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Catalytic, Kinetic, and Thermodynamic Properties of *Pleurotus Ostreatus* NRRL 3501 Laccase Immobilized on Clinoptilolite Nanoparticles and its Application in Dye Decolorization

Hala R. Wehaidy [1*], Shireen A. A. Saleh [1], Walaa A. Abdel Wahab [1], Samia A. Ahmed [1], Hanan F. Youssef [2], Heba M. El-Hennawi [3], Mohamed A. Abdel-Naby [1]

[1] Chemistry of Natural and Microbial products Department, National Research Centre, Dokki, Giza, Egypt
[2] Refractories, Ceramics and Building Materials Department, National Research Centre, Dokki, Giza, Egypt
[3] Dyeing, Printing and Textile Auxiliaries Department, National Research Centre, Dokki, Giza, Egypt

In Loving Memory of Late Professor Doctor ""Mohamed Refaat Hussein Mahran""

Abstract

Laccase from *Pleurotus ostreatus* was immobilized on different nanoparticles by physical adsorption and covalent binding. The immobilized enzyme on Na-clinoptilolite nanoparticles showed the highest recovered activity (71.75%) and thermal stability. X-Ray diffraction, surface morphology and the internal structure of the immobilization carrier (Na-clinoptilolite nanoparticles) were investigated. Compared to the free enzyme, the immobilized preparation exhibited higher optimum temperature, lower activation energy (*Ea*), lower deactivation constant rate (*kd*), higher $t_{1/2}$ and higher decimal reduction time values (*D*) within the temperature range of 50–70◦C. The thermodynamic parameters (ΔH^*, ΔG^*, ΔS^*) of irreversible thermal denaturation for the free and immobilized laccase were also evaluated. The values of enthalpy of activation (ΔH*), and free energy of transition state binding (ΔG^*_{E-T}) for substrate catalysis were lowered for the immobilized enzyme. Moreover, there was highly significant impact on improving the values of V_{max}/K_m, k_{cat}, k_{cat}/K_m, and ΔG^*_{E-S} for the immobilized enzyme. The immobilized laccase from *Pleurotus ostreatus* NRRL 3501 on Na-clinoptilolite nanoparticles was evaluated for dye decolorization. In batch experiments the immobilized enzyme was able to decolorize the following dyes R. Blue 203 (97%), A. red 299 (100%), and Acid mix (89%) after incubation for 120 min. In repeated use the immobilized enzyme was able to decolorize the dye mixture for 8 continuous cycles.

Keywords:: Laccase immobilization; Zeolite nanoparticles; sodium-clinoptilolite pellets; dye decolorization..

1. Introduction

Environmental pollution is a serious problem in the world. Land and water pollutants play a significant role in lowering the quality of life due to their detrimental effects on human health **[1].** Both soil and water contamination are caused by a variety of degradation-resistant dyes **[2]**. Enzymatic degradation of pollutants has become an important component of many sectors due to its effectiveness, lesser toxicity and greater availability **[3]**.

Laccases (EC.1.10.3.2 parabenzene diol oxidoreductase) are member of copper-containing oxidases which catalyze the breakdown of a wide variety of substrates, including lignin, aromatic amines, phenolic substrates, phosphates, ketones and ascorbates **[4-5]**. Laccases are able to convert substituted phenolic compounds into simpler ones by one-electron oxidation. This process is accompanied with the reduction of oxygen to water **[6]**.

Textile industry consumes large quantities of synthetic dyes and their residuals left in wastewater are considered as the main source of serious toxic environmental pollutants which threat human and plant life. These synthetic dyes exhibited high stability and are resistant to light, temperature, detergents, and microbial attack. Recently, the degradation of dyes using microbial enzymes has gained more attention. As most dyes are aromatic compounds, they can be degraded by microbial laccase oxidation **[7-9]**.

There are many difficulties associated with utilization of enzymes in industry because most enzymes are usually expensive and due to the high costs of their isolation and purification. Free enzymes are highly sensitive to temperature, pH and chemicals at trace levels. Most enzymes act in solutions which lead to product contamination and difficulty in their recovery from the reaction mixtures and reuse. The ideal solution to overcome these problems is the use of immobilized enzymes **[10]**. Laccase can be immobilized by a variety of techniques. With the advancement of nanotechnology, laccase was immobilized on silica nanoparticles **[11]**, nanotubes **[12]**, and on magnetic particles coated with chitosan **[13]**.

Zeolites are aluminosilicate rocks that have specific groups of uniformly–sized interconnected nanopores arranged in three-dimensional channel systems. Micro and mesoporous districts are characterizing zeolite constructions, where cations and molecules can reside or exchange, helping in many useful applications including catalysis, separation, and ion exchange fields **[14-15]**. Natural and synthetic zeolites have been utilized in agriculture **[16]**, detergents **[17]**, oil refining **[18]**, and as a purifying agent in wastewater treatment **[18-19]**. In addition to its role in the remediation of liquid radioactive wastes **[20]**.

Clinoptilolite (CLINO) is the most abundant component of the heulandite zeolite family in nature. Its average physical properties are 34% porosity, 2.16 meq/g ion exchange capacity, 2.15-2.25 g/cm specific gravity, and 1.15 gm/cm3 bulk density **[21]**. The theoretical unit cell of clinoptilolite is given as $(Na,K)_6(Al_6Si_{30}O_{72}).20H_2O$ **[22]**.

*Corresponding author e-mail: halolty2009@yahoo.com, hr.wehaidy@nrc.sci.eg, Hala R. Wehaidy
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Since the immobilization process offers the advantages of increasing enzyme stability, easy enzyme recovery and reuse. So, the main goal of this work is the development of an eco-friendly immobilized stabilized enzymatic system aiming at the removal of the hazardous micropollutants in textile wastewater. The enzymatic system is based on immobilization of laccase enzyme onto Clinoptilolite nanomaterial. This nanomaterial was evaluated in enhancing the catalytic and kinetic properties of laccase enzyme and also improving its thermal stability. The operational stability of the immobilized enzyme was evaluated also in repeated batch process.

2.Materials and methods

2.1. Microorganism

Pleurotus ostreatus NRRL 3501was obtained from the Microbiological Resources Center (Mircen, Faculty of Agriculture, Ain Shams University).

2.2. Laccase enzyme production:

Laccase enzyme production by *Pleurotus ostreatus* NRRL 3501 was conducted by submerged fermentation using the following medium (g/l): Wheat bran, 60; peptone, 3.0; $MgSO_4$.7H2O, 2.0; KH_2PO_4, 3.0 and glucose, 2.0 according to **Wehaidy et al.** with some modification **[23]**. Fermentation was conducted in 250 ml-Erlenmeyer flask containing 50 ml of the production media. After sterilization, medium was inoculated with 2 ml of inoculum suspension and incubated at 150 rpm and 28 °C for different incubation periods (5, 7 and 10 days). Samples were collected by centrifugation at 5000 rpm for 20 min at 4 °C. The supernatant was used as the crude laccase enzyme.

2.3. Determination of laccase activity

This was done according to **Bourbonnais et al. [24].** One unit of laccase activity was defined as activity of an enzyme that catalyzes the conversion of 1 µ mole of ABTS (ε420= 36,000 M^{-1} cm^{-1}) per minute.

2.4.Nanomaterials used for laccase immobilization

The prepared Zeolite nanoparticles (Zeolite-A and Zeolite-X) were prepared in lab and used as solid carriers for enzyme immobilization. The used zeolite samples of Clinoptilolite zeolite (CLINO) are of the commercial grade sodium form of clinoptilolite (Na-CLO), supplied by Yemen Zeolite Company, Yemen. It was supported as granules of different grain sizes, ranging from 1-5 cm.

2.5. Characterization of nanoparticles used for laccase immobilization

2.5.1.X-Ray Diffraction (XRD)

Clinoptilolite (CLINO) was analyzed by X-ray diffraction (XRD) using BRUKUR D8 ADVANCE with secondary monochromatic beam Cu Kα radiation at KV = 40 and mA = 40.

2.5.2. Scanning Electron Microscopy (SEM) and chemical microanalysis (EDs)

The surface morphology and the internal structure of Clinoptilolite (CLINO) was 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. EDs provides the microchemical analysis for the used nanomaterial.

2.6. Laccase immobilization by physical adsorption

0.05 g of the nano-carriers and 0.125 ml enzyme solution were incubated at 4°C for 24 h. The unbound enzymes were removed by washing with buffer.

2.7. Laccase immobilization by covalent binding

Nanoparticles were activated by 0.5 ml glutaraldehyde 2.5 % for 2 h at 30 °C. Subsequently, the activated carriers were washed several times with buffer solution. Then 0.05 g of activated nanoparticles were incubated with enzyme solution (0.125 ml) at 4 °C for 24 h. The unbound enzyme was removed by washing with buffer.

2.8. The catalytic, kinetic and thermodynamic properties of free and immobilized *Pleurotus ostreatus* NRRL 3501 laccase on clinoptilolite Zeolite nanoparticles

2.8.1. Optimum pH

The optimum pH of the reaction for the free and immobilized laccase was investigated at pH range from 2.0 to 6.0.

2.8.2. Optimum temperature

The optimal reaction temperature of the free and immobilized laccase was investigated in the temperature range 30 – 80◦C at the optimum pH for each enzyme preparation. The activation energy (*Ea*) was calculated from Arrhenius plot according to the following equation:

Slope = $-Ea/(2.303R)$,

Where: R = gas constant (8.314 $KJ.mol^{-1}$).

2.8.3. Effect of substrate concentration

Laccase was assayed with assay mixture contained varying amounts of substrate (ABTS) ranging from 1.0 to 7.0 mg/ml (w/v). The thermodynamic parameters

for substrate catalysis were determined as follows **[25]**:

as follows:

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

Where:

k_b Boltzmann's constant *(R/N)*=1.38×10^{-23} J K^{-1}

T Absolute temperature (K)

h Planck's constant =6.626×10^{-34} Js

N Avogadro's number =6.02×10^{23} mol^{-1}

R Gas constant=8.314 J K^{-1} mol^{-1}

ΔH^* (Enthalpy) = E_a – R*T* ---------- (2)

ΔG^* (Gibbs free energy of activation) =

$-RT \ln (k_{cat} h / k_b \text{ x } T)$ ---------- (3)

ΔS^* (Entropy) = $(\Delta H^* - \Delta G^*) / T$ ---------- (4)

Free energy of substrate binding

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

Free energy for transition state formation

$\Delta G^*_{E-T} = -RT \ln (k_{cat}/K_m)$ ---------(6)

2.8.4. Thermal stability of free and immobilized laccase enzyme

In the absence of substrate, the enzyme samples were incubated at the temperature range (50–70ºC) for 60 min. Enzyme sample was removed every 15 min and cooled. Then the residual activity was determined at the standard assay conditions. The deactivation rate constant (k_d) was calculated when Log residual activity (%) was plotted against time. The energy of activation for irreversible thermal inactivation $E_{a(d)}$ was determined from Arrhenius plot of $\ln K_d$ against 1/T in Kelvin. Thermodynamic parameters of irreversible inactivation of the enzyme were calculated as follows:

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

The values of ΔH^*, ΔG^*, and ΔS^* of irreversible inactivation were calculated by applying Eqs. 2, 3, and 4 with the modifications that in Eq. 2 $E_{a(d)}$ was used instead of E_a and in Eq. 3, K_d was used in place of k_{cat} .

$$Q_{10} = \text{antilog } E = (E \text{ x } 10/RT^2) \quad \text{---------- (8)}$$

Where $E = E_a$ = activation energy

Decimal reduction time (*D* value) was defined as the time required for 90% reduction in the initial enzyme activity at a specific temperature, and it was calculated as shown in Eq. (9):

$$D = 2.303/k_d \quad \text{---------- (9)}$$

2.9. Utilization of clinoptilolite immobilized laccase from *Pleurotus ostreatus* NRRL 3501 for dye decolorization

2.9.1. Utilization of immobilized laccase in oxidation of different dye solutions

The dye solutions of acid red 299 and reactive blue 203 were prepared by diluting 0.01 g of dye in one liter of distilled water. On the other hand the mix of commercial dyes used is the residual of the dyeing was diluted to the required concentration 0.01 g/L. The dye enzyme solution was prepared by adding equal amount of buffer (pH 5) and dye solution (1:1) ml to the enzyme then put it in shaking water bath at 50C◦ for intervals of time. The absorbance and wave length were measure using double-beam spectrophotometer Thermo Electron Corporation Unican 300, England.

Decolorization (%)= (initial absorbance – final absorbance / initial absorbance) × 100

2.9.2. Repeated use of immobilized enzyme

In this experiment, decolorization of dye was performed using immobilized *Pleurotus ostreatus* NRRL 3501 on clinoptilolite nanoparticles. The operational stability of the immobilized preparation was evaluated in repeated batch process.

2.10. Statistical analysis

All experiments were performed in triplicate, the average and standard deviation were calculated by Excel and used for the calculation of results throughout the work.

3. Results and discussion

3.1. Laccase enzyme production:

The fungus *Pleurotus ostreatus* NRRL 3501 is a well-known laccase producer. It proved to be a potent producer of laccase enzyme (56.4 U/ml) by submergrd fermentation after 5 days of incubation **(Table 1)**. However, the enzyme productivity was decreased after 7 and 10 days of incubation and that might be due to the nutrients depletion in the medium. The white rot fungus *Pleurotus ostreatus* belongs to a subclass of lignin-degrading microorganisms that produce laccases and manganese peroxidases **[26]**.

3.2. Characterization of CLINO nanoparticles

3.2.1. X-Ray Diffraction (XRD)

Clinoptilolite (CLINO) was analyzed by X-ray as shown in **Fig. 1** which represents the XRD mineral patterns for the nanomaterial. The peak intensities and positions are confirming the identity of the nanoparticle.

Table 1: Screening of some microbial strains for laccase enzyme production

Strain	Incubation period (day)	Laccase activity (U/ml)
Pleurotus ostreatus NRRL 3501	5	56.4 ± 0.95
	7	22.6 ± 0.58
	10	17.2 ± 0.15

*Values = Average of 3 replicates ± standard deviation.

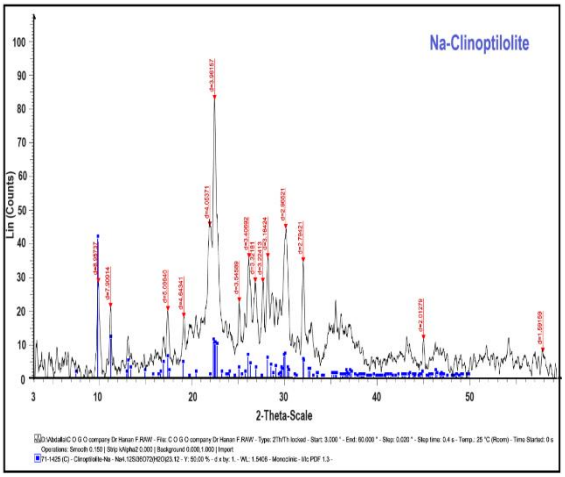


Fig. 1: XRD pattern for the used sodium-clinoptilolite pellets

3.2.2. Scanning Electron Microscopy (SEM)

The surface morphology and the internal structure of CLINO nanomaterial was investigated by scanning electron microscopy (SEM) as illustrated in Fig. 2. It shows platy crystals of different sizes for the CLINO that varies from 5-70 µm in length, and 2-20 µm in width.

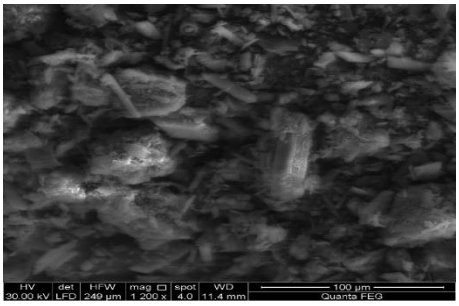


Fig. 2: SEM for Na-clinoptilolite

3.3. Immobilization of *Pleurotus ostreatus* NRRL 3501 laccase on nanomaterials

Pleurotus ostreatus NRRL 3501 laccase enzyme was immobilized by physical adsorption and covalent binding using Zeolite- A, Zeolite- X and clinoptilolite zeolite (CLINO) . In both physical and covalent immobilization, the highest Immobilization yield (71.75%) was obtained with clinoptilolite nanocarrier by covalent binding **(Table 2, 3).** Therefore, CLINO will be used for laccase immobilization by covalent binding throughout the upcoming work.

Table 2: Physical immobilization of laccase enzyme

Nanoparticle	Enzyme added Activity (U/g) A	Immobilized enzyme Activity (U/g) I	Immobilization yield = (I/A) %
Clinoptilolite zeolite	180.4	102.5 ± 0.5	56.82 ± 0.28
Zeolite-A	180.4	76 ± 0.58	42.13 ± 0.32
Zeolite-X	180.4	38.6 ± 0.2	21.39 ± 0.12

*All values = Average of 3 replicates ± standard deviation.

Table 3: Covalent immobilization of laccase enzyme

Nanoparticle	Enzyme added Activity (U/g) A	Immobilized enzyme Activity (U/g) I	Immobilization yield = (I/A) %
Clinoptilolite zeolite	88	63 ± 0.76	71.75 ± 0.7
Zeolite-A	88	16 ± 0.76	18.18 ± 0.87
Zeolite-X	88	18 ± 1	20.45 ± 1.1

All values = Average of 3 replicates ± standard deviation.

3.4. Chemical microanalysis (EDs) of CLINO

Fig. 3 and **Table 4** are showing the micro-chemical analysis of the CLINO sample. In **Table 4**, the calculated Si/Al ratio was 6.215 which exceeded the theoretical known ratio for the two elements in literature (Si/Al=5). So, this in hand sample is denoted as a high silica Na-CLO form. It is well known that, the amount of Al in any zeolite structure is the direct reflection of its alkali (Na or K) contents **[27]**. Therefore, there is a deficiency in the sodium amounts (3.60%) than the theoretical one (supposed to be 6%) in this structure.

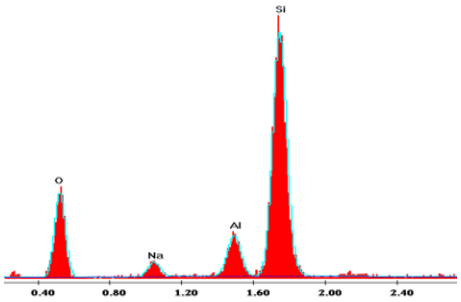


Fig. 3: EDAX for Na-clinoptilolite

Table 4: EDAX *analysis* of CLINO

Element	Weight%	Atomic %
O K	36.80	50.09
Na K	3.80	3.60
Al K	7.95	6.42
Si K	51.45	39.90
Total	100.00	100.00

3.5. The catalytic, kinetic and thermodynamic properties of free and immobilized *Pleurotus ostreatus* NRRL 3501 laccase on CLINO nanoparticles

3.5.1. Optimum pH

The results **(Fig. 4)** indicated that both free and immobilized enzyme were optimally active at pH 4.0. However, the immobilized enzyme was more tolerable to the changes of pH of the reaction. For example, the relative activity of the free enzyme at pH 2.5 and 8 were 60 and 36% compared to 68 and 78 % recorded for the immobilized form **(Fig. 4).** Similar observations were reported by other authors **[28-32].**

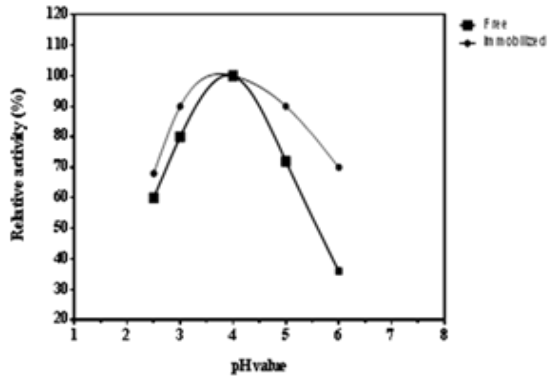


Fig. 4: Effect of pH of reaction on the activity of free and immobilized *Pleurotus ostreatus* NRRL 3501 laccase

3.5.2. Optimum temperature

The results showed that the optimum temperature for free and immobilized laccase was 70 ºC. Many authors also recorded the same optimum temperature for both the free and immobilized laccase enzyme **[31-36]**.

Arrhenius plots of temperature data **(Fig. 5)** showed that the calculated activation energy for the free enzyme was 24.8 (kJ.mol^{-1}) which is higher than that of the immobilized enzyme (18.95 kJ.mol^{-1}). Decreasing of the activation energy for the immobilized enzyme means that it had better catalytic efficiency since it required lower energy to make enzyme–substrate complex. This value for *Ea* of the immobilized laccase was much lower than that reported previously for other laccases (44.12 kJ mol^{-1}) **[37]**.

3.5.3. Effect of substrate concentration and determination of kinetic parameters *(K_m and V_{max})*

The kinetic parameters (K_m and V_{max}) of free and immobilized laccase from *Pleurotus* ostreatus NRRL 3501 were calculated from Lineweaver-Burk plots (**Fig 6)**. The enzymatic reaction for substrate catalysis was conducted at the optimal reaction conditions.

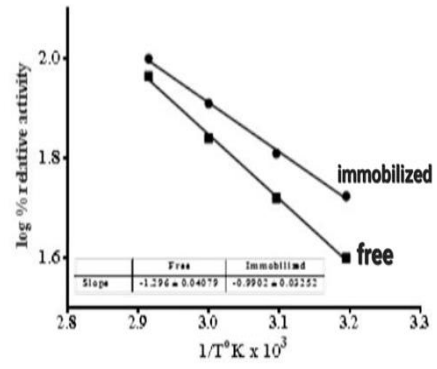


Fig. 5: Arrhenius Plots of temperature data for free and immobilized laccase

The K_m constant is a measure of the ability of enzyme to bind to the substrate. The lower the enzyme's affinity for the substrate, the higher the K_m value. This plays a role in conformational changes and steric hindrance in the protein molecule, which decreases the opportunity of formation of immobilized enzyme-substrate complex **[38].**

The results **(Fig 6)** indicated the free enzyme had lower K_m (0.76 mg ABTS ml^{-1}) compared to that of immobilized form (0.89 mg ABTS /ml) and this might be due to diffusion resistance of the substrate through the immobilization matrix and due to low availability of substrate to the immobilized enzyme active site. On the other hand, higher value was recorded for the maximum velocity of the reaction (V_{max}) for the free enzyme (571 U /mg protein) compared to the corresponding value of immobilized form (142.85 U /mg protein). Fixation of the enzyme on the immobilization matrix might result in increasing the rigidity of the enzyme, and reducing the catalytic activity of the immobilized enzyme **[39-40].** Subsequently, the maximum rate of the reaction of immobilized enzyme was lower than that of the free form.

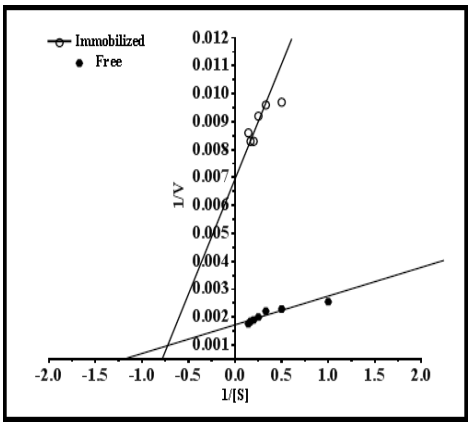


Fig. 6: Lineweaver–Burk plot of free and immobilized *Pleurotus ostreatus* NRRL 3501 accase

3.5.4. Thermal stability of free and immobilized *Pleurotus ostreatus* NRRL 3501 laccase

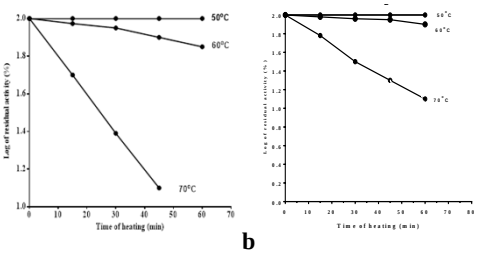
Thermal inactivation of free and immobilized *Pleurotus ostreatus* NRRL 3501 laccase was investigated at the range from 50 to 70 ºC. The data **(Fig 7 a & b, Table 5)** showed that both free and immobilized form were stable at 50ºC for 60 min. However, higher thermal stability were detected for the immobilized enzyme Thus, the thermal deactivation constant values (K_d) of free enzyme at 60°C and 70°C were 0.0025 min^{-1}, and 0.0066 min^{-1} respectively, against 0.00005, 0.0015 min^{-1} for the immobilized enzyme, at the same temperatures.

In addition, the calculated $t_{1/2}$ values at 60ºC, and 70ºC for the immobilized laccase were 5.0, and 4.4– times higher than the free enzyme. Furthermore, the

value of E_{ad} was calculated to be 376.8 KJmole^{-1} for immobilized enzyme and 166.28 KJmole^{-1} for the free enzyme **(Fig 8, Table 5)**. This indicates that more energy is required to inactivate the immobilized laccase **[41]**. Thus the conformational possibilities of the unfolded state were restricted and hence it implied to be more thermostable.

Table 5: Thermal properties of free and immobilized laccase from *Pleurotus ostreatus* NRRL 3501

Kinetic parameter	enzyme	
	Free	Immobilized
Optimum temperature	70ºC	70ºC
Ea(kJ^{-1} mol)	24.81	18.95
$t_{1/2}$ (min), at:		
50°C	Stable	Stable
60°C	680	3400
70°C	257.57	1133
Thermal deactivation constant		
K_d (min^{-1}), at:		
50°C	Stable	Stable
60°C	2.5x10^{-3}	0.5x10^{-3}
70°C	6.6x10^{-3}	1.5x10^{-3}
Decimal reduction time *(D)* min		
50°C	Stable	Stable
60°C	921.2	4606
70°C	348.9	1535.3
Deactivation energy E_{ad} (KJ/mol)	166.28	376.8



a **b**

Fig. 7: Thermal stability of Free (a) and immobilized (b) Pleurotus ostreatus NRRL 3501 laccase

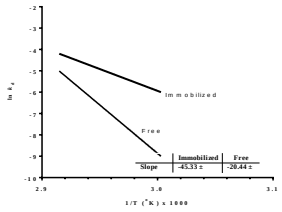


Fig. 8: Arrhenius plots of thermal denaturation (E_{ad}) of free and immobilized *Pleurotus ostreatus* NRRL 3501 laccase

Kikani and Singh reported that most stable enzymes, require higher energy for denaturation **[42]**. Therefore, we can say that the immobilized laccase is more thermostable than the free laccase.

3.5.5. The Thermodynamic Studies

As illustrated in **Table 6**, the turnover number (k_{cat}) of Free laccase (10.86 S^{-1}) was lower than that of immobilized form (13.58 S^{-1}).

Table 6: Thermodynamic parameters for substrate catalysis by free and immobilized laccase from *Pleurotus ostreatus* NRRL 3501

Parameter	Enzyme	
	Free	Immobilized
Km(mg/ml)	0.76	0.89
Vmax (U/mg protein)	571	142.85
K_{cat} (S^{-1})	10.86	13.58
K_{cat}/ K_m	14.28	15.25
Ka (1/Km)	1.31	1.123
ΔH^* (kJ.mol^{-1})	21.948	16.098
ΔG (kJ.mol^{-1})	77.6	77.963
ΔS (J.mol^{-1}.K^{-1})	-162.25	-180.36
$\Delta G_{E\text{-}T}$((kJ.mol^{-1})	-7.58	-7.77
$\Delta G_{E\text{-}S}$ ((kJ.mol^{-1})	-0.77	-0.33
V_{max} / K_m	751.3	160.5
Q_{10}	1.24	1.18

Moreover, the catalytic efficiency (k_{cat}/K_m) for ABTS decolonization by free laccase (14.28 S^{-1} mg ml^{-1}) was lower than that of the immobilized form (15.25 S^{-1} mg ml^{-1}) **(Table 6)**. The thermodynamic parameters of substrate (ABTS) catalysis by immobilized laccase from *Pleurotus ostreatus* NRRL 3501 showed pronounced changes, For example, the value of enthalpy of activation (ΔH^*) for immobilized laccase (16.095 kJ mol^{-1}) was lower than the recorded value free enzyme (21.948 kJ mol^{-1}). Similarly, the value of entropy of activation (ΔS^*) was lower for the immobilized enzyme by about 18.11 J mol K^{-1}. However, the magnitude of ΔG^* (the free energy of activation) was higher for the immobilized laccase by about 0.363 kJ mol^{-1}. The lower value of enthalpy (ΔH^*) and negative value of entropy (ΔS^*) recorded for immobilized laccase suggested the formation of an efficient and more ordered transition state of enzyme-substrate complex (ES*) **[43]**.

Riaz et al. reported that the feasibility and extent of an enzymatic reaction is best evaluated by measuring the change in ΔG^* for conversion of an enzyme-substrate complex into a product **[44]**. Thus, The low ΔG^* value suggest that the conversion of a transition state of enzyme-substrate complex into a product was more spontaneous. In the present study, the recorded value of $\Delta G^*_{E\text{-}T}$ immobilized laccase (-7.77 kJ.mol^{-1}) was lower than the free laccase (-7.58 kJ.mol^{-1}). Additionally, the free energy of substrate binding ($\Delta G^*_{E\text{-}S}$) for immobilized laccase (-0.33 kJ.mol^{-1}) was higher than free enzyme (-0.77 kJ/mol^{-1}). This indicates that immobilized enzyme needs lower amount of free energy (ΔG^*_{E-T}) to form activated (transition) complex and releases higher amount of transition state binding energy (ΔG^*_{E-S}) as compared with the free enzyme. Further, the catalytic efficiency (k_{cat}/K_m) of immobilized laccase was higher as compared to the free laccase **(Table 6)**.

The calculated values of thermodynamic parameters for irreversible inactivation **(Table 7)** indicated that immobilization of *Pleurotus ostreatus* NRRL 3501 laccase showed a drop in the values of both ΔS^* and ΔH^*. Furthermore, gradual increase of

temperature resulted in decreasing trend in the values of enthalpy and entropy of the immobilized *Pleurotus ostreatus* laccase, indicated its stabilization. For instance, the free lacccase recorded 22.12, 22.04, and 21.95 kJ mol^{-1} of ΔH^* (enthalpy of deactivation) at 50, 60, and 70°C. However, lower values of ΔH^* were recorded for the immobilized laccase (16.26. 16.18, and 16.10 kJ mol^{-1}, at 50, 60, and 70°C respectively). This could be attributed to conformational changes of the immobilized laccase which reflected a partially unfolded transition state. Moreover, the values of entropy of deactivation (ΔS^*) at 70°C for the immobilized lacccase was -276.23J $mol^{-1}k^{-1}$. However, higher values were recorded for the free laccase (-197.87 J $mol^{-1}k^{-1}$). The decrease in ΔS^* represents a lower difference in entropy between the transition state and ground state of reactants. This is commonly caused by multiple fixation of the enzyme to the immobilization carrier, which is commonly showing enzyme stabilization.

Table 7: Thermodynamic parameters for irreversible inactivation of free and immobilized laccase

Temperature (°C)	T (°K)	ΔH^* (kJ/ mol)	ΔG^* (kJ / mol)	ΔS^* (J /mol /K)
Free				
50	323	22.12	90.60	-212.01
60	333	22.04	91.69	-200.80
70	343	21.95	92.68	-197.87
immobilized				
50	323	16.26	110.42	-283.18
60	333	16.18	112.12	-279.78
70	343	16.10	113.70	-276.23

Singh et al [45] reported that the decreasing of ΔH^* and ΔS^* values with the immobilized enzyme indicated that the conformation changes of the immobilized enzyme was transformed in the direction of partially unfolded transition state.

3.6. Utilization of CLINO immobilized laccase for dye decolorization

3.6.1.Utilization of immobilized laccase in oxidation of different dye solutions

Table 8 illustrates the decolorization of reactive blue 203, acid red 299 and acid dye mixture by CLINO immobilized laccase. As shown in the table, the immobilized enzyme was able to decolorize R. Blue 203 (97%), and Acid dye mixture (89%) after incubation for 120 min. However, A. red 299 dye was completely decolorized (100% decolorization) after the same incubation period. This time of decolorization is much shorter than that investigated by other researchers for decolorization of some dyes by immobilized laccase (75% Methyl Green decolorization after 6 h, and 97% Congo red decolorization after 12 h) **[36,46]**.

3.6.2. Repeated use of immobilized enzyme

The results **(Fig. 9)** indicated the stability of the immobilized enzyme in repeated use. Thus, the immobilized enzyme could decolorize the dye mixture for 8 continuous cycles. The remaining activity was about 45% of the initial catalytic activity after 8 cycles. These results for repeated use were better than those investigated for laccases by other authors, for example, **Celikbicak et al.** observed that laccase activity was declined to 79% after five cycles of decolorization **[47]**. However, **Zheng et al.** observed that the activity of laccase was declined to 60% after 6 successive cycles **[48]**. This drop in enzyme activity might be attributed to deactivation of enzyme and leakage of enzyme molecules during the successive reuse. The opportunity of reuse of immobilized laccase is a very important factor for cost reduction in the industrial sector **[49].**

Table 8: Decolonization of dyes and dye mixture by CLINO immobilized laccase

Time (min.) / Dye used	Decolorization %			
	30 min	60 min	90 min	120 min
R. Blue 203	13	26	64	97
A. Red 299	70	73	89	100
Acid dye mixture	57	82	88	89

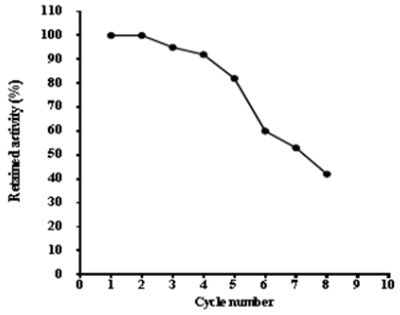


Fig 9: Operational stability of immobilized *Pleurotus ostreatus* NRRL 3501 laccase

4. Conclusion

Laccase immobilized on Na-clinoptilolite showed high thermal stability. Compared to the free laccase, the immobilized laccase had higher optimum temperature, lower activation energy (*Ea*), lower deactivation constant rate (*kd*) and higher $t_{1/2}$ within the temperature range of 50–70◦C. The values of enthalpy of activation (ΔH^*), and free energy of transition state binding (ΔG^*_{E-T}) for substrate catalysis were lowered for the immobilized enzyme. In batch experiments the immobilized enzyme was able to decolorize R. Blue 203, A. red 299, and Acid mix dye after incubation for 120 min. The immobilized enzyme was able to decolorize the dye mixture for 8 continuous cycles with a remaining activity 45% of the initial catalytic activity. Therefore, covalent immobilization of laccase on Na-clinoptilolite had improved the thermal stability and the thermodynamic

parameters of the enzyme. So, we can say that Na-clinoptilolite is an ideal carrier for laccase immobilization. Furthermore, the performance of immobilized enzyme system was very effective in dye decolorization and it is recommended to be used on large scale application in textile wastewater treatment.

Conflicts of interests:

The authors declare that there are no conflicts of interests.

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