Laccase immobilization via cross-linked aggregate preparation: Characterization, thermodynamic/kinetic properties and application in removal of bisphenol A from solutions

Adedeji Nelson Ademakinwa

Department of Physical and Chemical Sciences, Elizade University, Ilara-Mokin, Ondo-State.

E-mail: adedeji.ademakinwa@elizadeuniversity.edu.ng

Abstract

Fungal laccase from *Aureobasidium pullulans* was immobilized via cross-linked enzyme aggregate (CLEA) preparation under statistically optimized conditions. The stability of the CLEA to heat inactivation was studied via investigation of its thermodynamic and kinetic parameters. The immobilized enzyme was then deployed in the biodegradation of a bisphenol-A (BPA). The optimum conditions for CLEA preparation resulting in the highest immobilization yield were ammonium sulphate (60% v/v), glutaraldehyde (30 mM), pH (4.5), time (6 h) and temperature (55ºC). The CLEA retained about 51% of its activity after eight catalytic cycles. The optimum pH and temperature of the laccase CLEA were 5.5 and 60ºC respectively. The SEM indicated that the laccase CLEA was type II (unstructured). The data obtained from the heat inactivation kinetics and thermodynamic characterization indicated that the CLEA was stable to heat denaturation than the free enzyme. The kinetic parameters obtained for the CLEA with ABTS as substrate were 101.3 µM, 2.94 µmols-1mg-1 and 0.03 dm3s-1mg-1 for the *Km*, *Kcat* and *Kcat/Km* respectively. The optimum conditions for BPA biodegradation using the CLEA were temperature (55ºC), time (2 h), CLEA (1.0 mg) and BPA concentration (40 mg/L). After the 7th cycle, laccase CLEA retained about 63±2.3% biodegradation efficiency. A heat-resistant laccase CLEA was able to remove BPA from solutions under statistically optimized conditions. The laccase CLEA has properties for other futuristic applications.

Keywords: CLEA, laccase, RSM, biodegradation, Bisphenol A

Introduction

Laccases (multicopper oxidases) are useful enzymes mostly in the industry as they play roles such as in xenobiotic biodegradation, pulp and paper bleaching etc. They can perform these functions due to their low substrate specificity and potentials in the oxidation of a large number of substrates [1]. Substrate oxidation is often accompanied by the release of oxygen to water. Moreso, laccases are often regarded as being environmentally friendly, hence, its application in numerous field is quite understandable [2]. Laccases have been reported to be involved in the removal of pollutants such as endocrine-disrupting chemicals and dyestuff (EDC) [3, 4]. These EDC's are present in wastewater bodies and are known to cause deleterious effects on human and animal health [5].

Major hindrances to the application of laccases (in an aqueous form) industrially especially in xenobiotics biodegradation is the low yields and very high costs, loss of enzyme activity and poor reusability [6]. These problems are often solved via immobilizing the enzymes because the use of immobilized enzymes provides options for cost reduction, enzyme stability and enhanced reusability [7]. Several techniques of laccase immobilization for BPA biodegradation exists with the most prominent one being immobilization on a solid support [6]. This technique comes with usual problems such as low enzyme: solid support. This brings about the technique of deploying cross-linked enzyme aggregates (CLEA) [8, 9]. This technique is devoid of solid support as it involves precipitating the enzyme and then cross-linking it with a cross-linker such as glutaraldehyde. The production of laccase CLEA's has been reported in the literature. Laccase CLEA's are known to be thermostable and resistant to denaturants.

The preparation of laccase CLEA from the fungus, *Aureobasidium pullulans* with a focus on its application in the biodegradation of endocrine-disrupting compounds was the main objective of

this study. There are reports of production of extracellular laccase by this fungus [10, 11]. The utilization of laccase and mycelia from this fungus in the biodegradation of textile dyes has been reported [12]. This study is the first-ever for the immobilization of laccase via CLEA from this fungus. Despite the numerous advantages posed by using CLEA, very few reports exist in literature where laccase CLEA was produced for applications in BPA biodegradation. Only the report of Cabana et al. [3] established the application of CLEA in the removal of BPA using a fluidized bed reactor. Most recent studies on CLEA and its application focused on dye biodegradation [13]. Hence, this study provides needed information on the application of laccase CLEA in the removal of EDC's.

In this study, the use of response surface methodology via central composite rotatable design (CCRD) was utilized in both the preparation of the CLEA and its application in the biodegradation of BPA. During optimization of most bioprocesses, CCRD provides unique advantages such as easy monitoring of the several interactive variable effects [14].

Methods

Fungal growth and enzyme isolation

Aureobasidium pullulans was maintained on malt extract agar and sub-cultured every 4 days at 23±1°C. The fungus was inoculated into a laccase-producing medium containing (in g/L): CuSO4. 5H2O (4) Tyrosine (3.0), glucose (10), ZnSO4 (0.1), FeSO4 (0.05), MnSO4 (0.5), MgSO4 (0.5) yeast extract (5). Growth was monitored for 5 days and the mycelia was harvested after centrifugation at 6000 x g for 10 min. The mycelia (10 g) was homogenized using acid-washed sand (in a sterile and pre-chilled mortar/pestle) in 1:1 ratio [15] Thereafter, three-time volume of 10 mM citrate buffer pH 4.0 buffer (chilled at 4°C) was added to the homogenized mycelia and

it was stirred. All operations were carried out on ice at 4°C. The homogenate was centrifuged at 6000xg for 30 min at 4°C and the resulting supernatant served as the source of enzyme.

Laccase assay and protein concentration determination

Laccases was assayed using 13 mM ABTS as substrate [10]. ABTS oxidation was monitored at 460 nm. One unit of laccase activity was defined as the amount of enzyme that oxidizes one micromole of substrate to form the product ABTS+ in one min. Protein concentration was determined using bovine serum albumin as standard via methods described by Bradford [16].

Preparation of CLEA

Selection of precipitant

The choice of precipitant was determined over a concentration range of 0-70% v/v of each chilled precipitant (acetone, ethanol, ammonium sulphate, PEG-2000, PEG-5000). The precipitants were added to the enzyme solution in a 10 ml centrifuge tube and incubated for 8 h at 4°C. The enzymatic activity of the aggregates were determined with ABTS as substrate.

CLEA synthesis via optimization

In preliminary studies, the CLEA was synthesized by using the methods described by Cabana et al.3 and Ademakinwa et al.[17]. Briefly, 2 ml of 800 mU/mL of laccase was added to 4 ml of ammonium sulphate (50% v/v). After a 16 h incubation, the reaction mixture was centrifuged at 3500 x g for 10 min at 4°C. The pellet was collected and re-dissolved in minute amount of buffer (10 mM citrate-phosphate pH 4.0). Thereafter, 2 ml of glutaraldehyde (25 mM) was added to the re-dissolved pellet and it was allowed to cross-link for 2 h at 4°C. The resulting pellet was collected by centrifugation at 3500 x g for 10 min at 4°C. The pellet was washed in 10 mM citrate-phosphate buffer, pH 4.0 and stored at 4C. The immobilization yield was determined using the equation below

$$Immobilization\ yield(\%) = \frac{Specific\ activity\ of\ immobilized\ enzyme}{Specific\ activity\ of\ free\ enzyme} \quad [1]$$

To optimize CLEA preparation, response surface methodology via central composite design was adopted. Five independent variables (at five unique levels) were considered such as precipitant concentration, precipitation time, glutaraldehyde concentration, cross-linking time and temperature. The experimental design was generated using Design-Expert version 6.0.

The high and low levels of the process variables adopted for the RSM optimization were determined from results of preliminary experiments (not shown). The precipitation concentration 10 and 50% (v/v), precipitation time 4 and 16 h, glutaraldehyde concentration 2 and 30% v/v; cross-linking time 3 and 12 h and temperature 30 and 70°C. The CCRD is shown in Supplementary S1 and it illustrates the five variables studied at the five different levels, the observed and residual activity of the CLEA. The five different levels for the individual experiment were coded thus: -α, -1, 0, 1, +α.

$$\alpha = 2^{N/4}\ where\ N\ is\ the\ number\ of\ variable \quad [2]$$

Data analysis

Equation 1 illustrates the analysis of the experimental data for each independent variable by fitting of the second-order polynomial equation to the experimental data of each dependent variable.

$$X_i = \mu_0 + \sum_{i=1}^{n} \mu_i x_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \mu_{ij} x_j + \sum_{i=1}^{n} \mu_{ii} x_i^2 \quad [3]$$

$X_i\ is\ the\ response$ ($\%Immobilization\ yield$); $x_i\ represents\ the\ independent\ variables$ considered in this study, i.e. x1: precipitant concentration [% v/v], x2: precipitation time [h], x3: glutaraldehyde concentration [% v/v]; x4: cross-linking time [h] and x5: temperature [C]. $\mu_0\ represent\ the\ value\ of\ the\ fitted\ response\ at\ the\ central\ points$. The liner, interactive and quadratic terms are designated by $\mu_i, \mu_{ij}\ and\ \mu_{ii}\ respectively$.

CLEA characterization

Effect of pH and temperature

The effect of pH on the free enzyme and CLEA was determined using several buffers: 10 mM citrate-phosphate buffer pH 3.0-5.0, phosphate buffer 6.0-8.0, Tris-HCl buffer 9.0-11.0. The effect of temperature on CLEA was determined at temperatures ranging from 20 to 90°C with ABTS as substrate.

pH and heat stability studies

CLEA was incubated at optimum pH for 8 h and the residual activity determined every 1 h with ABTS as the substrate. For the heat stability studies, the CLEA was incubated at 40 to 80°C for 6 h and the residual activity was estimated.

Heat inactivation kinetics of the free enzyme and CLEA

The irreversible heat inactivation process is often kinetically described by a first-order reaction, see equation (4)

$v = k_d E$ [2], [4]

where v is defined as the rate of inactivation of the enzyme and E is the concentration of the active form of the enzyme. The k_d is obtained from the slopes of the plot of the activity coefficient, ρ, against time at different temperature range (40-80°C) i.e. $In\ \rho\ vs\ t$. From the Arrhenius plot, $In\ k_d\ vs\ 1/T$, the energy of thermal denaturation, E_d, was estimated from the slopes of the straight lines. Other thermodynamic parameters were estimated from the equations stated below as described by Melikoglu et al. [18].

$\Delta H_d^{\ *} = E_d - RT$ [5]

$\Delta G_d^{\ *} = -RTIn(\frac{k_d h}{k_b T})$ [6]

$\Delta S_d^{\ *} = \frac{\Delta H^* - \Delta G^*}{T}$ [7]

161 ΔH_d^* : *Change in enthalpy*

162 ΔG_d^*: Gibb's free energy

163 ΔS_d^*: Change in entropy

164 R: universal gas constant (= 8.314 J/mol/K)

165 h: Planck's constant (= 6.63 x 10-34)

166 k_b: Boltzmann constant (= 1.38 x 10-23)

167 The half-life of the enzyme, $t_{1/2}$, was determined according to the equation described below.

168 $t_{1/2} = 0.693/k_d$ [8]

169 **Determination of kinetic parameters**

170 The kinetic parameters for the immobilized laccase were determined using ABTS (25-1500 μM)
171 as substrate. The kinetic parameters (Km, Kcat and Vmax) were determined from the Michealis-
172 Menten plot (v against S) using the GraphPad Prism software version 7.0.

173 **SEM and FTIR studies**

174 The surface structure of the CLEA was determined by FTIR spectroscopy in a transmittance
175 mode while the structural features of the CLEA was investigated using SEM. For the SEM
176 studies, the dried CLEA was coated with gold, viewed under 2500 X magnification under 10 kV
177 using scanning electron microscope model TESCAN. For the FTIR studies, the dried CLEA was
178 ground in a mortar containing KBr (in 1:1) and screwed to pellets [17]. Scans were recorded
179 from 4000-400 cm in transmittance mode using a Shimadzu FTIR-8400S spectrophotometer.

180 **Biodegradation efficiency of BPA by CLEA**

181 The potential of the CLEA to biodegrade BPA was investigated under response surface
182 methodology via CCRD (described earlier) only that in this case, the response is %
183 biodegradation efficiency and the independent variables are concentration of the BPA (mg/L),

time of incubation (h), temperature (C) and CLEA amount (mg). Supplementary S2 provides the information on the CCRD experimental design and it illustrates the four variables studied at five different levels. As seen in equation 3,

X_i *is the response* (% *Biodegradation efficiency*); x_i *represents the independent variables* considered in this study, i.e. x1: BPA concentration, [mg/L], x2: time of incubation [h], x3: CLEA amount [mg]; x4: temperature [C]. μ_0 *represent the value of the fitted response at the central points*. The liner, interactive and quadratic terms are designated by μ_i, μ_{ij} *and* μ_{ii} *respectively*.

$$\% \boldsymbol{Biodegradation\ Efficiency} = \left(\mathbf{1} - \frac{[\boldsymbol{BPA}]_t}{[\boldsymbol{BPA}]_0}\right) \boldsymbol{x}\ \mathbf{100} \qquad \textbf{[9]}$$

Where $[BPA]_t$ *and* $[BPA]_o$ represent the BPA concentration at a specified time after treatment with CLEA and the initial BPA concentration respectively.

2.4.1 Model validation

To validate the model, the optimum conditions obtained in the experimental runs were repeated at least three times and the biodegradation efficiency determined.

2.4.2. Reusability of CLEA in BPA biodegradation

The CLEA was used in the biodegradation of BPA at the optimum conditions for several catalytic cycles. The experiment was repeated thrice and the biodegradation efficiency determined.

Result and discussion

Selection of best precipitating agent for laccase prior to cross-linking

It was observed that acetone had the least recovery at all concentrations studied (0-75% v/v). The reduced recovery might be due to the acetone inducing certain changes in structural properties of the laccase, which resulted in a loss of enzymatic activity. Ammonium sulphate had the highest recovery of about 80% at 65% (v/v). The choice of precipitant for laccase is

necessary because of the recovery obtained after precipitation and the change in structural conformation/enzymatic features afterwards [8]. The conclusion that ammonium sulphate was the best precipitating agent prior to crosslinking was also reported by Yang et al. [19].

Statistical optimization of the preparation of laccase CLEA

From Supplementary S1, it was observed that the optimum conditions resulting in the highest yield (73%) for the laccase CLEA were ammonium sulphate (60% v/v), glutaraldehyde (30 mM), pH (4.5), time (6 h) and temperature (55ºC). The second order RSM obtained via ANOVA of the immobilization yield is provided in Table 1. The model F value of 36.31 indicated that the model was significant which implied that there was a 0.01% chance that this model could occur due to noise. The standard deviation, coefficient of variation, adequate precision and coefficient of determination values were 1.27, 1.92, 23.80 and 0.96 respectively. The p-value obtained was less than 0.0001and it indicated that the model was significant. Other significant parameters included from the viewpoint of the p-value were X_1, X_2, X_3, X_4, X_5, $X_1X_2, X_1X_3, X_1X_4, X_1X_5, X_2X_3, X_2X_5 and\ X_4X_5$. The implications of the significance of these variables is that they provide information that these variables affect the immobilization yield. Other parameters were not significant (p>0.05) indicating that those variables had no profound effect on the immobilization yield of the laccase CLEA.

A second-order polynomial equation was obtained that described the link between the variables studied and the response (immobilization yield). The equation is described as:

$$Immobilization\ Yield\ (\%) = 70.91 - 3.96X_1 + 0.57X_2 + 1.29X_3 - 0.83X_4 + 0.46X_5 - 1.57X_1^2 - 0.37X_2^2 - 1.57X_3^2 - 1.39X_4^2 - 0.69X_5^2 + 0.46X_1X_2 - 0.27X_1X_3 - 0.85X_1X_4 + 0.38X_1X_5 + 0.26X_2X_3 - 0.52X_2X_4 - 0.060X_2X_5 - 0.31X_3X_4 + 0.39\ X_3X_5 + 0.24X_4X_5$$

The variables were coded such that, x1: precipitant concentration [% v/v], x2: precipitation time [h], x3: glutaraldehyde concentration [% v/v]; x4: cross-linking time [h] and x5: temperature [ºC]. To critically understand the interactions between the variables considered in this study and how it influences the response (immobilization yield), response surface plots (Supplementary S3)

were obtained for two variables while the others are kept at a constant (central levels). For example, the effects of pH and time (while precipitant and glutaraldehyde concentration and temperature were kept constant at their central levels) on the CLEA immobilization yield is provided in Fig. 1. It was observed that the immobilization yield gradually increased as both the time and pH values were increased until the optimum was reached. Further increased above the optimum values led to decreases in the response.

Model Validation

The immobilization yield obtained after repeating the experiments under the optimum conditions predicted by the model was 75.3 ± 2.8% which was very close to that predicted by the model. The immobilization yield obtained for laccase CLEA was higher than the values obtained for laccase CLEA from *Cerrena sp. [*19] and *Coriolopsis polyzona [*3] which had values of 68.1% and 60.2% respectively.

Reusability of CLEA

Supplementary S4 shows the potential of the laccase CLEA to catalyze ABTS oxidation over several cycles of catalysis. After eight reaction cycles, the resulting immobilization yield was 51%. A gradual decrease in the rate of catalysis was observed after the fifth cycle and this might be due to substrate mass-transfer limitations that result in a decreased rate of ABTS oxidation by the CLEA.

Laccase CLEA characterization

Effect of pH and temperature

The optimum pH obtained for the free laccase and CLEA were 4.5 and 5.5 respectively (Fig.2). The optimum pH of most fungal laccases falls within the acidic region [20]. The pH of the CLEA increased by a unit when compared with the free enzyme. This increase could be due to the

changes in the microenvironment of the immobilized enzyme. This observation was in agreement with the reports of several authors [21, 22]. The optimum temperature for the free laccase and CLEA were 40 and 60ºC respectively (Fig. 3). Most immobilized laccases have an optimum temperature that falls within 40 and 60ºC [20].

pH and temperature stability

The pH stability is shown in Supplementary S5. It was noted that after 4 h incubation the free laccase retained about 100% of its initial activity at pH 4.5. This decreased after 5 h incubation with a loss of about 5%. Upon the increase of the incubation time to 8 h, the free laccase retained about 85% of its residual activity. The results obtained in this study was in agreement with what Lassaoune et al. [21] obtained for free laccase where the enzyme retained about 85.2% after incubation. Meanwhile, the CLEA retained more than 95% of its initial enzymatic activity after 8 h incubation at pH 5.5

The thermostability of the free laccase is shown in Supplementary S6. It was observed that after 8 h incubation at 40ºC, the free laccase retained about 80% of its initial activity. It was observed that at very high temperatures (60-80ºC) more than 80% of the initial activity was lost. Supplementary S8 illustrates the thermostability of the laccase CLEA. It was noted that after 8 h incubation, at 40-50ºC, the laccase CLEA retained more than 90% of its initial activity. Increase of the temperature from 50-60ºC still showed that the immobilized enzyme retained more than 75% of its initial activity after 8 h incubation. A drastic drop to about 55% of its initial activity was observed after the incubating temperature was increased to 80ºC after 8 h incubation. It was concluded that the laccase CLEA was more thermostable than the free enzyme. The thermostability of CLEA or other immobilized enzymes over their free enzyme has been reported in recent literature [17, 21, 23-25]

Heat inactivation studies

Table 2 shows the summary of the kinetic and thermodynamic parameters for both the free and immobilized laccase. Both enzyme form's enzymatic activity decayed in a typical first-order heat inactivation pattern. This inactivation pattern is similar to other enzymes produced by this fungus [15, 23-25].

The values of the first-order thermal denaturation constant, k_d, for the CLEA (0.0113-0.0791 h-1) and the free laccase (0.0317-0.1995 h-1) is indicated in Table 2. These k_d values were initially obtained from the plot of $In\left(\frac{E}{Eo}\right) vs\ time$ (see supplementary S7 and S9) and they increased as the temperature was gradually raised. At any temperature range considered, the k_d values obtained for the CLEA lower than the free enzyme. This also implies that the immobilized enzyme displayed a greater degree of thermostability at each temperature increase than the free enzyme. Several authors have reported that immobilized forms of the enzyme tend to be more thermally stable than the free enzyme [23, 24].

From Table 2, the half-life ($t_{1/2}$)values for both free laccase and laccase CLEA decreased progressively while the heat inactivation constant increased as the temperature was gradually increased from 40 to 80°C. A higher $t_{1/2}$value is required if the enzyme is to applied industrially [26]. The laccase CLEA prepared in this study had very high $t_{1/2}$ even at higher temperatures and hence could be useful in numerous industrial applications.

The values energy of thermal inactivation, E_d, for both enzyme forms were obtained from the plot indicated in Fig. 4. E_d values obtained for both free and immobilized laccase were 39.9 ($R^2 = 0.86$) and 42.4 ($R^2 = 0.96$) kJ/mol/K for free laccase and CLEA respectively. It was observed that the E_d values for the CLEA was higher than the free enzyme. This further attests to the fact

301 that the free enzyme was relatively less stable to thermal inactivation compared to the laccase
302 CLEA.
303 The E_d is directly related to the enthalpy of thermal inactivation, ΔH_d. The ΔH_d values provide
304 information on the overall energies necessary to cause heat inactivation of proteins. Therefore,
305 it can be suggested that a high value of ΔH_d *and* E_d indicates increased resistance to heat [27].
306 The laccase CLEA had higher values of ΔH_d compared to the free enzyme (Table 2).
307 The degree of heat-mediated inactivation of enzymes is quite dependent on the entropy of
308 thermal inactivation, $\Delta S_{d.}$ ΔS_d values indicate the energies required for the transition from a
309 native (N) to a denatured state (U) (N↔ $I \leftrightarrow U$). Every thermal inactivation process leads to
310 changes in the enzyme conformation leading to increased orderliness and randomness (or
311 positive values for ΔS_d) [28]. In this study, the negative values obtained for ΔS_d for both free
312 laccase (-162.3 to -155.0 J/mol/K) and CLEA (-155.6 to -155.5 J/mol/K) is suggestive of even more
313 increased resistance to disorderliness. This means that both enzyme forms were resistant to
314 heat inactivation. A comparative look into the ΔS_d values obtained for both free and laccase
315 CLEA, it can be concluded that the process of CLEA preparation (precipitating with ammonium
316 sulphate and crosslinking with glutaraldehyde) allowed for more orderliness which
317 concomitantly increased its thermal stability. This conclusion was also reached for protease [28],
318 rhodanese [24], cellulase [23].
319 The values for Gibb's free energy (ΔG_d)obtained for the free laccase (88.1 to 91.7 kJ/mol) and
320 laccase CLEA (88.5 to 94.4 kJ/mol) is provided in Table 3. The ΔG_d values obtained for both the
321 free and laccase CLEA were quite very close. This is indicative of thermostability.

When all thermodynamic and kinetic parameters were taken into consideration, it was confidently asserted that the CLEA was very stable to thermal denaturation even at very high temperatures (50-60ºC) compared to the free laccase.

3.4.4 Kinetic Parameters for laccase CLEA

The K_M for free laccase (106.2 μM) and CLEA (101.3 μM) was obtained from the Michaelis-Menten plot ($v\ against\ S$) with ABTS as substrate. These values were about the same. This observation was quite similar to the report of Cabana et al. [3] for free laccase and laccase CLEA. The K_{cat} obtained in this study for the free laccase and the laccase CLEA were 1.94 and 2.94 (μmol/s/mg) respectively. The rate of catalysis by the CLEA was approximately twice that of the free enzyme as observed from the K_{cat} values. The values obtained in this study is quite lower than what was obtained for laccase CLEA from *C. polyzona [*3] (which was six times higher than the free enzyme). Another kinetic parameter of great importance is the catalytic efficiency, ${K_{cat}}/{K_M}$. From the ${K_{cat}}/{K_M}$ values obtained for the free (0.04 $dm^3 s^{-1} mg^{-1}$) and immobilized laccase (0.06 $dm^3 s^{-1} mg^{-1}$), it can be concluded that the catalytic efficiency in the CLEA is approximately twice that of the free enzyme. This suggests the laccase CLEA had a better overall catalytic potential than the free enzyme.

3.4.5 SEM and FTIR studies on CLEA

The FTIR spectra of the laccase CLEA is shown in supplementary S10. Several characteristic peaks were noted such as the peaks at 1647 cm-1 which is indicative of the amide I (carbonyl stretching C=O). Another peak that represents the bending and stretching of both C-N and N-H [17]. The SEM of the CLEA is indicated in Fig. 5. Typically, CLEA structure from SEM falls into two categories, the ball type (type 1) and unstructured (type 2) [29]. From the SEM, it was evident that the laccase CLEA falls into type 2.

3.5 RSM-modelled biodegradation of BPA using laccase CLEA

The optimum conditions predicted by the mathematical model were BPA (40 mg/L), time (2 h), temperature (55°C) and CLEA amount (1.0 mg) (Supplementary S2). The optimum conditions resulted in a biodegradation efficiency of 99.9%. Asagdol et al. [30] reported the removal of BPA with an efficiency of 88.3% by the free laccase which was lower than that obtained for the immobilized enzyme used in this study or reported elsewhere [21]. The results obtained in this study was quite similar to the findings of Lassaoune et al. [21] where it was reported that laccase immobilized by cross-linking prior to entrapment in alginate had a biodegradation efficiency of 99%.

From Table 3, the R2, standard deviation and, CV were 0.97, 5.91 and 11.39 respectively. The model-F value obtained in this study was 34.56 and the model was deemed significant ($p<0.0001$). A model-F value that is as large as the one obtained in this study has a 0.01% chance it occurred due to noise. Significant model terms (based on the values of "Prob>F" less than 0.05) where A, C, D, A2, B2, D2 and AD. The final equation in terms of the coded factor was:

$$\%\text{Biodegradation Efficiency} = +65.68 - 21.92A - 1.77B + 8.50C - 5.87D - 3.63A^2 - 4.01B^2 - 1.25C^2 - 8.41D^2 - 1.31AB - 2.47AC + 3.74AD + 1.48BC + 0.51BD - 1.37CD$$

The response surface plots are shown in supplementary S11. S11 (a) examines the effects of time and temperature on the BE. It was observed that increasing the temperature increased the BE by the CLEA. Meanwhile, when the optimum time was reached for maximum BE by the CLEA, a gradual decrease in the BE was noted. S11 (b) illustrates the effects of time and BPA concentration on the BE of the CLEA. It was noted that a steady decline in the BE was observed as the BPA concentration was increased above the optimum value. It can be deduced that

perhaps, the CLEA enzymatic activity had been inhibited either by the substrate (BPA) or the products formed.

3.5.1 Model validation of BPA biodegradation by laccase CLEA

The optimum conditions predicted by the model were validated under the following experimental conditions: temperature (55°C), time (2 h), CLEA (1.0 mg) and BPA concentration (40 mg/L). The biodegradation efficiency obtained after the experiment was conducted thrice was 98.4±1.2.

3.6. Reusability of CLEA in BPA biodegradation

Laccase CLEA under the optimum conditions removed BPA from solutions while retaining about 63±2.3% biodegradation efficiency after the 7th cycle (Supplementary S12). The gradual loss of enzymatic activity by the CLEA might be associated with mass-transfer limitations

4.0 Conclusions

Response surface methodology provided a unique mathematical/statistical model for both the preparation of laccase CLEA and its application in the removal of BPA. From this study, the optimum conditions for CLEA preparation resulting in the highest immobilization yield were ammonium sulphate (60% v/v), glutaraldehyde (30 mM), pH (4.5), time (6 h) and temperature (55°C) while the optimum conditions for BPA biodegradation using the CLEA were temperature (55°C), time (2 h), CLEA (1.0 mg) and BPA concentration (40 mg/L). The thermodynamic and kinetic properties of the CLEA indicated the biocatalyst was thermally stable and resistant to heat inactivation. This property is necessary for biocatalysts of industrial importance. The laccase CLEA retained about 63±2.3% of BPA biodegradation efficiency after seven rounds of catalysis. The CLEA could be deployed in possible large-scale BPA removal and other futuristic biotechnological applications.

Ethical Approval: Ethics approval not required for this study.

-Consent to Participate: Not applicable

-Consent to Publish: Not applicable

-Authors Contributions: Ademakinwa Adedeji Nelson carried out the Investigation, Writing, Conceptualization, Methodology, Software, Data curation, Writing- Original draft preparation.

-Funding: The author has no relevant financial or non-financial interests to disclose.

-Competing Interests: The author declares that there are no conflicts of interests

-Availability of data and materials: Yes

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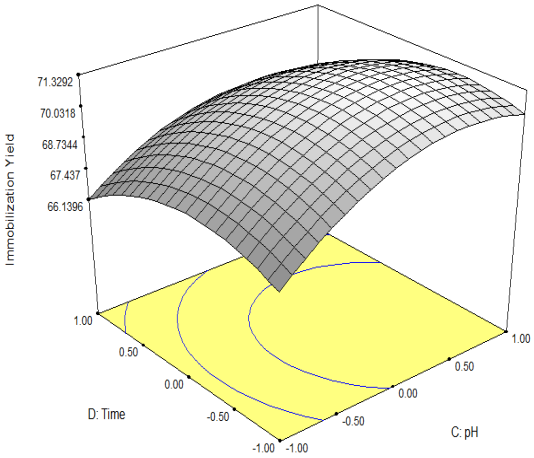


Fig.1. Response surface plots for the preparation of CLEA. Effect of time and pH (A). The Figures indicating the effects of glutaraldehyde and precipitant (B) precipitant and pH (C) temperature and time (D) and temperature and pH on the immobilization yield of the laccase CLEA are shown in the supplementary section.

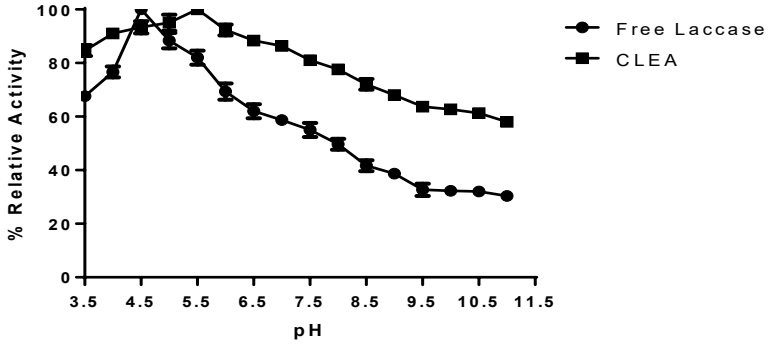
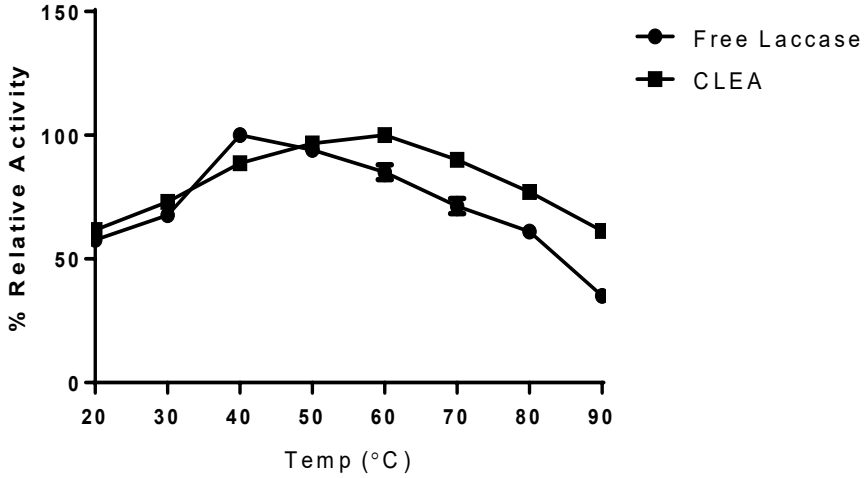


Fig. 2. Effect of pH on free an immobilized laccase



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555 **Fig. 3.** Effect of temperature on free and immobilized laccase

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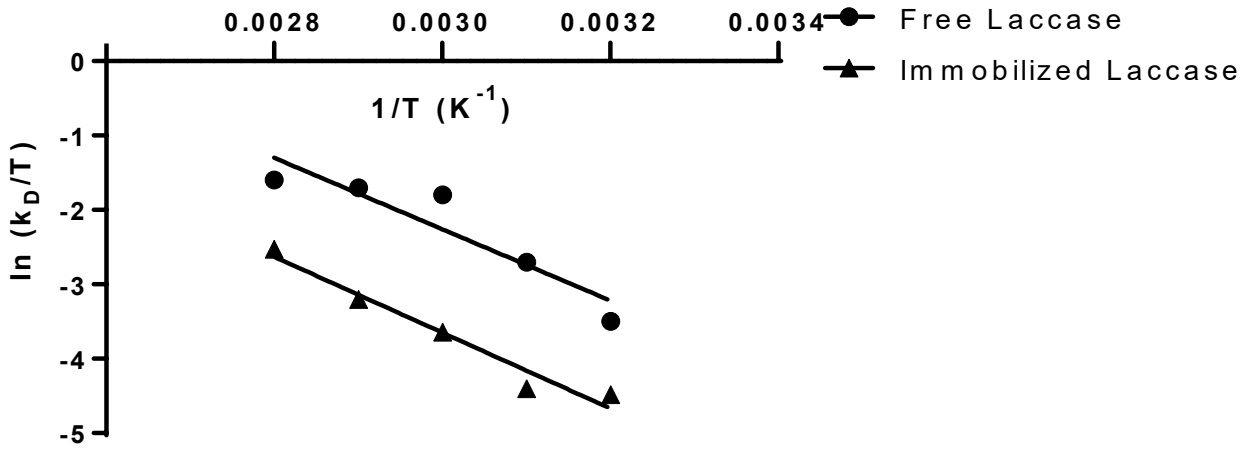

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559 **Fig. 4**. Arrhenius-type plot to estimate the activation energy of thermal denaturation (E_d^*) for free and
560 immobilized laccase

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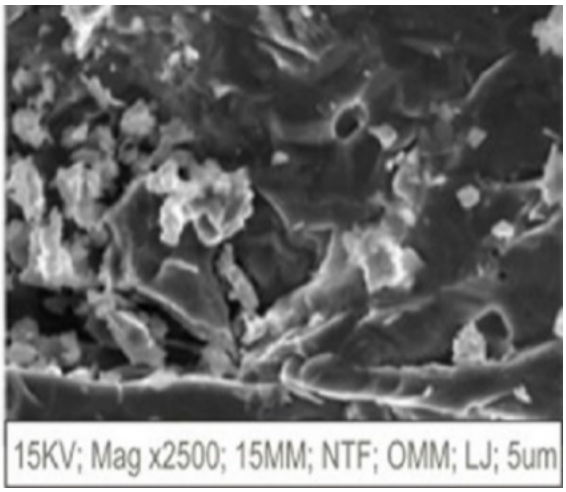

Fig. 5. Scanning electron microscopy of the laccase CLEA

Table 1: Regression analysis and analysis of variance (ANOVA) table for the immobilization yield (%) of laccase CLEA preparation

Source	Sum of squares	df	Mean square	F-value	p-value prob > F	
Model	1168.20	20	58.41	36.31	<0.0001	Significant
X_1	680.29	1	680.29	422.85	<0.0001	Significant
X_2	14.02	1	14.02	8.71	0.0062	Significant
X_3	71.66	1	71.66	44.54	0.0002	Significant
X_4	29.74	1	29.74	18.48	0.0024	Significant
X_5	9.36	1	9.36	5.82	<0.0001	Significant
X_1X_2	136.11	1	136.11	84.60	0.043	Significant
X_1X_3	7.42	1	7.42	4.61	<0.0001	Significant
X_1X_4	137.32	1	137.32	85.36	<0.0001	Significant
X_1X_5	107.19	1	107.19	66.63	0.0003	Significant
X_2X_3	26.79	1	26.79	16.65	0.004	Significant
X_2X_4	6.83	1	6.83	4.25	0.2499	Not Significant
X_2X_5	2.27	1	2.27	1.41	0.0007	Significant
X_3X_4	23.15	1	23.15	14.39	0.1036	Not Significant
X_3X_5	4.54	1	4.54	2.82	0.2527	Not Significant
X_4X_5	2.19	1	2.19	1.36	0.0276	Significant
X_1^2	8.66	1	8.66	5.38	0.7915	Not Significant
X_2^2	0.11	1	0.11	0.071	0.1775	Not Significant
X_3^2	3.07	1	3.07	1.91	0.0930	Not Significant
X_4^2	4.91	1	4.91	3.05	0.2859	Not Significant
X_5^2	1.90	1	1.90	1.18		
Residual	46.66	29	1.61			
Lack of fit	46.66	22	2.12			
Pure error	0.00	7	0.00			
Cor Total	1214.86	49				

$Std.\,Dev = 1.27;\ R^2 = 0.96; Adj\ R^2 = 0.94; C.V. = 1.92; Adeq\ Precision = 23.79$

599 Table 2. Summary of the kinetic and thermodynamic parameters for free and immobilized laccase

T (K)	k_d (h^{-1})	$t_{1/2}$ (h)	E_d (kJ/mol/K)	ΔH_d^* (kJ/mol)	ΔG_d^* (kJ/mol)	ΔS_d^* J/mol/K
40	#0.0317 $R^2 = 0.96$	#21.8	#39.9 $R^2 = 0.86$	#37.3	#88.1	#-162.3
	*0.0113 $R^2 = 0.94$	*61.1		*39.8	*88.5	*-155.6
50	#0.0645 $R^2 = 0.98$	#10.8	*42.4 $R^2 = 0.96$	#37.2	#88.6	#-159.1
	*0.0128 $R^2 = 0.95$	*54.1		*39.7	*91.0	*-158.8
60	#0.1718 $R^2 = 0.98$	#4.0		#37.1	#86.7	#-149.0
	*0.026 $R^2 = 0.94$	*26.3		*39.6	*91.9	*-157.1
70	#0.1822 $R^2 = 0.99$	#3.8		#37.0	#89.3	#-152.5
	*0.0401 $R^2 = 0.94$	*17.3		*39.6	*93.6	*-157.4
80	#0.1955 $R^2 = 0.89$	#3.5		#37.0	#91.7	#-155.0
	*0.0791 $R^2 = 0.97$	*8.8		*39.5	*94.4	*-155.5

600 #- Free enzyme

601 *- CLEA

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611 Table 3: Regression analysis and analysis of variance (ANOVA) table for the immobilization yield (%) of
612 laccase CLEA preparation

Source	Sum of squares	df	Mean square	F-value	p-value	

					prob > F	
Model	16877.51	14	1205.54	34.56	< 0.0001	Significant
A-BPA	11530.10	1	11530.10	330.56	< 0.0001	Significant
B-Time	75.57	1	75.57	2.17	0.1617	
C-Temp	1735.02	1	1735.02	49.74	< 0.0001	Significant
D-CLEA	826.26	1	826.26	23.69	0.0002	Significant
A2	360.85	1	360.85	10.35	0.0058	Significant
B2	439.98	1	439.98	12.61	0.0029	Significant
C2	42.93	1	42.93	1.23	0.2847	
D2	1940.95	1	1940.95	55.65	< 0.0001	Significant
AB	27.28	1	27.28	0.78	0.3905	
AC	97.71	1	97.71	2.80	0.1149	
AD	224.37	1	224.37	6.43	0.0228	
BC	35.18	1	35.18	1.01	0.3312	
BD	4.17	1	4.17	0.12	0.7342	
CD	30.24	1	30.24	0.87	0.3665	
Residual	523.20	15	34.88			
Lack of fit	523.20	10	52.32			
Pure error	0.000	5				
Cor Total	17400.71	29				

$Std.Dev = 5.91;\ R^2 = 0.97; Adj\ R^2 = 0.94; C.V. = 11.39; Adeq\ Precision = 20.99$

S1 Central composite rotatable experimental design variables for the optimization of immobilization yield of laccase CLEA preparation

	Precipitant Conc. (v/v) (x_1)	Glutaraldehyde (mM) (x_2)	pH (x_3)	Time (h) (x_4)	Temperature ℃ (x_5)	Immobilization Yield (%)	
						Experimental	Observed
1	-1(50)	-1(30)	-1(3.5)	-1(4)	-1(30)	67.87	67.52
2	1(65)	-1(30)	-1(3.5)	-1(4)	-1(30)	60.72	60.15
3	-1(50)	1(70)	-1(3.5)	-1(4)	-1(30)	68.58	68.37
4	1(65)	1(70)	-1(3.5)	-1(4)	-1(30)	62.15	62.85
5	-1(50)	-1(30)	1(5.5)	-1(4)	-1(30)	54.23	57.34
6	1(65)	-1(30)	1(5.5)	-1(4)	-1(30)	62.15	61.50
7	-1(50)	1(70)	1(5.5)	-1(4)	-1(30)	70.73	71.84
8	1(65)	1(70)	1(5.5)	-1(4)	-1(30)	67.87	65.25
9	-1(50)	-1(30)	-1(3.5)	1(8)	-1(30)	67.87	68.54
10	1(65)	-1(30)	-1(3.5)	1(8)	-1(30)	59.30	57.96
11	-1(50)	1(70)	-1(3.5)	1(8)	-1(30)	68.30	67.51
12	1(65)	1(70)	-1(3.5)	1(8)	-1(30)	59.30	58.58
13	-1(50)	-1(30)	1(5.5)	1(8)	-1(30)	70.94	69.92
14	1(65)	-1(30)	1(5.5)	1(8)	-1(30)	57.15	58.08
15	-1(50)	1(70)	1(5.5)	1(8)	-1(30)	70.94	69.73
16	1(65)	1(70)	1(5.5)	1(8)	-1(30)	57.15	59.74
17	-1(50)	-1(30)	-1(3.5)	-1(4)	1(55)	67.87	66.55
18	1(65)	-1(30)	-1(3.5)	-1(4)	1(55)	60.72	60.68
19	-1(50)	1(70)	-1(3.5)	-1(4)	1(55)	68.58	67.16
20	1(65)	1(70)	-1(3.5)	-1(4)	1(55)	62.15	63.14
21	-1(50)	-1(30)	1(5.5)	-1(4)	1(55)	71.01	70.53
22	1(65)	-1(30)	1(5.5)	-1(4)	1(55)	62.15	63.30
23	-1(50)	1(70)	1(5.5)	-1(4)	1(55)	70.95	72.19

24	1(65)	1(70)	1(5.5)	-1(4)	1(55)	67.87	67.11
25	-1(50)	-1(30)	-1(3.5)	1(8)	1(55)	67.94	68.74
26	1(65)	-1(30)	-1(3.5)	1(8)	1(55)	59.30	59.47
27	-1(50)	1(70)	-1(3.5)	1(8)	1(55)	66.08	67.27
28	1(65)	1(70)	-1(3.5)	1(8)	1(55)	59.30	59.45
29	-1(50)	-1(30)	1(5.5)	1(8)	1(55)	70.95	71.48
30	1(65)	-1(30)	1(5.5)	1(8)	1(55)	62.15	61.15
31	-1(50)	1(70)	1(5.5)	1(8)	1(55)	70.95	71.06
32	1(65)	1(70)	1(5.5)	1(8)	1(55)	62.30	62.58
33	-2.38(40)	0(30)	0(4.5)	0(6)	0(45)	70.88	71.48
34	2.38(70)	0(30)	0(4.5)	0(6)	0(45)	52.87	52.63
35	0(60)	-2.38	0(4.5)	0(6)	0(45)	66.44	67.49
36	0(60)	2.38	0(4.5)	0(6)	0(45)	70.88	70.19
37	0(60)	0(30)	-2.38(3.0)	0(6)	0(45)	58.58	58.95
38	0(60)	0(30)	2.38(7.0)	0(6)	0(45)	65.08	65.07
39	0(60)	0(30)	0(4.5)	-2.38(2)	0(45)	63.58	65.02
40	0(60)	0(30)	0(4.5)	2.38(10)	0(45)	62.15	61.08
41	0(60)	0(30)	0(4.5)	0(6)	-2.38(20)	64.30	65.87
42	0(60)	0(30)	0(4.5)	0(6)	2.38(70)	69.30	68.08
43	0(60)	0(30)	0(4.5)	0(6)	0(45)	73.21	72.89
44	0(60)	0(30)	0(4.5)	0(6)	0(45)	72.98	73.12
45	0(60)	0(30)	0(4.5)	0(6)	0(45)	73.66	72.99
46	0(60)	0(30)	0(4.5)	0(6)	0(45)	74.21	73.87
47	0(60)	0(30)	0(4.5)	0(6)	0(45)	73.00	72.67
48	0(60)	0(30)	0(4.5)	0(6)	0(45)	72.86	73.10
49	0(60)	0(30)	0(4.5)	0(6)	0(45)	72.87	73.03
50	0(60)	0(30)	0(4.5)	0(6)	0(45)	73.00	72.65

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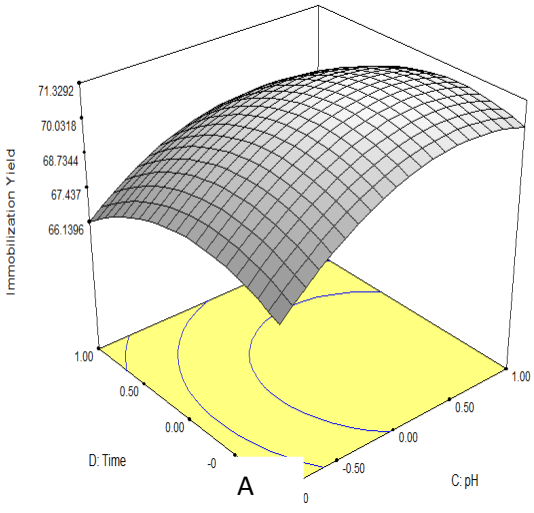
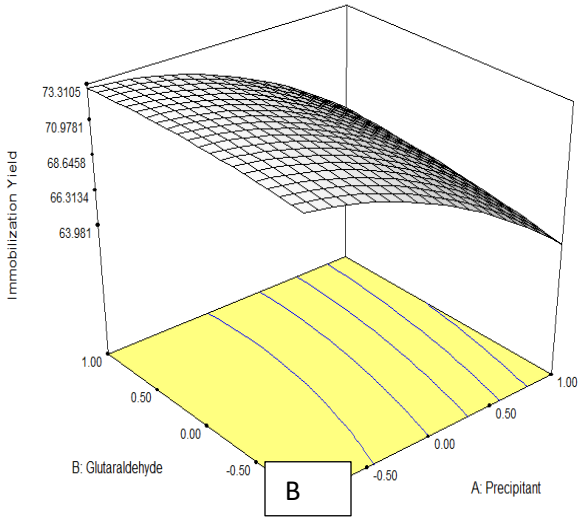
634 S2: Central composite rotatable experimental design variables for the optimization of biodegradation of
635 BPA by laccase CLEA

	BPA (mg/L) (x_1)	Time (h) (x_2)	Temp (C) (x_3)	CLEA (mg) (x_4)	Biodegradation (%)	
					Experimental	Observed
1	1(40)	-1(2)	1(50)	-1(1.0)	73.8	70.0
2	-2(25)	0(3)	0(40)	0(1.5)	31.5	26.2
3	1(40)	1(4)	1(50)	1(2.0)	64.4	65.1
4	0(35)	2(5)	-1(30)	0(1.5)	18.1	16.1
5	-1(30)	1(4)	0(40)	-1(1.0)	87.0	91.7
6	1(40)	-1(2)	-1(30)	1(2.0)	35.7	38.1
7	1(40)	-1(2)	-1(30)	-1(1.0)	99.9	92.7
8	-1(30)	-1(2)	-1(30)	-1(1.0)	26.5	33.8
9	2(45)	0(3)	1(50)	0(1.5)	56.9	52.5
10	0(35)	0(3)	0(40)	0(1.5)	20.8	23.7
11	0(35)	0(3)	0(40)	0(1.5)	56.2	49.5
12	0(35)	0(3)	0(40)	0(1.5)	17.5	15.3
13	0(35)	0(3)	0(40)	-2(0.5)	70.9	68.6
14	1(40)	-1(2)	0(40)	1(2.0)	27.8	30.8
15	0(35)	-2(1)	1(50)	0(1.5)	63.6	71.1
16	-1(30)	-1(2)	0(40)	1(2.0)	28.4	27.1
17	0(35)	0(3)	1(50)	2(2.5)	90.3	95.0
18	0(35)	0(3)	0(40)	0(1.5)	10.5	7.3
19	0(35)	0(3)	2(60)	0(1.5)	52.1	53.0
20	1(40)	1(4)	0(40)	-1(1.0)	45.7	46.1
21	-1(30)	-1(2)	1(50)	1(2.0)	34.0	43.7
22	1(40)	1(4)	-1(30)	1(2.0)	85.8	77.8
23	-1(30)	1(4)	1(50)	1(2.0)	42.8	43.6

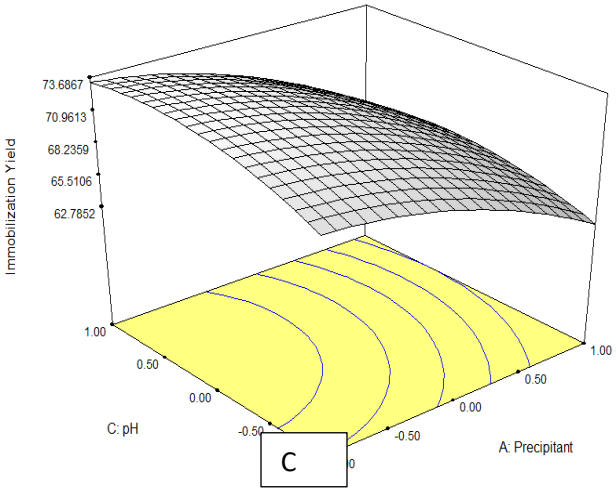
24	1(40)	1(4)	-1(30)	-1(1.0)	19.8	20.9
25	-1(30)	1(4)	1(50)	-1(1.0)	65.6	65.8
26	0(35)	0(3)	0(40)	0(1.5)	65.6	65.8
27	-1(30)	1(4)	1(50)	1(2.0)	65.6	65.8
28	0(35)	0(3)	-2(20)	0(1.5)	65.6	65.8
29	0(35)	0(3)	0(40)	0(1.5)	65.6	65.6
30	-1(30)	-1(2)	-1(30)	-1(1.0)	65.6	65.6


Immobilization Yield
71.3292
70.0318
68.7344
67.437
66.1396
D: Time
C: pH
1.00
0.50
0.00
-0.50
-1.00

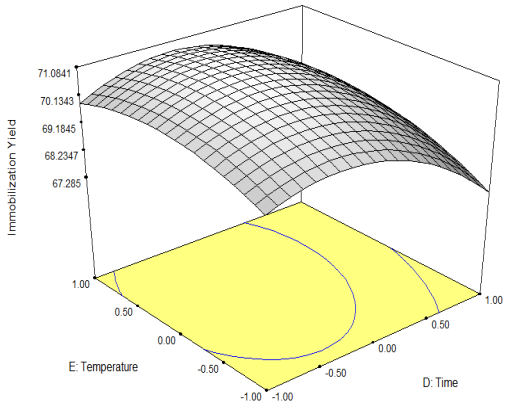
A


Immobilization Yield
73.3105
70.9781
68.6458
66.3134
63.981
B: Glutaraldehyde
A: Precipitant
1.00
0.50
0.00
-0.50

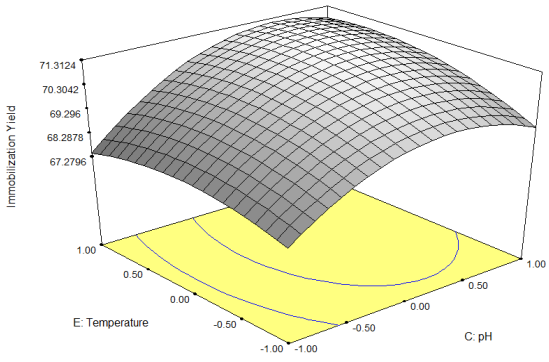
B


Immobilization Yield
73.6867
70.9613
68.2359
65.5106
62.7852
C: pH
A: Precipitant
1.00
0.50
0.00
-0.50

C

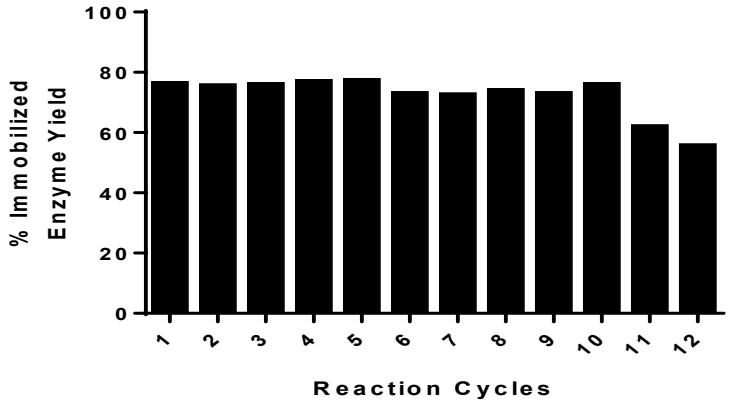

Immobilization Yield
71.0841
70.1343
69.1845
68.2347
67.285
E: Temperature
D: Time
1.00
0.50
0.00
-0.50
-1.00

D

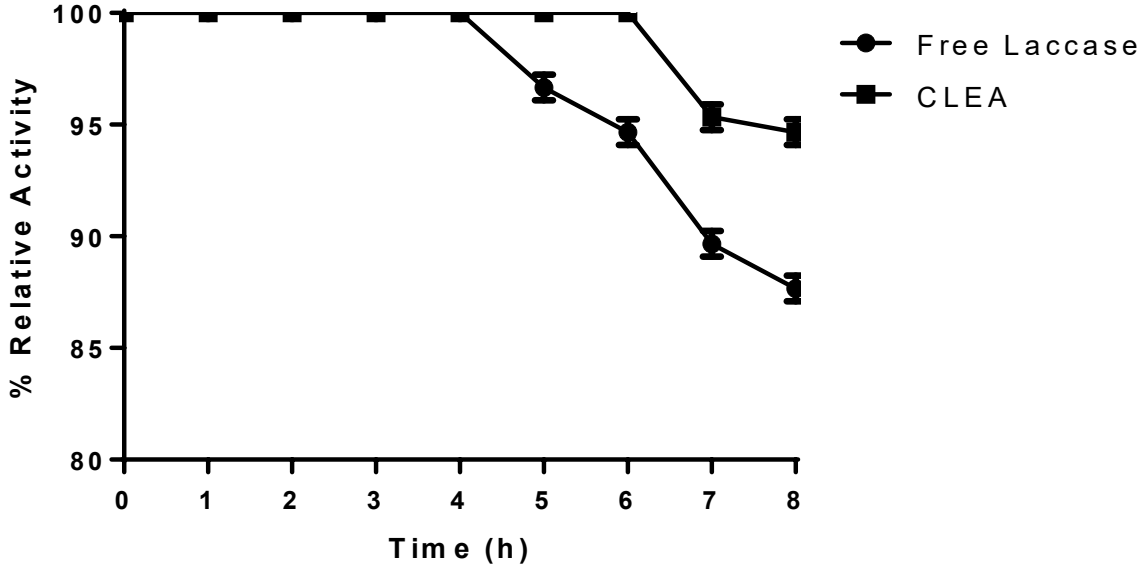

Immobilization Yield
71.3124
70.3042
69.296
68.2878
67.2796
E: Temperature
C: pH
1.00
0.50
0.00
-0.50
-1.00

E

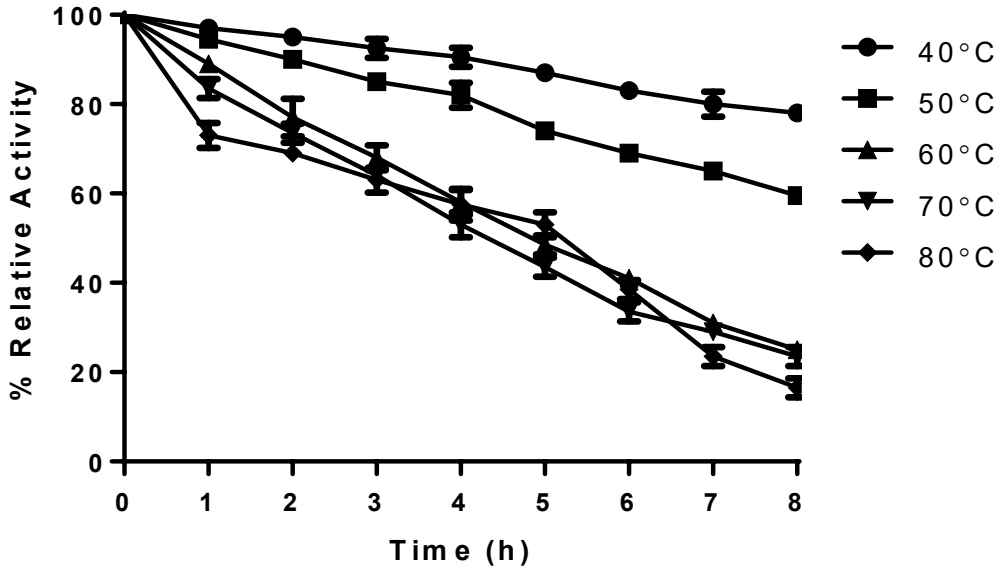
S3: Response surface plots for the preparation of CLEA. Effect of time and pH (A) glutaraldehyde and precipitant (B) precipitant and pH (C) temperature and time (D) and temperature and pH on the immobilization yield of the laccase CLEA



S4: Reusability of CLEA



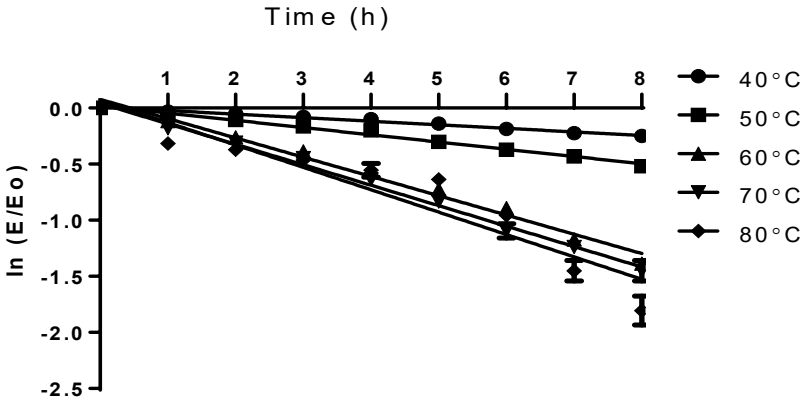
S5: pH stability of free and immobilized laccase



670

671 S6: Thermostability of free laccase

672



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675 S7: Plot of In (E/Eo) against 1/T for free laccase

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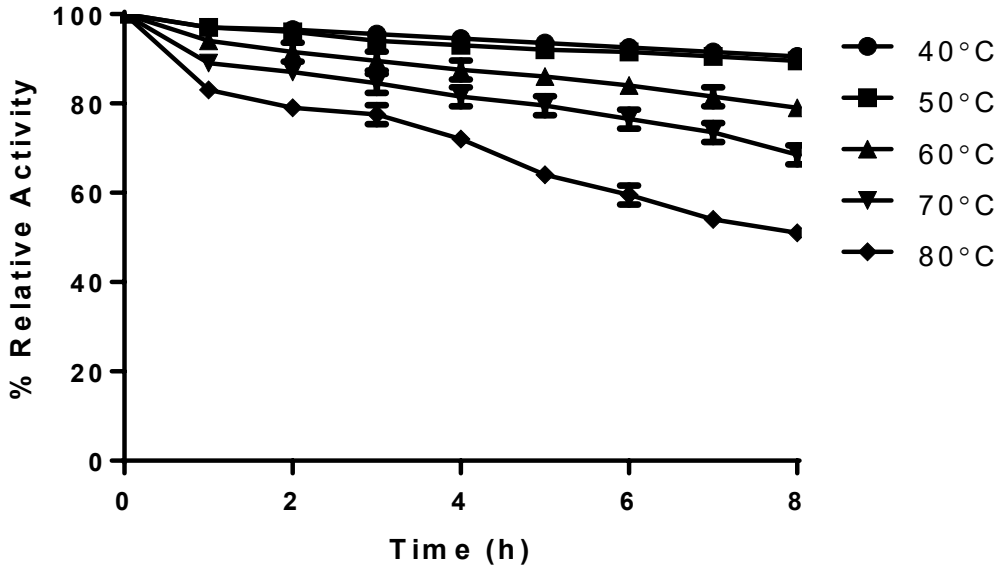

684

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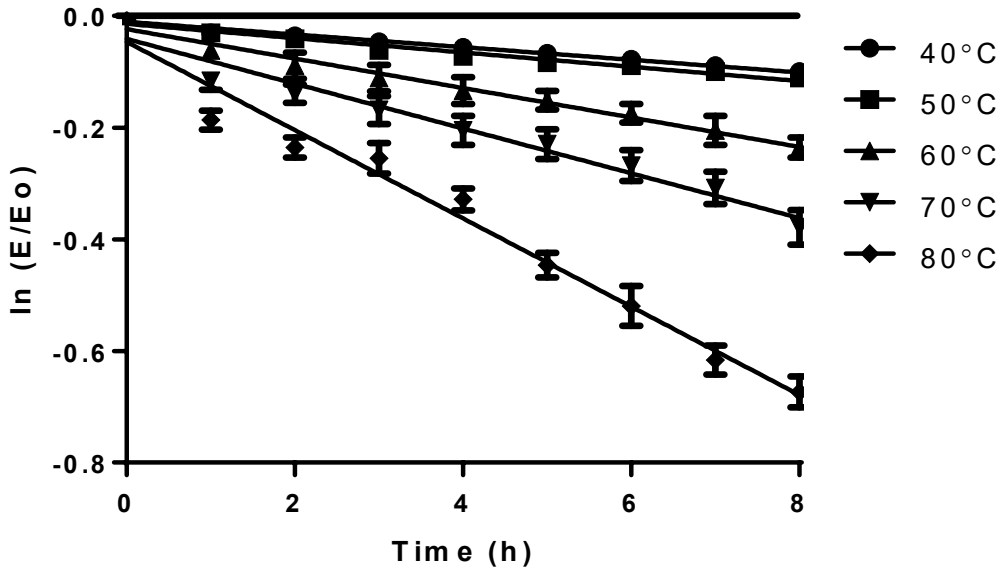
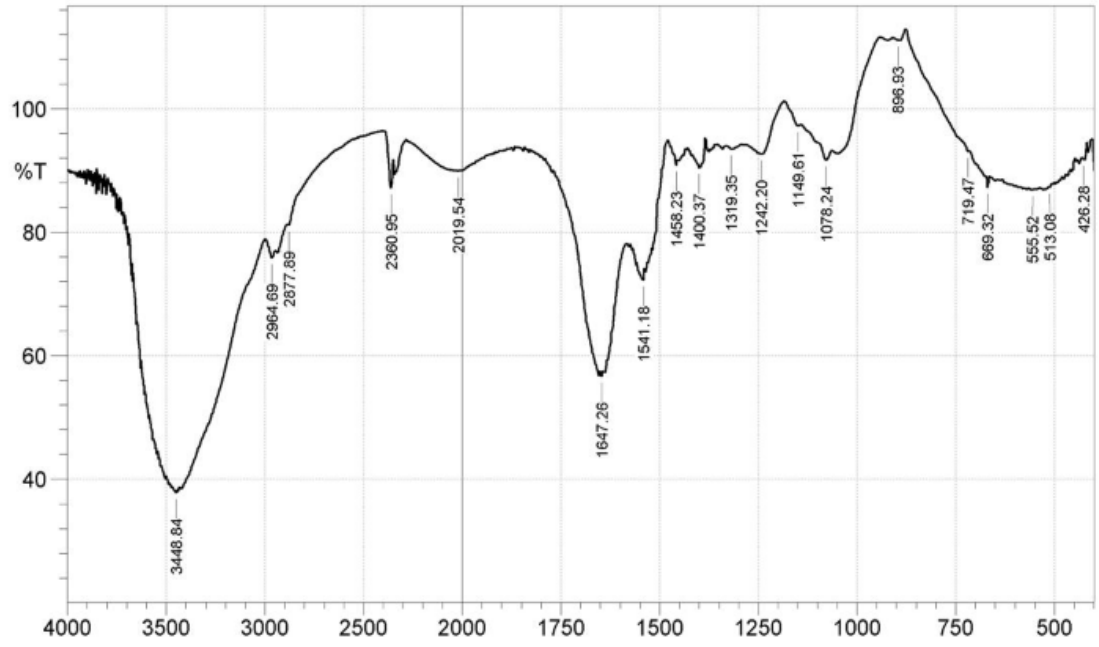
686 S8: Thermostability of laccase CLEA



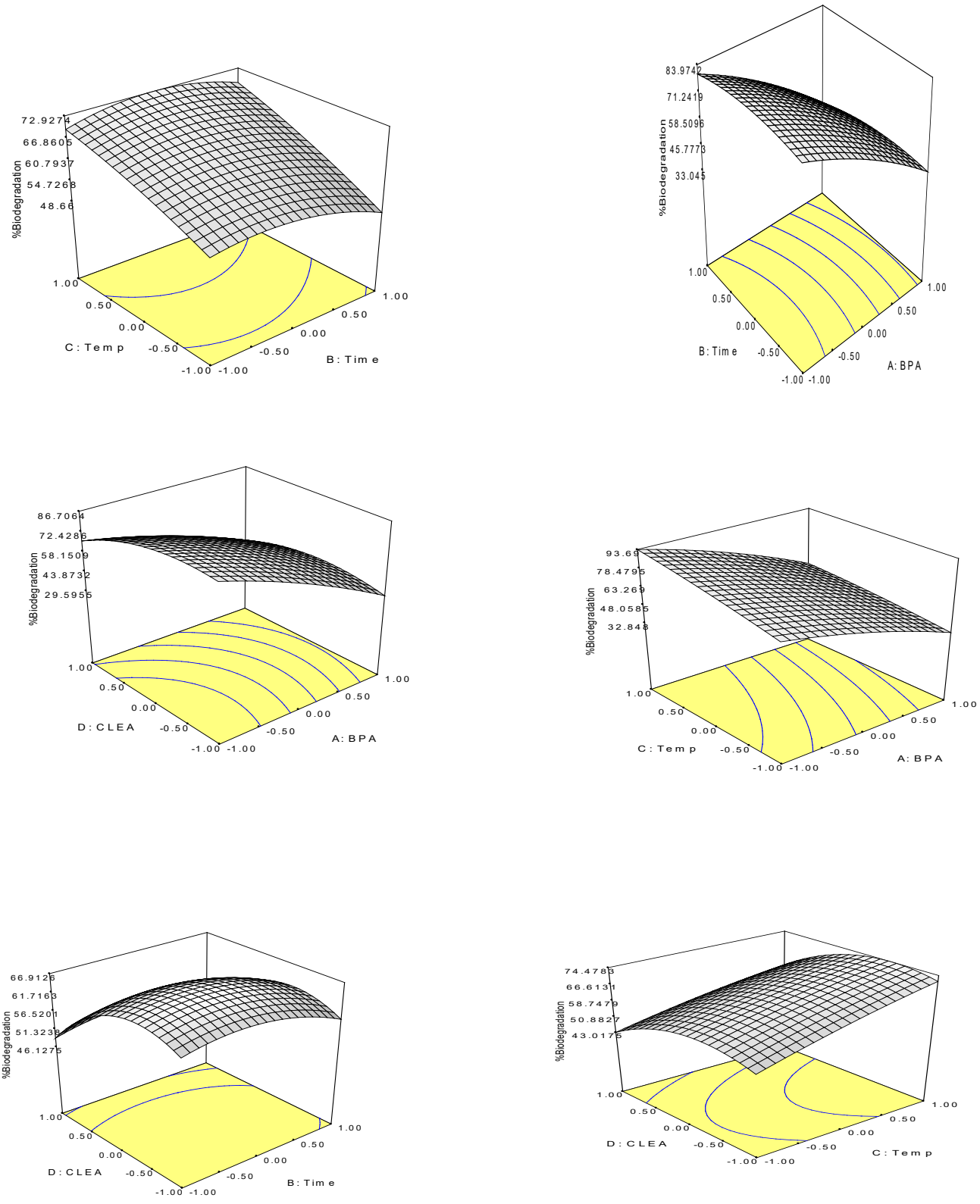
687

688 S9: Plot of In (E/Eo) against 1/T for immobilized laccase

689

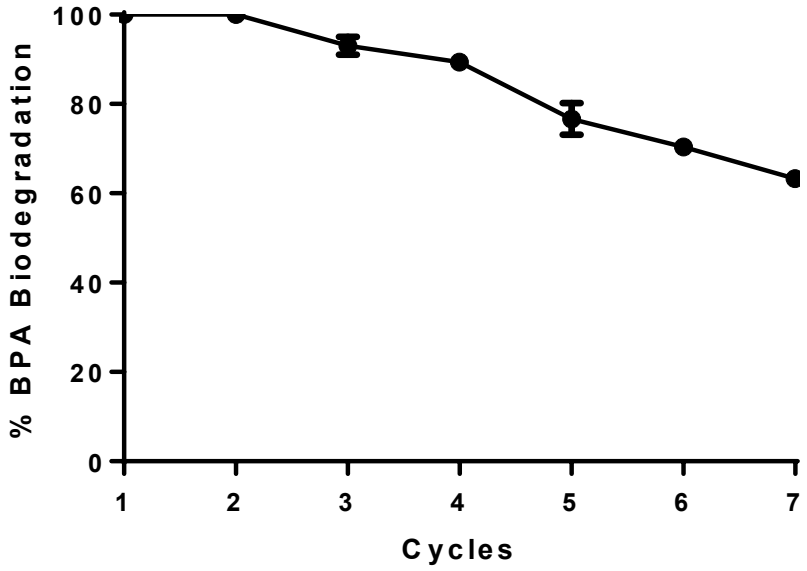


S10: Laccase CLEA FTIR



S11: Effect of (A) temperature x time (B) time x [BPA] (C) CLEA amount x [BPA] (D) temperature x [BPA] (E) CLEA amount x time and (F) CLEA amount x temperature on the biodegradation efficiency of CLEA on BPA.

711



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713 S12: Reusability of CLEA in BPA biodegradation

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