Biocatalysis and Agricultural Biotechnology 19 (2019) 101135

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Nanoporous Zeolite-X as a new carrier for laccase immobilization and its application in dyes decolorization

Hala R. Wehaidy[a,*], Mohamed A. Abdel-Naby[a], Heba M. El-Hennawi[b], Hanan F. Youssef[c]

[a] Natural and Microbial Products Chemistry Department, National Research Centre, Dokki, Giza, Egypt
[b] Dyeing, Printing and Textile Auxiliaries Department, National Research Centre, Dokki, Giza, Egypt
[c] Refractories, Ceramics and Building Materials Department, National Research Centre, Dokki, Giza, Egypt

ARTICLE INFO

Keywords:
Zeolite-X
Laccase immobilization
Dye decolorization
Thermal stability
Thermodynamic parameters

ABSTRACT

Laccase enzyme from *Polyporus durus* ATCC 26726 was immobilized on nanoporous Zeolite-X (ZX), with 83% immobilization yield. The immobilization process protected laccase against pH and temperature changes. The activation energy was lowered after immobilization. Compared to the free enzyme the immobilized enzyme displayed higher K_m value and lower V_{max}. The immobilized laccase exhibited higher half-life values ($t_{1/2}$), higher decimal reduction time (*D*-value) and lower deactivation rate constant (K_d) within the temperature range of 50–70 °C. The thermodynamic analysis for substrate oxidation indicated that the enthalpy (ΔH^*), entropy (ΔS^*), and free energy of transition state (ΔG^*_{E-T}) were lower for immobilized laccase. Furthermore, there was an increase in turnover number (k_{cat}) after immobilization. The immobilized laccase could decolorize two dyes and two synthetic wastewater solutions. It retained 100% activity against AB 225 dye after 7 successive decolorization cycles and it could be reused for 11 times with 48% activity loss.

1. Introduction

Laccases (EC 1.10.3.2) are copper-containing oxidases, which were first discovered in the Japanese lacquer tree, *Rhus vernicifera* (Giardina et al., 2010; Morozova and Shumakovich, 2007). This enzyme has been applied for dyes decolorization (Rodríguez Couto and Toca Herrera, 2006). The reaction is easy to operate because it consumes O_2, at room temperature (Zamora et al., 2003; Zille et al., 2003). In addition, the separation of products is difficult. In wastewater treatment, laccase has low stability and high prices which discourage its use (Jořenek and Zajoncová, 2015). Further, the uses of free enzymes have limited industrial applications due to their low thermal and operational stability resulting from susceptibility for deactivation in the presence of other chemicals. The uses of the immobilized enzyme provide several advantages included the improvement of thermal stability and possible reuse (Abdel-Naby et al., 1999c; Wang et al., 2008).

The textile industry generates large volumes of liquid effluent pollutants (Bokare et al., 2008; Sun et al., 2009). Synthetic dyes are the largest of all textile dyestuffs produced, and their residuals left in wastewater have been considered as the main source of environmental pollution (Telke et al., 2008; Vandevivere et al., 1998). The enzymatic treatment of dye pollutants is preferred due to its mild reaction conditions which will not generate toxic products (Gholami-Borujeni et al., 2011a, 2011b; Li et al., 2015).

Zeolites are crystalline hydrated aluminum silicates with a framework structure of interconnected SiO_4 and AlO_4 tetrahedra to form three-dimensional network skeleton of uniformly-sized interconnected pores and channels in the molecular dimensions of 0.3–1.4 nm range. Synthetic Zeolites are preferred than their natural counterparts for industrial applications, such as adsorption, catalysis and cation exchange, due to the uniform pore sizes, relative ease of manufacture, low costs and can be tailored for specific shape and size. The pore size ranges of some synthetic molecular sieves are 3.5–4.5 Å for LTA (Z-A) Zeolite, 4.5–6.0 Å for ZSM-5 and 6.0–8.0 Å for Zeolite X and Y (Youssef et al., 2015).

Synthetic Faujasite-type zeolite is commonly prepared from pure chemical sources of sodium aluminate and sodium silicate. It has a general formula of $Na_2O.Al_2O_3.nSiO_2.\ xH_2O$. Depending on the silica-to-alumina ratio, Faujasite zeolites are either of the X- (Si/Al is less than 3) or Y-type (Si/Al = 3–5) (Htun et al., 2012). Zeolites constitute an important group of silicates that hosts large percentages of water within their impervious structure. This aqueous environment is useful for the enzyme immobilization to retain its active conformational structure. Further, grafted zeolite (with different composition) could be used for covalent immobilization of laccase (Celikbicak et al., 2014).

In the present study, Faujasite-NaX (also called Zeolite Na-X or

* Corresponding author.
E-mail address: halolty2009@yahoo.com (H.R. Wehaidy).

https://doi.org/10.1016/j.bcab.2019.101135
Received 5 February 2019; Received in revised form 3 April 2019; Accepted 14 April 2019
Available online 16 April 2019

simply ZX) was prepared from refined kaolin, using the microwave hydrothermal technique. The obtained product was evaluated as a novel carrier for laccase immobilization to mainly improve its catalytic properties and thermal stability. The reusability of the immobilized enzyme in dyes decolorization and synthetic wastewater treatment processes was also evaluated.

2. Materials and methods

2.1. Laccase enzyme

In the current study, the used laccase enzyme was produced in the lab from *Polyporus durus ATCC* 26726 by submerged fermentation for 7 days using the following medium (g/l): Wheat bran, 60; Yeast extract, 3.0; $MgSO_4.7H_2O$, 2.0; KH_2PO_4, 3.0 and glucose, 2.0 and 1 mM $CuSO_4$ was added to the fermentation medium at the 5th day of fermentation (Wehaidy et al., 2018). The crude culture filtrate was partially purified with acetone precipitation at 40% concentration and used for the preparation of the immobilized enzyme.

2.2. Laccase assay

Laccase activity was built on the oxidation of the substrate 2,2-azino–bis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) (Bourbonnais et al., 1995). The reaction mixture contained the following; 600 µl sodium acetate buffer (0.1 M, pH 4.0), 300 µl ABTS (3 mg/ml), 300 µl culture filtrate or 50 mg of immobilized enzyme and 1400 µl distilled water. The prepared mixture was incubated for 5 min at 30 °C. The color was monitored spectrophotometrically at 420 nm. One unit of laccase activity was defined as the activity of an enzyme that catalyzes the conversion of 1 µ mole of ABTS ($\varepsilon 420 = 36{,}000\ M^{-1}\ cm^{-1}$) per minute.

2.3. Preparation of zeolite slurry

50 ml of NaOH (3 M) was divided into two equal parts; to one part, 5 g of metakaolinite (calcined kaolin at 800 °C/4 h) was added on stirring for 10 min (solution 1) whereas, a 40 ml of ludox40 (additional silica source) was gradually added to the second part with vigorous stirring for 1 h (solution 2). The two solutions were then mixed with vigorous stirring (800 rpm) for 24 h to obtain the gel.

2.4. Microwave hydrothermal treatments (M-H)

In the microwave method (M-H), 20 ml of the previously prepared fresh gel was loaded into the 100 ml capacity Xpress vessels of microwave digestion system (Mars 5, Model XP-1500, CEM Corp., Matthews, NC). The system operated at a frequency of 2.45 GHz and can operate at 1–100% of 1600 W power, and heat-treated for 1 h at 110 °C. The synthetic product was then collected, washed several times with distilled water to remove the excess alkali and dried at 100 °C overnight. The clean dry product was then being ready to be identified.

2.5. XRD characterization of prepared zeolite

The mineral composition of the synthetic product was characterized by X-ray diffraction (XRD) method using BRUKUR D8 ADVANCE with secondary monochromatic beam Cu Kα radiation at KV = 40 and mA = 40.

2.6. SEM and EDX identification for zeolite

The surface morphology and microstructure of both ZX and ZX-immobilized laccase were investigated by Scanning Electron Microscopy (SEM) model Quanta 250 FEG (Field Emission Gun) attached with EDX Unit (Energy Dispersive X-ray Analyses), with accelerating voltage 30 K.V., magnification 14× up to 1000000 and resolution for Gun.1n). FEI Company, Netherlands. The chemical composition was analyzed by EDX microanalysis.

2.7. Enzyme immobilization

A measure of 250 µl (contains 4213 IU and 0.2 mg protein) of the partially purified laccase was mixed with 50 mg of zeolite powder and the mixture was left overnight at 4 °C. At the end of the incubation period, the zeolite-laccase mixture was washed twice with distilled water and used for laccase assay. The following formula was used:

The immobilization yield = [I /(A – B)] x 100

Where:

I = the immobilized enzyme activity.
A = the total activity of enzyme added.
B = the amount of residual enzyme activity in the washing solution.

In all experiments, a blank of boiled laccase enzyme was used.

2.8. Properties of free and immobilized enzyme

2.8.1. Optimum temperature

The effect of temperature on the activity of free (4213 IU) and immobilized laccase (3160 IU) was studied by carrying out the reaction at different temperatures (from 30 to 80 °C). The activation energy (*Ea*) for both the free and immobilized laccase was calculated from Arrhenius plot as follows:

$$\text{Slope} = E_a / 2.303\,R,$$

Where R is the gas constant (R = 8.314 J/K.mol).

2.8.2. Optimum pH

To determine the optimum pH for the free and immobilized laccase. The enzymes were assayed at different pH range (pH 3.0–7.0) and at the optimum reaction temperature. 0.1 M acetate buffer (pH 3 to 5), 0.1 M phosphate buffer, (pH 6 to 7), 4213 IU of free enzyme and 3160 IU of immobilized enzyme were used.

2.8.3. Effect of substrate concentration

ABTS was used as a substrate at different concentrations (1–10 mg/ml). Each enzyme preparation (4213 IU of free enzyme and 3160 IU of immobilized enzyme) was assayed for activity with these substrate concentrations at the optimum assay conditions. The Michaelis–Menten constant (K_m) and the maximum velocity (V_{max}) of free and immobilized laccase were calculated from Lineweaver–Burk Plot. The specificity constant (V_{max}/K_m), the turnover number (k_{cat}), free energy of transition state binding (G^*_{E-T}) and free energy of substrate binding (G^*_{E-S}) were determined as described by Abdel-Naby et al. (2017) as follow:

$$K_{cat} = (k_b T / h) \text{ x } e^{(-\Delta H*/RT)} \text{ x } e^{(\Delta S*/R)} \quad (1)$$

Where:

k_b Boltzmann's constant $(R/N) = 1.38 \times 10^{-23}\ J\ K^{-1}$
T Absolute temperature (K)
h Planck's constant = 6.626×10^{-34} Js
N Avogadro's number = $6.02 \times 10^{23}\ mol^{-1}$
R Gas constant = $8.314\ J\ K^{-1}\ mol^{-1}$

$$\Delta H^* \text{ (Enthalpy)} = E_a - RT \quad (2)$$

$$\Delta G^* \text{ (Gibbs free energy of activation)} = -RT\ ln\ (k_{cat}\ h / k_b \text{ x } T) \quad (3)$$

$$\Delta S^* \text{ (Entropy)} = (\Delta H^* - \Delta G^*) / T \quad (4)$$

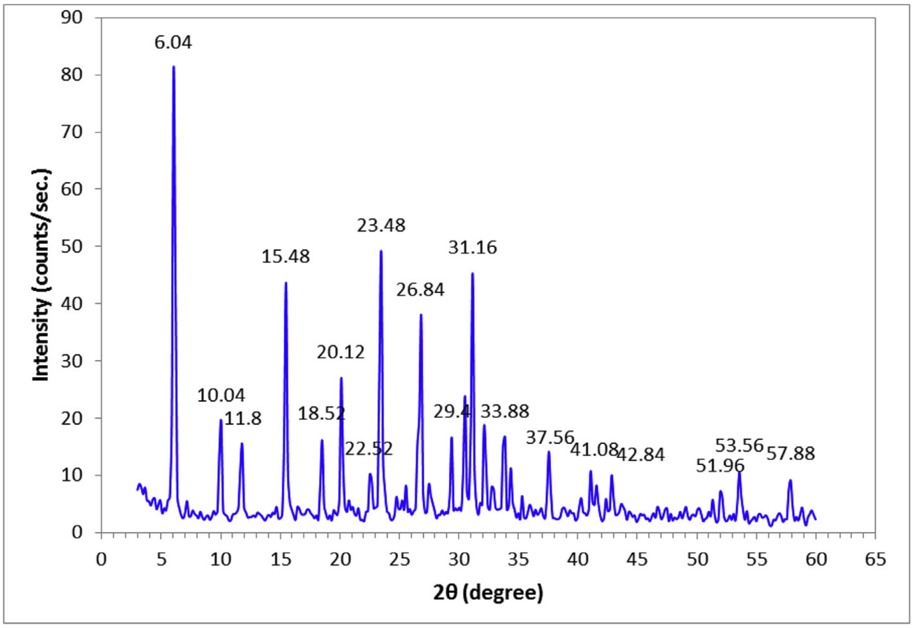


Fig. 1. XRD for Faujasite-NaX prepared at 110 °C/2 h under microwave heating.

Free energy of substrate binding $\Delta G^*_{E\text{-}S} = -RT \ln K_a$, where $K_a = 1/k_m$ (5)

Free energy for transition state formation $\Delta G^*_{E\text{-}T} = -RT \ln (k_{cat}/K_m)$ (6)

2.8.4. Thermal stability

This was approached by heating the enzymes (the same units as in the previous experiments) at 50, 60 and 70 °C, in absence of the substrate, for different incubation periods (up to 90 min). Thereafter, the residual activity was assayed at the optimum pH and temperature for each enzyme preparation. A sample was taken from the reaction every 15 min and placed immediately in an ice bath. The thermally treated enzyme samples were assayed for laccase activity as described earlier. The activity without heating was taken as 100%. The process of thermal inactivation followed the kinetics of a first-order model. *D* values (decimal reduction time), half-life values ($t_{1/2}$) and deactivation rate constant (k_d) within the temperature range of 50–70 °C were calculated from the semi-logarithmic plot of residual activity as a function of time. The thermodynamic parameters at different incubation temperatures were determined as described by as follow (Ramos et al., 2011; Abdel-Naby et al., 2017):

$$K_d = (k_b T / h) \text{ x } e^{(-\Delta H*/RT)} \text{ x } e^{(\Delta S*/R)} \quad (7)$$

$$Q_{10} = \text{antilog } E = (E.\ 10/RT^2)$$

Where $E = E_a$ = activation energy.

2.9. Decolorization activity of immobilized laccase against some dyes

In this part, the decolorization efficiency of immobilized laccase was investigated against two anthraquinones dyes (Sunzol Brilliant Blue 19 (RBBR, Reactive blue 19 or RB 19) and Dystar. Supralan Blue 2 R (C. I. Acid blue 225 or AB 225) supplied from Egypt colors, Heliopolis, Cairo).

The optimal decolorization conditions for each dye were also investigated. The reaction mixture containing 1 ml dye (100 mg/l) in acetate buffer solution (pH 4) was incubated with immobilized laccase (50 mg containing 3160 IU) with shaking at 150 rpm at the optimum reaction conditions. The decolorization efficiency was expressed in terms of percentage and calculated by the decrease in absorbance at λ_{max} of each dye (590 nm for RB 19 and 584 for AB 225) as follows:

$$\% \text{ Decolorization} = [(A_i - A_o) / A_i] \times 100$$

Where A_i is the initial absorbance of dye, and A_o is the final absorbance of the dye after decolorization. The optimum conditions for efficient dye decolorization activity were also investigated.

2.10. Decolorization activity of immobilized laccase against synthetic wastewater solutions

The decolorization activity of immobilized laccase against two synthetic wastewater solutions A and B was investigated at the optimum reaction conditions. Synthetic wastewater solution A (100 mg/l RB 19, 100 mg/l AB 225, 90 g/l sodium sulfate, and 20 g/l sodium carbonate) and solution B (100 mg/l RB 19, 100 mg/l RV 5 (Sunzol Brilliant Violet 5 R) a metal complex azo dye, 90 g/l sodium sulfate, and 20 g/l sodium carbonate) were prepared in the lab as described by other author (Yesilada et al., 2014; Wehaidy et al., 2018).

2.11. Reusability of immobilized laccase in dye decolorization

The reusability of immobilized laccase was determined by using the immobilized enzyme for degradation of AB 225. After each reaction cycle, the laccase immobilized particles were washed 3 times with distilled water to remove any residual substrate or products. Then the immobilized enzyme was transferred into fresh dye solution for the next cycle, and the remaining enzyme activity was determined.

All experiments were performed in triplicate, and the means were used for the calculation of results throughout the work.

2.12. Storage stability

Both the free and immobilized laccase were stored in 0.1 M acetate buffer (pH 4) at 4 °C for two months. A sample of the free and the immobilized laccase has been withdrawn every week and assayed for enzyme activity.

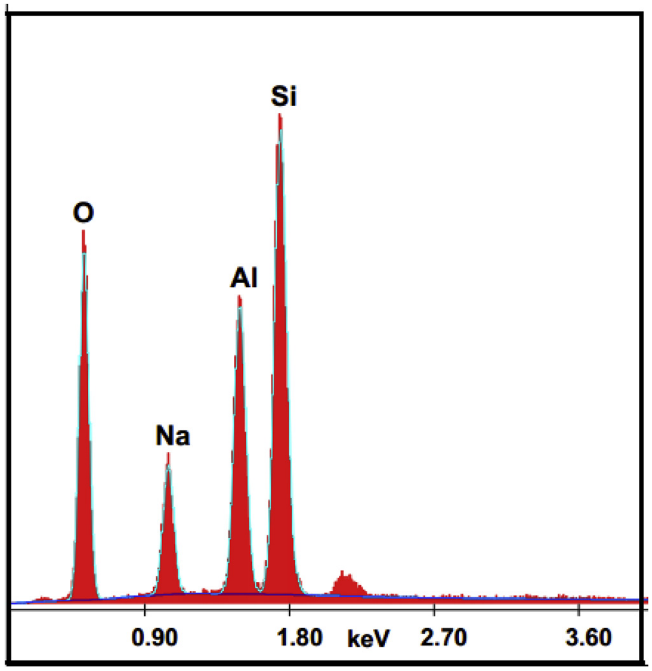


Fig. 2. EDX microanalysis for Faujasite-NaX zeolite.

3. Results and discussion

3.1. X-zeolite characterization

3.1.1. X-ray diffraction

The formed zeolite was characterized by XRD method. Fig. 1 represented a distinctly strong and complete set of peaks for the micronized faujasite-NaX zeolite, with $Na_2Al_2Si_4O_{12}.8H_2O$ chemical formula (Si/Al = 2.0). The complete set of sharp and strong peaks confirmed the structure of the X-zeolite type with high crystallinity. The absence of interfering phases indicated the high purity of the product.

3.1.2. SEM and EDX microanalysis

The SEM microstructure and EDX microanalysis of the produced ZX chemical composition was presented in Fig. 2 & 3a, along with Table 1. The detected Si/Al ratio (nearly equals 2.0) agreed well with the previous XRD data. The crystals were of uniformly distributed, having an average particle size of less than 10 µm.

Fig. 3b illustrates the crystals of ZX after enzyme immobilization. It is obvious that the morphology of the particles surface is different in comparison with ZX alone (Fig. 3a). The enzyme appeared as a white cloud covering the surface of ZX crystals and the intermolecular spaces and changed the roughness of ZX molecules. This could prove that the laccase enzyme was effectively immobilized on the surface of zeolite. This might be due to the high surface area of the carrier since the nanopores and the surface properties of ZX were very effective factors in the immobilization process. Many authors used SEM for comparing the surface morphological changes before and after immobilization (Mahmoud et al., 2011; Dehghanifard et al., 2013; Lettera et al., 2016).

Table 1
Microanalysis for faujasite-NaX zeolite chemical composition.

Element	wt %	At %	K-Ratio	Z	A	F
O K	39.35	52.10	0.1469	1.0330	0.3612	1.0007
NaK	9.59	8.84	0.0480	0.9668	0.5147	1.0057
AlK	17.73	13.92	0.1230	0.9620	0.7134	1.0113
SiK	33.33	25.14	0.2186	0.9900	0.6626	1.0000
Total	100.00	100.00				

3.2. Laccase immobilization on the synthesized zeolite material

The partially purified laccase enzyme (40% acetone fraction) was immobilized by physical adsorption on the synthesized zeolite with an immobilization yield of 83%.

3.3. Characterization of ZX immobilized laccase

The following experiments were conducted to evaluate the role of ZX as a carrier for laccase immobilization.

3.3.1. The optimum temperature of free and immobilized laccase

Both the free and immobilized laccase were optimally active at 70 °C. This result agrees well with many authors who recorded the same optimum temperature of action for both the free and the immobilized laccase enzyme (Pye and Chance, 1976; Leonowicz et al., 1988; Berka et al., 1997; D'Annibale et al., 1999; Kunamneni et al., 2008; Dehghanifard et al., 2013). At higher temperatures, the enzyme activity decreased for both forms. However, the immobilized laccase maintained higher relative activity than that recorded for the free laccase. For example, at 80 °C the relative activity of the immobilized enzyme was 85% which was higher than the free enzyme (50%). These results proved the positive effect of the immobilization on enzyme stability.

The calculated values of activation energy (*Ea*) of the free and immobilized enzyme using Arrhenius plot were found to be 12.43 kJ/mol and 7.22 KJ/mol, respectively (Fig. 4). The reduction in *Ea* after immobilization indicates the high catalytic efficiency of immobilized laccase by lowering down the required energy to make the activated enzyme-substrate complex. Lowering *Ea* of laccase after immobilization was previously reported (Ratanapongleka and Punbut, 2017). The recorded value for *Ea* of the immobilized enzyme was lower than that reported previously for other laccases (44.12 kJ mol^{-1}) (Weiwei et al., 2012).

The effect of temperature on the rate of substrate oxidation by the free and immobilized *Polyporus durus* ATCC 26726 laccase was measured in terms of Q_{10}. The values of Q_{10} indicated whether the catalytic reactions are controlled by temperature or by other factors. In general, catalytic reactions of the enzymes show Q_{10} value between 1 and 2. However, deviation from this value is an indicator of the involvement of other factors (do not include temperature) in controlling the catalytic rate (Elias et al., 2014). The Q_{10} value for dye decolorization by both free and immobilized enzyme was found to be 1.0, indicating that the catalytic reaction is temperature dependent.

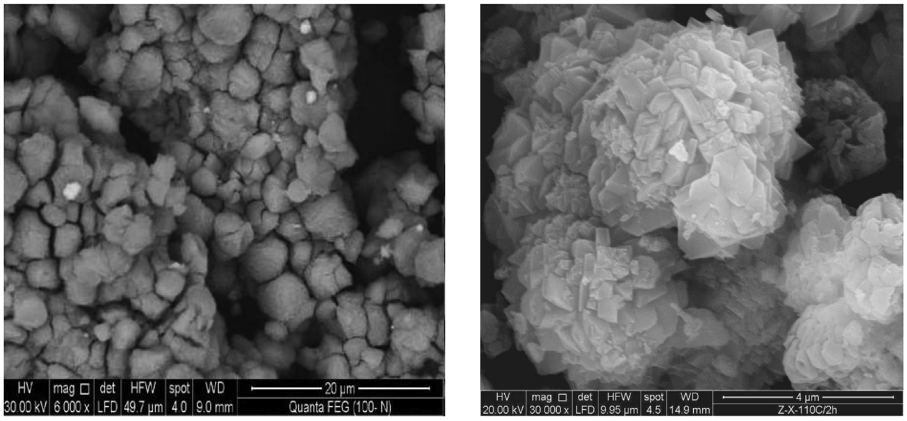
3.3.2. Optimum pH

Both the free and immobilized laccase has an optimum pH of 4.0. At higher pH values (pH 5–7) the immobilized enzyme was tolerant for the changes of pH of the reaction (Fig. 5). This means that the immobilized laccase is more stable against pH change, but the ionization of the amino acid residues at the active site remains unaffected by the immobilization process. Similar results were reported by other authors (Kunamneni et al., 2008; Dehghanifard et al., 2013; Srivastava, 1999; Abdel-Naby, 1999a; Catapane et al., 2013).

3.3.3. Thermal stability

The data of thermal properties of free and immobilized *Polyporus durus* ATCC 26726 laccase indicated that the immobilization process significantly improved the thermal stability of laccase enzyme. Thus, after heat treatment at 60 °C for 30 min, the retained activity of the free enzyme was 5%. However, the recovered activity of the immobilized enzyme reached 93.5% at the same treatment. Further, after heat treatment at 70 °C the free enzyme lost its activity at 15 min, however, the immobilized enzyme retained 76% activity after 90min. These results indicate that ZX immobilized laccase is more thermostable than laccase immobilized on grafted zeolite (retained about 42% activity after 90 min at 70 °C, Celikbicak et al., 2014). When the logarithm of

a:



b:

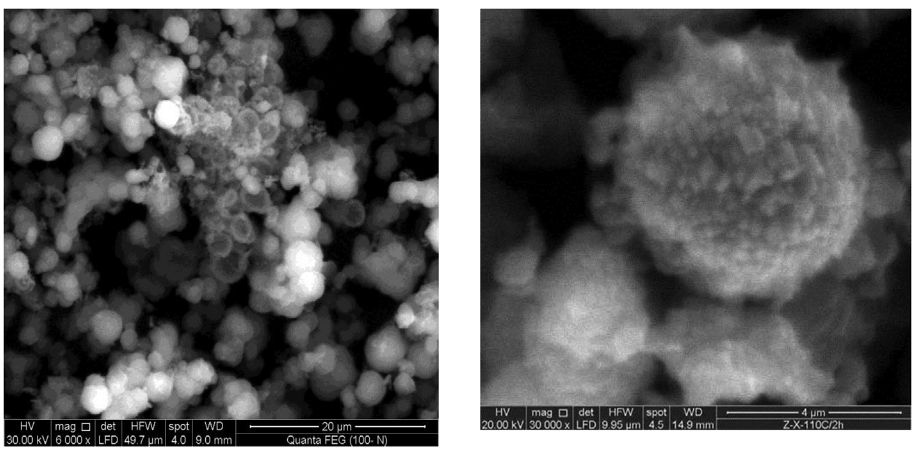


Fig. 3. a: SEM for ZX prepared under microwave heating, b: ZX loaded with immobilized laccase.

the retained activity was plotted against time (Fig. 6), the free and immobilized enzymes gave straight-line plots. This means that the thermal inactivation process of the two forms of the enzyme corresponded to the theoretical curves of a simple first-order reaction. The thermal properties of the free and immobilized laccase were recorded in Table 2. The calculated values of $t_{1/2}$ for the free enzyme at 50, 60 and 70 °C (77.5, 60 and 4.7 min, respectively) however, higher values were recorded for the immobilized enzyme (698, 281 and 140.9 min, respectively). Lower value of $t_{1/2}$ was previously recorded for immobilized laccase by other authors ($t_{1/2}$ value of immobilized laccase was 90 min at 65 °C as calculated by Lettera et al. (2016)). The calculated kd at 70 °C for the free laccase was 0.066 min^{-1} which was 30-time faster than the immobilized enzyme (0.0022 min^{-1}). Further, after heat treatment at 70 °C, D-value of the immobilized enzyme was 30-time more than the free enzyme. Based on the previous results, the physical adsorption of *Polyporus durus* ATCC 26726 laccase on ZX appeared to enhance its thermal stability. In general, the thermal parameters recorded for the immobilized *Polyporus durus* ATCC 26726 indicated more stability than immobilized laccase reported by other authors (for example, $t_{1/2}$ at 60 °C = 18.6 h (Bezerra et al., 2015), $t_{1/2}$ at 70 °C = 0.07 h (Tavares et al., 2015)). The enhanced thermal stability of laccase by immobilization is an advantage for its industrial application due to the high temperatures needed in the industrial processes (Berka et al., 1997; Elias et al., 2014).

3.3.4. *Effect of substrate concentration*

Linweaver-Burk plots (Fig. 7) of the free enzyme gave K_m of 1.7 mg/ml, with ABTS, which is lower than that of the immobilized enzyme (2.3 mg/ml). The calculated value of V_{max} of the free enzyme was 36000 U/ml which is higher than that of the immobilized enzyme (30000 U/g carrier).

The increase of the K_m value after immobilization may be due to mass transfer limitations of the substrate into the immobilization matrix and low accessibility of substrate to the active site of the immobilized enzyme. Increasing the K_m value of other enzymes after the immobilization was similarly reported by various authors (Tavares et al., 2015; Berrio et al., 2007; Kusano et al., 1989; Abdel-Naby et al., 1999b). Fixation of the enzyme on the immobilization matrix might cause decreasing in the enzyme flexibility, which leads to a decrease in the catalytic activity of the immobilized form (Sampaio et al., 2016; Erarslan et al., 1996). Consequently, the maximum reaction rate of the immobilized laccase was lower than that of the free form.

The kinetic parameters of ABTS oxidation by the free and immobilized *Polyporus durus* ATCC 26726 laccase on x-zeolite are recorded in Table 3. The calculated value of k_{cat} of the immobilized enzyme (31.186 S^{-1}) was about 40.47% higher than the free laccase (22.2 S^{-1}). Further, the catalytic efficiency (k_{cat}/K_m) for the immobilized enzyme (13.6 S^{-1} mg ml^{-1}) was slightly higher than the free form (13.0 S^{-1} mg ml^{-1}). Lower values of k_{cat}/K_m (0.5 S^{-1} mg ml^{-1}) were recorded for *Cerrena unicolor* immobilized on cellulose-based carrier Granocel −4000 (Rekuc et al., 2009).

The thermodynamic data of ABTS oxidation by the free and immobilized laccase are recorded in Table 3. The enthalpy of activation (ΔH^*) for the immobilized form (4.372 kJ mol^{-1}) was lower than the

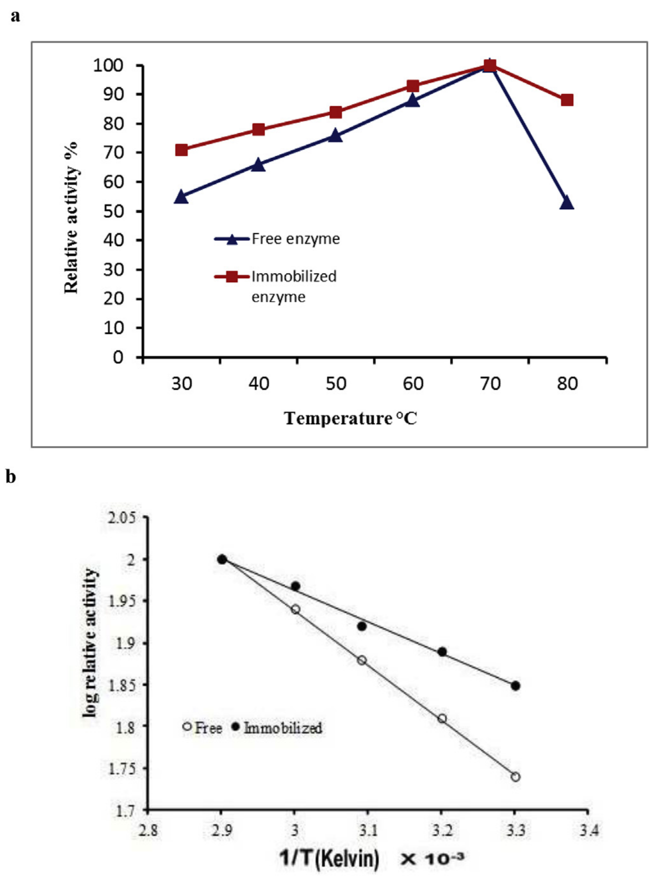


Fig. 4. (a) Optimum temperature of the free and immobilized laccase. (b) Arrhenius plots of temperature data of the free and immobilized laccase (T: temperature in Kelvin).

value of the free enzyme (9.58 kJ mol^{-1}). Further, the value of ΔS^* was lower for ZX bound laccase by about 43.46 Jmol^{-1}k^{-1}. However, ΔG^* was higher for the immobilized form by about 9.7 k Jmol^{-1}. The lower value of ΔH^* and negative value of ΔS^* recorded for the immobilized form (Table 3) indicated that the transition state of enzyme-substrate complex (ES^*) was stable and more ordered (Bhatti et al., 2007). The

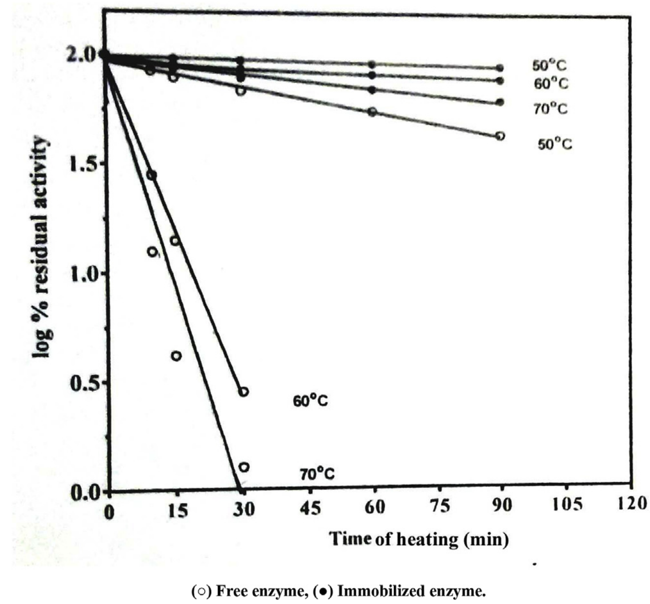


Fig. 6. First-order plots of the effect of thermal inactivation of free and immobilized laccase.

negative value for ΔS^* usually indicates that entropy decreases on the formation of the transition state. In general, the change in the value ΔG^* is the best parameter for evaluating the conversion of the activated ES^* complex into a product. Thus, the low value of ΔG^* indicates the spontaneously of conversion of the transition state complex into products (Riaz et al., 2007). The calculated value of $\Delta G^*_{\text{E-T}}$ for immobilized laccase (−7.44 kJ mol^{-1}) was lower than the free enzyme by about 410 J mol^{-1}. In addition, the free energy of substrate binding ($\Delta G^*_{\text{E-S}}$) for the immobilized enzyme (2.473 kJ mol^{-1}) was higher than the free laccase (1.456 kJ mol^{-1}). This indicated that laccase immobilized on ZX required a lower amount of free energy ($\Delta G^*_{\text{E-T}}$) to form the activated (transition) complex. However, a higher amount of ΔG^*_{E-S} was released as compared with the free laccase. This means that the higher catalytic efficiency of the immobilized laccase was due to the stabilization of transition state (Ramos et al., 2011). The thermodynamic parameters of the immobilized laccase indicated that the immobilization of laccase onto nanoporous ZX promoted an increase in the

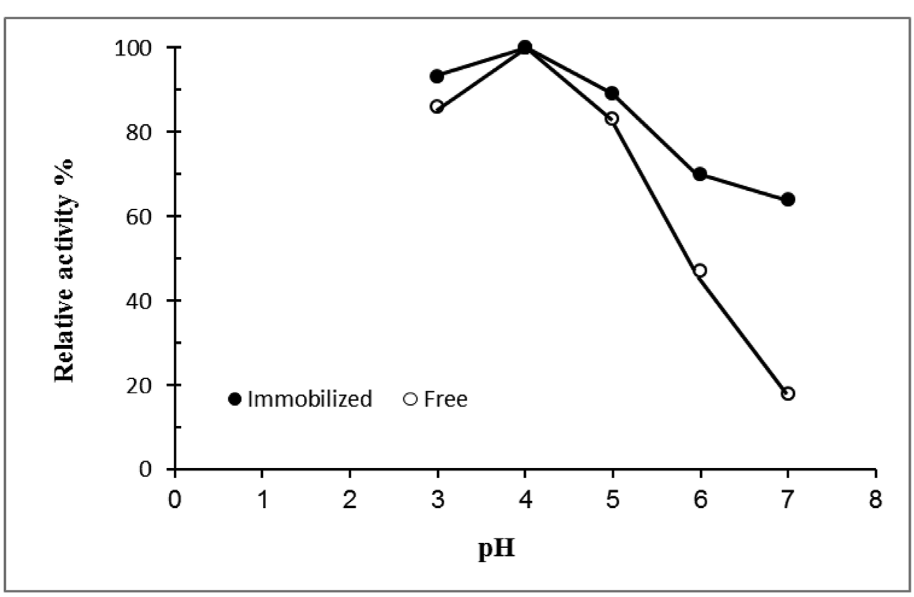


Fig. 5. Optimum pH of the free and immobilized laccase (○) Free enzyme, (●) Immobilized enzyme.

Table 2
Thermal properties of free and immobilized *Polyporus durus* ATCC 26726 laccase.

Property	Free enzyme	Immobilized enzyme
Optimum temperature (°C)	70	70
Half-life ($t_{1/2}$,min) at:		
50°C	77.5	698.2
60°C	60	281
70°C	4.7	140.9
Deactivation rate constant (min^{-1}) at:		
50°C	0.004	0.00044
60°C	0.0051	0.0011
70°C	0.066	0.0022
Decimal reduction time		
D-value (min) at:		
50°C	475	4318.18
60°C	115.5	535.45
70°C	8.92	267.72

catalytic efficiency and enzymatic stability.

3.4. Decolorization activity of immobilized laccase against some dyes

In this part, the decolorization activity of x-zeolite immobilized laccase was evaluated against two dyes (AB 225 and RB 19). The optimum conditions for an effective decolorization process were also investigated.

3.4.1. Effect of reaction time on decolorization activity

The decolorization activity of immobilized laccase on RB 19 and AB 225 was investigated at 50 °C. Fig. 8 represents the effect of time on the % decreases in the absorbance of dyes. For the AB 225 100% decolorization activity was obtained after 15 min incubation. However, with RB 19 100% decolorization activity was reached after 45 min. This time is shorter than that reported by other authors for dyes decolorization by immobilized laccase (1 h for 93% decolorization of RBBR and 82% decolorization of AB 25) (Li et al., 2015), (75% Methyl Green decolorization within 6 h) (Kunamneni et al., 2008), (97% and 90% Congo red decolorization activity after 12 and 60 h, respectively) (Telke et al.,

Table 3
Thermodynamic and kinetic parameters of substrate catalysis by free and immobilized *Polyporus durus* ATCC 26726 laccase.

Parameter	Free	Immobilized
Activation energy *Ea* (KJ/mol)	12.432	7.224
K_m (mg/ml)	1.7	2.3
V_{max} (U/mg protein)	36	30
K_{cat} (S^{-1})	22.2	31.186
K_{cat}/K_m	13.0	13.6
Ka (1/Km)	0.6	0.42
ΔH (kJ/mol)	9.58	4.372
ΔG (kJ/mol)	92.153	101.852
ΔS (J/mol.K)	−240.73	−284.19
$\Delta G_{E\text{-}T}$ (kJ/mol)	−7.03	−7.44
$\Delta G_{E\text{-}S}$ (kJ/mol)	1.456	2.473
V_{max}/K_m	21.68	12.60
Q_{10}	1.0	1.0

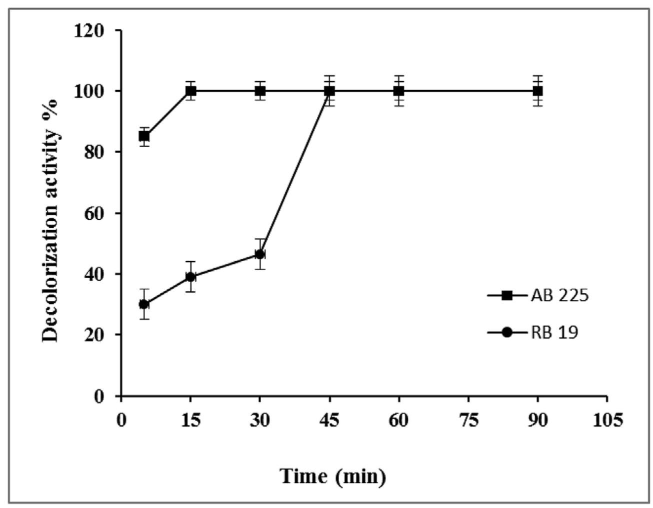


Fig. 8. Effect of decolorization time on the decolorization activity (% decreases in absorbance of dyes).

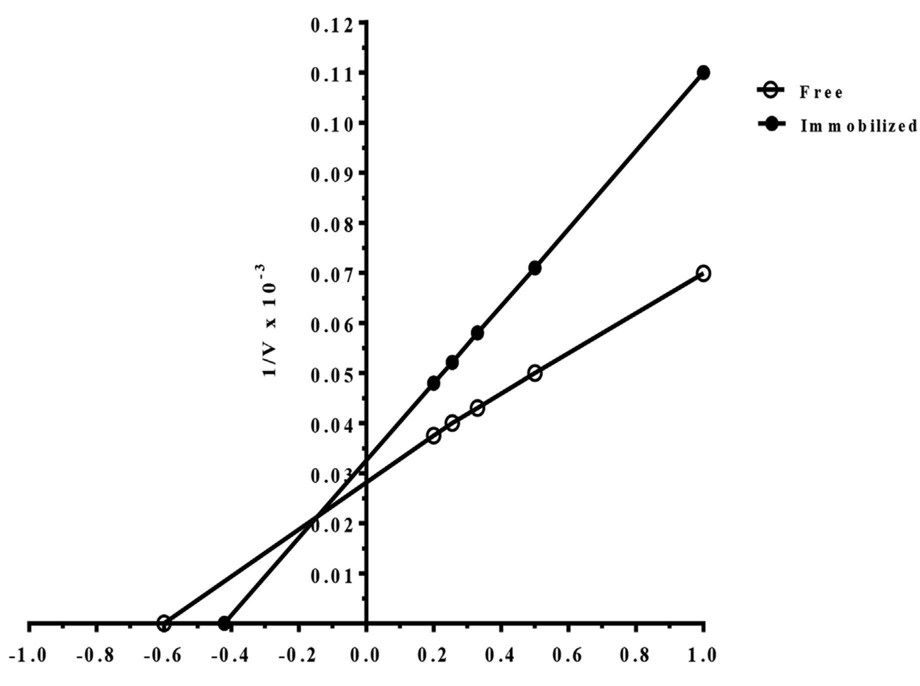


Fig. 7. Lineweaver–Burk plot for the determination of Michaelis kinetic. constants (V_{max} and K_m) for Free (○) and immobilized (●) laccase.

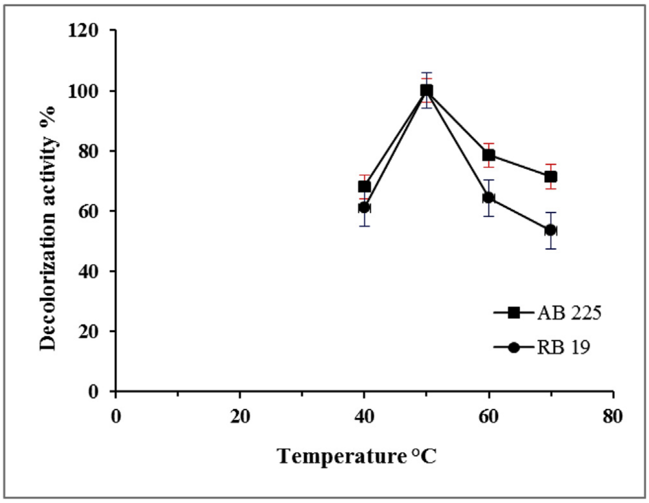


Fig. 9. Effect of temperature on the decolorization activity (% decreases in dyes absorbance).

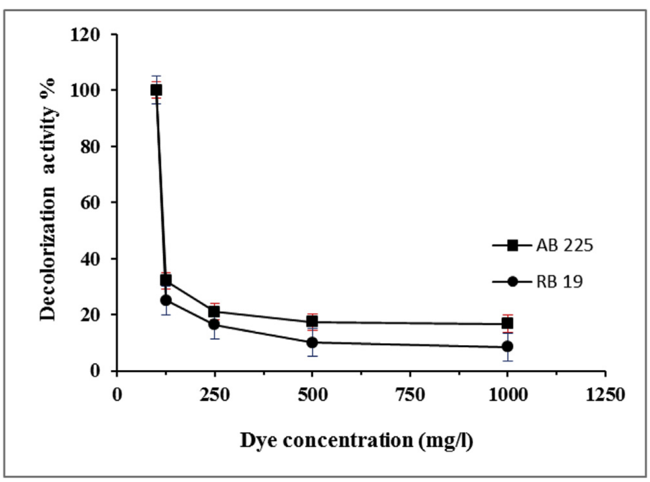


Fig. 10. Effect of initial dye concentration on the decolorization activity (% decreases in dyes absorbance). The decolorization of RB 19 was conducted at 50 °C for 45 min. The decolorization of AB 225 was conducted at 50 °C for 15 min.

2010) and (85% RR-120 after 90 min) (Celikbicak et al., 2014).

3.4.2. Effect of reaction temperature on decolorization activity

In this experiment, the effect of temperature on RB 19 and AB 225 dye decolorization was investigated at pH 4.0. As indicated in Fig. 9 the maximum decolorization activity on both RB 19 and AB 225 dye was achieved at 50 °C. Other researchers reported a lower optimum decolorization temperature for immobilized laccase, 45 °C (Ranimol et al., 2018); 35 °C (Thakur et al., 2015); 20–30 °C (Li et al., 2015; Ilk et al., 2016).

3.4.3. Effect of initial dye concentration on decolorization activity

The decolorization activity of laccase enzyme on different concentrations of RB 19 and AB 225 (100–1000 mg/l) was investigated at the optimum reaction conditions. The results (Fig. 10) illustrated that the maximum decolorization activity (100%) of both dyes was achieved with a dye concentration of 100 mg/l. As the dye concentration increases the decolorization efficiency decreases. Yesilada et al. (2014) also observed the maximum decolorization activity at a dye concentration of 100 mg/l and lowering in decolorization activity at higher concentrations. This observation was previously reported by other

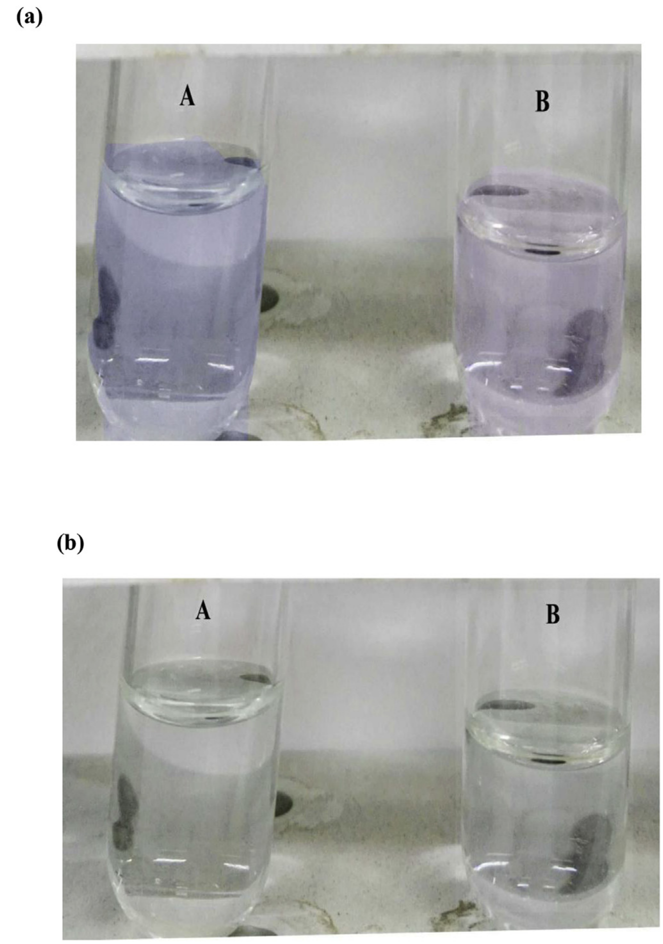


Fig. 11. (a) Synthetic wastewater solutions A (100 mg/l RB 19, 100 mg/l AB 225, 90 g/l sodium sulfate, and 20 g/l sodium carbonate) **and B** (100 mg/l RB 19, 100 mg/l RV 5, 90 g/l sodium sulfate, and 20 g/l sodium carbonate).
(b) Synthetic wastewater solutions A and B after decolorization by immobilized laccase.

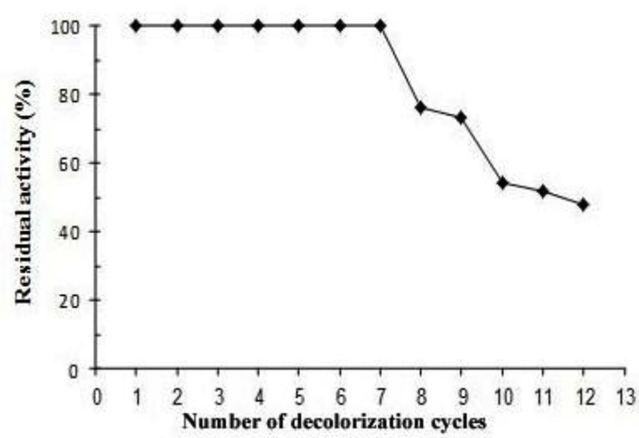


Fig. 12. Reusability of immobilized laccase in AB 225 dye decolorization.

authors as the decolorization was decreased or remains constant with increasing dye concentration (Thakur et al., 2015; Ilk et al., 2016). This could be attributed to that the higher substrate concentrations had a poisoning effect on the Nano biocatalyst and overpassed the equilibrium state of dye concentration-Nano biocatalyst dosage (Katuri et al., 2009; Mohajershojaei et al., 2014, 2015; Kashefi et al., 2019).

3.5. Effect of immobilized laccase on decolorization of synthetic wastewater solutions

The data shown in Fig. 11 indicated that both wastewater solutions A and B could effectively be decolorized (100% decolorization) by the immobilized laccase after 45 min incubation period at 50 °C. The decolorization effect of laccase enzyme against synthetic wastewater solutions was previously investigated by other authors (Nilratnisakorn et al., 2008; Yesilada et al., 2014). From these data, it is obvious that the immobilized laccase can be used in textile industries for the wastewater treatment.

3.6. Reusability of immobilized laccase in dye decolorization

The possibility of regeneration of immobilized laccase for repeated use is the most important factor for reducing the overall costs in industrial applications (Fernandez-Fernandez et al., 2013). As indicated in Fig. 12, the immobilized laccase retained 100% activity against AB 225 for 7 successive cycles. However, in the eighth cycle, the activity was decreased to 76%. The activity dropped to about 52% after 11 cycles. These results were better than that reported by other researchers, for example, Celikbicak et al. (2014) reported that the enzyme activity was decreased to 79% after five cycles of reuse. However, Zheng et al. (2016) observed that the relative activity of laccase was dropped to 90% after 3 cycles and reached 60% after 6 cycles of reuse. The decrease in enzyme activity after successive reuse could be due to enzyme inactivation and leakage of enzyme molecules during each cycle.

3.7. Storage stability

The immobilized laccase maintained 100% activity and 80% activity after storage at 4 °C for one month and two months, respectively. However, at the same storage conditions, the activity of the free enzyme was dropped to 80% and 40%, respectively. Thus, the storage stability of laccase has been improved after immobilization. Other authors also reported an increase in storage stability of laccase after immobilization (Kunamneni et al., 2008; Ilk et al., 2016; Zheng et al., 2016).

4. Conclusions

This study demonstrates that immobilization of *Polyporus durus* ATCC 26726 laccase on nanoporous ZX with high immobilization yield (83%). The immobilization of the enzyme on nanoporous ZX most probably resulted in conformational changes in the enzyme which subsequently altered the catalytic and thermodynamic properties of the immobilized enzyme. The analysis of thermodynamic data indicated that the immobilization improved both entropy and enthalpy of deactivation, thus confirming the superiority of the immobilized enzyme over the free one. The immobilized laccase was successfully used for the decolorization of RB 19, AB 225 dyes and two synthetic wastewater solutions. In addition, the immobilized preparation retained 100% of its original activity after being used for 7 successive decolorization cycles. This may provide a great advantage in reducing costs in industrial applications. Furthermore, the immobilization on nanoporous ZX offered additional advantages: it is easily prepared, non-toxic, inexpensive compared to other nanomaterials, has a large surface area and excellent loading capacity, the chemical and physical properties of ZX are stable during and after being used for several times even at high temperature. These results may provide new guidelines for laccase engineering. There are numerous reports on the immobilization of microbial laccase to improve the function, structure and stability and in turn the structure–stability–function relationships of the enzyme. However, the immobilization of *Polyporus durus* ATCC 26726 laccase on the nanoporous ZX prepared in our lab induces favorable changes in the stability and catalytic properties of the enzyme in a more simple, inexpensive and eco-friendly way. Thus, we suggest that, the nanoporous ZX prepared in our lab is better to be used for laccase immobilization and suits various needs of the biotechnology.

Conflicts of interest

The authors declare that they have no conflict of interest.

References

Abdel-Naby, M.A., 1999a. Immobilization of *Paenibacillus macerans* NRRL B-3186 cyclodextrin glucosyltransferase and properties of the immobilized enzyme. Process Biochem. 34, 399–405.

Abdel-Naby, M.A., Abdel-Mohsen, S.I., Abdel Fattah, A.M., Abdel Fattah, A.F., 1999b. Preparation and some properties of immobilized *Penicillium funiculosum* 258. Process Biochem. 34 (4), 391–398.

Abdel-Naby, M.A., Hashem, A.M., Esawy, M.A., Abdel-Fattah, A.F., 1999c. Immobilization of *Bacillus subtilis* α-amylase and characterization of its enzymatic properties. Microbiol. Res. 153 (4), 319–325.

Abdel-Naby, M.A., Ahmed, S.A., Wehaidy, H.R., El-Mahdy, S., 2017. Catalytic, kinetic and thermodynamic properties of stabilized *Bacillus stearothermophilus* alkaline protease. Int. J. Biol. Macromol. 96, 265–271.

Berka, R.M., Schneider, P., Golightly, E.J., Brown, S.H., Madden, M., Brown, K.M., Halkier, T., Mondorf, K., Xu, F., 1997. Characterization of the gene encoding an extracellular laccase of *Myceliophthora thermophila* and analysis of the recombinant enzyme expressed in *Aspergillus oryzae*. Appl. Environ. Microbiol. 63, 3151–3157.

Berrio, J., Plou, F.J., Ballesteros, A., Martinez, A.T., Martinez, M.J., 2007. Immobilization of *Pycnoporous coccineus* laccase on Eupergit C: stability increase and treatment of oil mill wastewaters. Biocatal. Biotransform. 25.

Bezerra, T.M.S., Bassan, J.C., Santos, V.T.O., Ferraz, A., Monti, R., 2015. Covalent immobilization of laccase in green coconut fiber and use in clarification of apple juice. Process Biochem. 50, 417–423.

Bhatti, H.N., Rashid, M.H., Asgher, M., Nawaz, R., Khalid, A.M., Perveen, R., 2007. Chemical modification results in hyperactivation and thermostabilization of *Fusarium solani* glucoamylase. Can. J. Microbiol. 53, 177–185.

Bokare, A.D., Chikate, R.C., Paknikar, K.M., 2008. Iron-nickel bimetallic nanoparticles for reductive degradation of azo dye Orange G in aqueous solution. Appl. Catal. B Environ. 79, 270.

Bourbonnais, R., Paice, M.G., Reid, I.D., Lanthier, P., Yaguchi, M., 1995. Lignin oxidation by laccase isozymes from T.versicolor and role of the mediator ABTS in kraft lignin depolymerization. Appl. Environ. Microbiol. 61, 1876–1880.

Catapane, M., Nicolucci, C., Menale, C., Mita, L., Rossi, S., Mita, D.G., Diano, N., 2013. Enzymatic removal of estrogenic activity of nonylphenol and octylphenol aqueous solutions by immobilized laccase from Trametes versicolor. J. Hazard Mater. 248–249, 337–346.

Celikbicak, O., Bayramoglu, G., Yılmaz, M., Ersoy, G., Bicak, N., Salih, B., Arica, M.Y., 2014. Immobilization of laccase on hairy polymer grafted zeolite particles: degradation of a model dye and product analysis with MALDI–ToF-MS. Microporous Mesoporous Mater. 199, 57–65.

D'Annibale, A., Stazi, S.R., Vinciguerra, V., Mattia, E.D., Sermanni, G.G., 1999. Characterization of immobilized laccase from *Lentinula edodes* and its use in olive-mill wastewater treatment. Process Biochem. 34, 697–706.

Dehghanifard, E., Jafari, A.J., Kalantary, R.R., Mahvi, A.H., Faramarzi, M.A., Esrafili, A., 2013. Biodegradation of 2,4-dinitrophenol with laccase immobilized on nano-porous silica beads. Iran. J. Environ. Health Sci. Eng. 10, 25.

Elias, M., Wieczorek, G., Rosenne, S., Tawfik, D.S., 2014. The universality of enzymatic rate–temperature dependency. Trends Biochem. Sci. 39, 1–7.

Erarslan, A.L., Bozoglu, T.F., Ray, B., 1996. The stabilization of biocatalytic proteins against inactivation. In: Lactic Acid Bacteria : Current Advances in Metabolism, Genetics and Applications, Bozoglu ed. Springer-verlag, Berlin, Germany, pp. 381–398.

Fernandez- Fernandez, M., Sanroman, M.A., Moldes, D., 2013. Recent developments and applications of immobilized laccase. Biotechnol. Adv. 31, 1808–1825.

Gholami-Borujeni, F., Mahvi, A.H., Naseri, S., Faramarzi, M.A., Nabizadeh, R., Alimohammadi, M., 2011a. Application of immobilized horseradish peroxidase for removal and detoxification of azo dye from aqueous solution. Res. J. Chem. Environ. 15 (2), 217–222.

Gholami-Borujeni, F., Mahvi, A.H., Nasseri, S., Faramarzi, M.A., Nabizadeh, R., Alimohammadi, M., 2011b. Enzymatic treatment and detoxification of acid orange 7 from textile wastewater. Appl. Biochem. Biotechnol. 165 (5–6), 1274–1284.

Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., 2010. Laccases: a never-ending story. Cell. Mol. Life Sci. 67, 369–385.

Htun, M.M.H., Htay, M.M., Lwin, M.Z., 2012. Preparation of zeolite (NaX,Faujasite) from pure silica and alumina sources. In: International Conference on Chemical Processes and Environmental Issues July 15-16, Singapore, pp. 212–216.

Ilk, S., Demircan, D., Saglam, S., Saglam, N., Rzayev, Z.M.O., 2016. Immobilization of laccase onto a porous nanocomposite: application for textile dye degradation. Turk. J. Chem. 40, 262–276.

Jořenek, M., Zajoncová, L., 2015. Immobilization of laccase on magnetic carriers and its use in decolorization of dyes. Chem. Biochem. Eng. Q. 29 (3), 457–466.

Kashefi, S., Borghei, S.M., Mahmoodi, N.M., 2019. Covalently immobilized laccase onto graphene oxide nanosheets: preparation, characterization, and biodegradation of azo dyes in colored wastewater. J. Mol. Liq. 276, 153–162.

Katuri, K.P., Mohan, S.V., Sridhar, S., Pati, B.R., Sarma, P.N., 2009. Laccase-membrane reactors for decolorization of an acid azo dye in aqueous phase: process optimization. Water Res. 43, 3647–3658.
Kunamneni, A., Ghazi, I., Camarero, S., Ballesteros, A., Francisco, J., Plou, F.J., Alcalde, M., 2008. Decolorization of synthetic dyes by laccase immobilized on epoxy-activated carriers. Process Biochem. 43 (2), 169–178.
Kusano, S., Shiraishi, T., Takahashi, S.I., Fujimoto, D., Sakano, Y., 1989. Immobilization of *Bacillus acidopullulyticus* pullulanase and properties of the immobilized pullalanase. J. Ferment. Technol. Bioeng. 68, 233–237.
Leonowicz, A., Sarkar, J.M., Bollag, J.M., 1988. Improvement in stability of an immobilized fungal laccase. Appl. Microbiol. Biotechnol. 29, 129–135.
Lettera, V., Pezzella, C., Cicatiello, P., Piscitelli, A., Giacobelli, V.G., Galano, E., Amoresano, A., Sannia, G., 2016. Efficient immobilization of a fungal laccase and its exploitation in fruit juice clarification. Food Chem. 196, 1272–1278.
Li, W., Sun, H., Zhang, R., 2015. Immobilization of laccase on a novel ZnO/SiO2 nanocomposited support for dye decolorization. Mater. Sci. Eng. 87.
Mahmoud, D.A.R., Refaat, H.W., Abdel-Fattah, A.F., Mahdy, E.M.E., Shousha, W. Gh, 2011. Novel application of Luffa cylindrica in production of fructose. Austr. J. Basic Appl. Sci. 5 (12), 2127–2137.
Mohajershojaei, K., Khosravi, A., Mahmoodi, N.M., 2014. Decolorization of dyes using laccase enzyme from single and binary systems. Desalin. Water Treat. 52, 1895–1902.
Mohajershojaei, K., Mahmoodi, N.M., Khosravi, A., 2015. Immobilization of laccase enzyme onto titania nanoparticle and decolorization of dyes from single and binary systems. Biotechnol. Bioproc. Eng. 20, 109–116.
Morozova, O.V., Shumakovich, G.P., 2007. Laccase-mediator systems and their applications: a review. Appl. Biochem. Microbiol. 43, 523.
Nilratnisakorn, S., Thiravetyan, P., Nakbanpote, W., 2008. Synthetic reactive dye wastewater treatment by narrow-leaved cattail: studied by XRD and FTIR. Asian J. Energy Environ. 9, 231–252.
Pye, E.K., Chance, B., 1976. Investigations of the physical properties of immobilized enzymes. Methods Enzymol. 44, 357–372.
Ramos, E.L., Mata-Gómez, M.A., Rodriguez-Duran, L.V., Belmares, R.F., Rodrigues-Herrera, R., Aguilar, C.N., 2011. Catalytic and thermodynamic properties of a tannase produced by *Aspergillus Niger* GH1 grown on polyurethane foam. Appl. Biochem. Biotechnol. 165, 1141–1151.
Ranimol, G., Venugopal, T., Gopalakrishnan, S., Sunkar, S., 2018. Production of laccase from *Trichoderma harzianum* and its application in dye decolourisation. Biocatal. Agric. Biotechnol. 16, 400–404.
Ratanapongleka, K., Punbut, S., 2017. Removal of acetaminophen in water by laccase immobilized in barium alginate. Environ. Technol. Mar. 21, 1–10.
Rekuc, A., Jastrzembska, B., Liesiene, J., Bryjak, J., 2009. Comparative studies on immobilized laccase behaviour in packed-bed and batch reactors. J. Mol. Catal. B Enzym. 57, 216–223.
Riaz, M., Perveen, R., Javed, M.R., Nadeem, H., Rashid, M.H., 2007. Kinetic and thermodynamic properties of novel glucoamylase from *Humicola sp*. Enzyme Microb. Technol. 41, 558–564.
Rodríguez Couto, S., Toca Herrera, J.L., 2006. Industrial and biotechnological applications of laccases: a review. Biotechnol. Adv. 24, 500.
Sampaio, L.M.P., Padrão, J., Faria, J., Silva, J.P., Silva, C.J., Dourado, F., Zille, A., 2016. Laccase immobilization on bacterial nanocellulose membranes: antimicrobial, kinetic and stability properties. Carbohydr. Polym. 145 (10), 1–12.
Srivastava, R.A.K., 1999. Studies on stabilization of amylase by covalent coupling to soluble polysaccharides. Enzym. Microb. Technol. 13, 164–170.
Sun, J.H., Shi, S.H., Lee, Y.F., Sun, S.P., 2009. Fenton oxidative decolorization of the azo dye Direct Blue 15 in aqueous solution. Chem. Eng. J. 155, 680.
Tavares, A.P.M., Silva, C.G., Drazic, G., Silva, A.M.T., Loureiro, J.M., Faria, J.L., 2015. Laccase immobilization over multi-walled carbon nanotubes: kinetic, thermodynamic and stability studies. J. Colloid Interface Sci. 454, 52–60.
Telke, A., Kalyani, D., Jadhav, J., Govindwar, S., 2008. Kinetics and mechanism of Reactive Red 141 degradation by a bacterial isolate *Rhizobium radiobacter* MTCC 816. Acta Chim. Slov. 55, 320.
Telke, A.A., Joshi, S.M., Jadhav, S.U., Tamboli, D.P., Govindwar, S.P., 2010. Decolorization and detoxification of Congo red and textile industry effluent by an isolated bacterium *Pseudomonas s*p. SU-EBT. Biodegradation 21, 283–296.
Thakur, V., Kumar, P., Verma, A., Chand, D., 2015. Decolorization of dye by alginate immobilized laccase from *Cercospora* SPF-6: using compact 5 stage plug flow reactor. Int. J. Curr. Microbiol. App. Sci. 4 (1), 183–200.
Vandevivere, P.C., Bianchi, R., Verstraete, W., 1998. Review: treatment and reuse of wastewater from the textile wet-processing industry: review of emerging technologies. Chem. Technol. Biotechnol. 72, 289.
Wang, P., Fan, X., Cui, L., Wang, Q., Zhou, A., 2008. Decolorization of reactive dyes by laccase immobilized in alginate/gelatin blent with PEG. J. Environ. Sci. 20 (12), 1519–1522.
Wehaidy, H.R., El-Hennawi, H.M., Ahmed, S.A., Abdel-Naby, M.A., 2018. Comparative study on crude and partially purified laccase from *Polyporus durus* ATCC 26726 in decolorization of textile dyes and wastewater treatment. Egypt. Pharm. J. 17 (2), 94–103.
Weiwei, H., Yuxiang, Y., Bin, W., Hongming, Y., Yani, Z., Xiangnong Chin, L., 2012. Degradation of 2,4-DCP by the immobilized laccase on the carrier of Fe3O4@SiO2-NH2. J. Chem. 30, 2849–2860.
Yesilada, O., Birhanli, E., Ercan, S., Özmen, N., 2014. Reactive dye decolorization activity of crude laccase enzyme from repeated-batch culture of *Funalia trogii*. Turk. J. Biol. 38, 103–110.
Youssef, H.F., Hegazy, W.H., Abo-almaged, H.H., El-Bassyouni, G.T., 2015. Bioinorganic Chemistry and Applications. pp. 12.
Zamora, P.P., Pereira, C.M., Tiburtiusm, E.R.L., 2003. Appl. Catal. B Environ. 42, 131.
Zheng, F., Cui, B.K., Wu, X.J., Meng, G., Liu, H.X., Si, J., 2016. Immobilization of laccase onto chitosan beads to enhance its capability to degrade synthetic dyes. Int. Biodeterior. Biodegrad. 110, 69–78.
Zille, A., Tzanov, T., Gübitz, G.M., Paulo, A.C., 2003. Immobilized laccase for decolorization of reactive black 5 dyeing effluent. Biotechnol. Lett. 25, 1473.