is slightly lower than free laccase but not significantly. For the acetone, all CLEAs had a higher residual activity than free laccase except CLEA-1.867-50.5. And for the methanol, only CLEA-1.5-100 and CLEA-1.867-50.5 lost more apparent activity than free laccase and the other CLEAs.

3.5. Stability against Wastewater Effluent. To confirm the usability of our biocatalysts in a wastewater treatment bioprocess, the stability of CLEAs and free laccase has been determined by exposing the biocatalysts to a sample of WWTP effluent for which the characteristics are shown in Table 6.

The residual activities of free laccase and CLEAs after a 24-hour exposure to this wastewater effluent are shown in Figure 3.

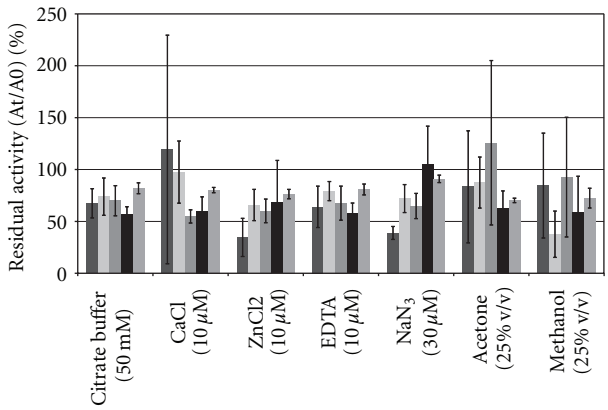


Figure 2: Residual activity of free laccase and CLEAs after 4 hours of incubation with various chemical denaturants at a pH of 3 and 20°C. From left to right: CLEA-1.0-136 (dark gray), CLEA-1.5-100 (light gray), CLEA-1.867-50.5 (gray), CLEA-1.0-50.5 (black), and free laccase (gray). Values represent means of triplicate results ± standard deviation.

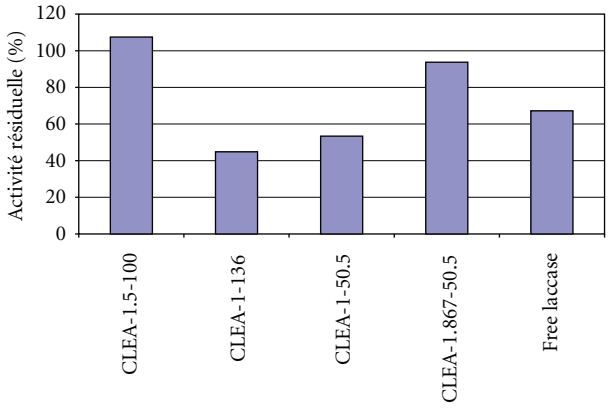


Figure 3: Residual activity of free laccase and CLEAs after 24 hours of incubation in a wastewater effluent collected from the WWTP of Mont St-Grégoire (Qc, Canada).

Free laccase seems to be quite resistant to the wastewater effluent chosen (67.2% residual activity) but CLEA-1.5-100 and CLEA-1.867-50.5 have a residual activity of 107.5% and 93.8%, respectively. The other two samples are less stable to wastewater effluent than free laccase by 14% for CLEA-1.0-50.5 and 22% for CLEA-1.0-136.

3.6. SEMs. Figure 4 shows the SEMs of chitosan (Figure 4(a)) and CLEA-1.0-50.5 (Figure 4(b)). The chitosan is more than 50 μm long according to Figure 4(a) while the laccase-based CLEA is approximately 10 μm long (Figure 4(b)). The laccase aggregates seem to be attached to the chitosan backbone. The structure of the CLEA appears to be amorphous and relaxed rather than compact and uniform.

3.7. Particle Size. The size of the CLEAs produced has been determined by PCS, and the results are presented in Table 7. The CLEA-1.5-100 and CLEA-1.0-136 are approximately 35% smaller than CLEAs-1.867-50.5 and CLEA-1.0-50.5. The standard deviation is between 800 and 1200 nm.

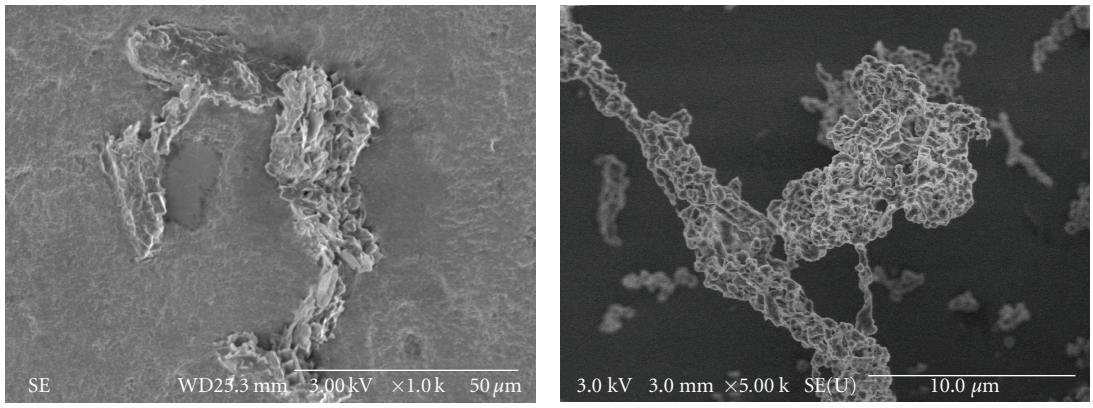
Table 6: Characteristics of the effluent taken at the Mont St-Grégoire WWTP after the settling basin.

Type of contaminant	Concentration
Total phosphorus (mg/L)	1.8
Dissolved COD (mg/L)	36
Particles in suspension (mg/L)*	820
Nitrogen-NH_3 (mg-N/L)	0.61
Nitrites (mg-N-NO_2/L)	2.53
Nitrates (mg-N-NO_3/L)	8.12
Oils and greases (mg/L)	24.1

* Before using this sample, it has been filtrated through a 0.02 μm filter so we assume there are no particles in suspension left.

4. Discussion

It is important to determine good reaction conditions to produce efficient biocatalysts due to the possible denaturation of the enzyme or mass transfer limitations associated with the cross-linking agent [23]. The activator EDAC is known to react with carboxylic acids, like aspartic acid or glutamic acid found in laccase, to form O-acylisourea intermediates. These active intermediates can then react with a nucleophilic species such as a primary amine to form an amide bond [24]. In the present case, the primary amine is found in chitosan but also in the amino acid lysine present in laccase. A high concentration of the activator can reduce CLEAs activity according to the specific activities of CLEAs prepared with 200 mM or more of EDAC. Bindhu and Abraham [25] observed the same phenomenon when immobilizing horseradish peroxidase on chitosan. It is probably caused by the nonspecific activation of carboxyl groups provoking a perturbation in the tridimensional structure of the enzyme. Activity reduction is also observed when the amount of chitosan used is higher than 1.0 g/L. The dilution of laccase concentration by chitosan or a steric hindrance phenomena caused by the addition of the biopolymer can explain the loss of specific activity. The tridimensional structure of the CLEAs could also limit the diffusion of the substrate to the catalytic site therefore reducing the specific activity [26]. The specific activities of chitosan-based CLEAs obtained are lower than the specific activity of free laccase except for CLEA-1.0-136 and CLEA-0.2-200. D'Annibale et al. [26] used chitosan to immobilize laccase with GLU, and the biocatalysts retained 45% of the initial activity (specific activity of immobilized enzyme divided by the specific activity of free enzyme). Zhang et al. [23] produced laccase-chitosan biocatalysts that retained 52.2% of initial activity. The laccase-chitosan CLEAs retained up to 259% of initial specific activity. Considering that chitosan can account for an important mass in the biocatalyst, the retention of activity of 259% can indicate a hyperactivation of laccase when attached to chitosan [27]. Laccase attached to chitosan by Cabana et al. [27] also exhibited a hyperactivation of 265%.



(a) (b)

Figure 4: SEMs of (a) chitosan and (b) CLEA-1.0-50.5.

Table 7: Particle sizes of CLEAs as determined by PCS.

	CLEA-1.5-100	CLEA-1.0-136	CLEA-1.867-50.5	CLEA-1.0-50.5
Average (nm)	1694.3	1716.5	2313.7	2308.6
Standard deviation (nm)	900.9	835.9	1171.6	860.3

An important factor to consider for specific activity optimization is the temperature of the formation of the CLEAs. The CLEAs prepared at 4°C had the highest activity, comparable to those prepared at 20°C. The CLEAs formed at 30°C however are less active. It is probably because the laccase was thermally degraded therefore yielding less active CLEAs. The optimal reaction time seems to be at 24 hours, but longer times have not yet been tested. Higher specific activity could be obtained for longer reaction time. The agitation seems to reduce the influence of reaction time. The CLEAs prepared under agitation at 150 RPM have a comparable specific activity for all the reaction times tested.

The thermal stability of CLEAs was significantly higher than the stability of free laccase. It can be explained by the formation of chemical bonds between chitosan and laccase. Bonds can also be formed between different molecules of laccase and inside the same enzyme. These decrease the mobility of the enzyme, therefore providing a greater resistance to thermal degradation, often caused by drastic conformational change [8, 28]. It can also be explained by the presence of chitosan which may somehow coat the enzyme. CLEAs prepared with GLU by Cabana et al. [5] had a residual activity between 20% and 40% after a 24-hour period under the same denaturing conditions.

The kinetics of thermal degradation of free laccase are accelerated, and these soluble enzymes present almost no activity after 24 hours of incubation under denaturing conditions while the CLEAs stayed active for a longer period of time. The experiment was run over 48 hours, and CLEAs were still active after two days of thermal degradation while laccase lost almost all its activity within the first 24 hours. The same tendency was observed by Cabana et al. [5] but the CLEAs prepared in that previous study were degraded faster than the chitosan-based CLEAs.

To our knowledge, no studies have determined the amount of substrate theoretically oxidized by laccase under thermal degradation. By using this approach, the thermal stability and the initial specific activity are integrated into one parameter describing the global performance of a biocatalyst. The model proposed by Aymard and Belarbi [21] is ideal for it describes well the kinetics of thermal degradation of our CLEAs. This model was integrated to obtain the total amount of substrate a laccase can oxidize when exposed to the denaturing conditions tested. It appears that, for each unit of laccase activity, the CLEAs can oxidize more molecules of ABTS than free laccase after 24 hours. The fast decay of free laccase activity can explain these results. The amount of substrate oxidized by the CLEAs prepared by Cabana et al. [5] with GLU was calculated on a 24-hour period and compared the chitosan-based CLEAs. The CLEAs made without a coprotein oxidized 0.642 mmol/U of ABTS while the CLEAs with 0.01, 0.1, and 1 mg/U of bovine serum albumin oxidized, respectively, 0.738, 0.792, and 0.954 mmol/U of ABTS. CLEA-1.867-50.5 and CLEA-1.5-100 oxidized more substrate (resp., 1.094 and 1.103 mmol/U of ABTS) while CLEA-1.0-136 and CLEA-1.0-50.5 oxidized less substrate than all the CLEAs from Cabana et al. [5].

The kinetic study, based on the Michaelis-Menten constant (K_m), shows that the different CLEAs have almost the same affinity for the substrate (ABTS). However, the CLEAs are better biocatalysts when the turnover number is used for comparison (k_{cat}), and free laccase is better when it is the biocatalytic efficiency that is the base of the comparison (k_{cat}/K_m). In terms of kinetic properties, CLEA-1.867-50.5 is not as good as the others. CLEAs prepared with GLU in Cabana's group proved to have a turnover number 6 times higher than that of free laccase [5] but CLEA-1.0-136 has a k_{cat} 15 times higher than that of the free laccase. This can be

explained by the hyperactivation of laccase when in contact with chitosan [27].

The stability of CLEAs and free laccase has been measured in solutions containing various chemicals known to denature enzymes. These chemicals inhibit or denature laccase in various ways. The chloride salts are known to inhibit laccase activity by raising the ionic strength of the solution [28]. The solvents lower the strength of hydrophobic interactions so the equilibrium is shifted to the denaturated state [28]. Sodium azide binds to the active site of laccase and modifies its structure [29], and EDTA is a chelator that can take out the copper ions present in the catalytic site of the laccase [30]. The amorphous structure of CLEAs reduces the mass transfer of EDTA to the catalytic site of the enzymes [5]. The CLEAs structure does not limit the diffusion of smaller molecules like chloride ions or sodium azide to the catalytic site but their rigidity reduces their denaturation by hydrophobic interactions. Therefore, the stability of CLEAs is not significantly higher than free laccase when exposed to salts or chelators but is slightly increased towards solvents.

Little research has been done to determine the stability of laccase in a real wastewater effluent. Auriol et al. [31, 32] used real wastewater effluents to test the capacity of commercial laccase from the WRF *Trametes versicolor* to transform the endocrine disrupting chemicals estrone, estriol, and estradiol, but the stability of the enzyme was not evaluated. Laccase can be used in wastewater effluents to eliminate phenolic compounds, and it is important to have a good stability in this complex medium. These results show that some CLEAs had a higher stability than free laccase probably because the denaturants present in the effluent cannot get to the catalytic site of the CLEAs due to their amorphous structure. The rigidity of the CLEAs is another factor in the enhancement of stability towards organic solvents. Since free laccase and CLEAs proved to be quite stable in a wastewater effluent, they are good candidate to eliminate phenolic compounds.

According to Schoevaart et al. [33], CLEAs can be separated in two types based on their structure. Type 1 aggregate has a uniform structure and has a diameter around 1 μm. Type 2 aggregates are usually smaller with a diameter around 0.1 μm. According to this classification, chitosan-based CLEAs have a structure similar to that of type 1 CLEAs. The results obtained concerning the particle size determined by PCS of the CLEAs confirm our observations with the SEM. The particles have a diameter of about 1700–2300 nm. An interesting fact is that the CLEAs prepared with more EDAC are smaller. This could be explained by a more important cross-linking level of the CLEAs formed. The small CLEAs have a higher surface/volume ratio that helps diffusion to the catalytic site of laccase and therefore makes them more active than the larger CLEAs (Table 6).

5. Conclusion

The results presented in this study demonstrate a novel method for making CLEAs with laccase by using chitosan as the cross-linking agent. The CLEAs formed are stable, active biocatalysts which are suitable for use in environmental and industrial bioprocesses. The method used should be applicable to many other enzymes although this was not demonstrated in this study. Chitosan used here for the first time as the cross-linking agent used to form CLEAs has considerable advantages from environmental and worker safety points of view over the traditional chemicals traditionally used. Because chitosan is obtained from aquatic organisms byproducts, its use is in good conformity with sustainable development. Its abundance and renewable nature make it an attractive candidate for the production of insolubilized enzyme, while its biocompatibility makes it an environmentally harmless reactant that does not put the health of the workers at risk.

Abbreviations

CLEAs: Cross-linked enzyme aggregates
WRF: White rot fungus
GLU: Glutaraldehyde
EDAC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
ABTS: 2,2_-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)
SEMs: Scanning electron micrographs
PCS: Photon correlation spectroscopy.

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