



## Purification and Studying Some Characters of Laccase Enzyme from Irradiated *Pleurotus Ostreatus* ATCC 56270

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Received 11<sup>th</sup> May 2018 Accepted 19<sup>th</sup> Feb. 2019 Purification of laccase enzyme by anion exchange chromatography using DEAE sepharose and sephacryl HR are successful in purifying laccase. SDS- PAGE is the determined of laccase molecular weight which is 75KDa. The optimum pH for activity was 5 and for stability was 6.5-7.5. The optimum temperature for activity was 35°C and stability of the enzyme was stable up to 55°C, the enzyme retained 100% of its activity after 20 minutes incubation, 85% of its activity after 1h incubation at the same temperature. The activation energy ( $E_{act}$ ) of the purified laccase between 20°C and 45°C at its optimum pH was 16.3 k Jmol<sup>-1</sup>. The apparent  $K_m$  value of the enzyme for ABTS was estimated to be 1.4 mM under standard assay conditions,  $k_{cat}$  value of ABTS was calculated to be 3450 min<sup>-1</sup> and  $V_{max}$  is the maximum initial rate in the Michaelis-Menten equation (1.2 μmol L<sup>-1</sup> min<sup>-1</sup>). Microencapsulation of laccase exhibited that free laccase (35U/ml) has higher activity than encapsulated laccase (24.3 U/ml).

**Keywords:** Laccase, *Pleurotus ostreatus*, Purification, Properties, Microencapsulation

### Introduction

Laccase (EC 1.10.3.2) is a blue copper protein, but it also falls within the broader description of polyphenol oxidases. Polyphenol oxidases are copper proteins with the common feature of being able to oxidize aromatic compounds with molecular oxygen as the terminal electron acceptor [1].

Laccases exists in 3 different classes as botanical, fungal and bacterial laccases. It is presented in numerous plants such as white-rot fungi. However, the fungal strains to be used should be properly inexpensive, sensitive and fast methods and screenings, and the productions of laccase should be their functions resistance and functional in the industrial field where they will be applied [2].

Their property could be to detoxify a range of pollutants. They have been used in many industries for several purposes. Application of laccase in food industry processing includes medical and health care. Recently, the design of biosensors and nanotechnology has been among the applications of laccase [3].

Currently, this enzyme is the central subject of many worldwide research groups, due to scientific curiosity and its high potential in numerous applications in biotechnology and bio-analytical chemistry. Laccases can oxidize a number of natural and synthetic substrates using oxygen, producing water as the only by-product and they have been used in a variety of industrial and environmental applications. Laccases which are able to oxidize recalcitrant compounds like lignin,

which makes them attractive for their uses in various biotechnological processes as is a versatile gas [4].

Reports have suggested that the use of free enzymes in traditional enzyme applications has many drawbacks which can be overcome by immobilization processes [5]. In some materials, the enzymes are encapsulated. This includes polymers, liposomes, and sol-gels or hydro-gels, depending on their sensing conditions. The enzymes are stabilized via encapsulation with new methods and materials, and their activity may be maintained for a longer time, and even the selectivity can possibly be enhanced. Compared to immobilization/entrapment techniques, micro-encapsulation has many advantages. The microcapsule is composed of a semi permeable, spherical, thin and strong membranous wall [6]. Moreover, compared to an entrapment matrix, there is no solid or gelled core in the microcapsule and its small diameter helps to reduce mass transfer limitations. The substrate can diffuse through the semi permeable membrane easily.

Due to their wide specificity, laccase is used in diverse biotechnological applications such as bio-bleaching, paper pulping, textile refining, dye decolorization, juice and wine clarification, organic synthesis, medical diagnostic tools and bioremediation of soil contaminated [5].

Laccases are important and promising enzymes for various industrial applications. However, their constitutive production from white rot fungi is very low. This greatly hinders their industrial exploitation. The present work aims at purification of laccase and studying its characters. A trial for immobilizing the enzyme by microencapsulation method have also been studied.

## Materials and Methodes

### *Organism*

Fungus *Pleurotus ostreatus* (ATCC 56270) was purchased from Microbiological Resources Centre (Cairo MIRCEN), Egypt (Al-Hagar et al., 2014). It was exposed to 0.5 KGy Gamma irradiation and used in the present study.

### *Medium*

Laccase production by the white rot fungus *Pleurotus ostreatus* ATCC 56270 has been investigated under solid state fermentation. The highest yield of laccase was recorded in case of using lignocellulosic waste (rice bran) with tap water in the presence of copper sulphate 0.005 g/l

as inducer according to AL-Hagar et al. [7]. The growth was allowed under the following optimum conditions: 30°C; inoculums size of 2ml (6×10<sup>5</sup> spore/2ml); pH 4.5 the enzyme was assayed according to Niku-Paavola et al. [8] using (2,2-azino-bis-[3-ethyl-benzo-thiazolin-6 sulphonate]) ABTS.

### *Purification of laccase from Pleurotus ostreatus*

#### *Laccase assay*

Laccase activity was determined by spectrophotometer Milton Roy spectronic 1201 as described by Niku-Paavola et al [8] using ABTS as a substrate. The total protein was estimated following the method of Lowry et al. [9] using bovine serum albumin as a standard protein.

#### *Determination of the activation energy*

Plots of the reaction rate vs temperature and the log of the rate (ln k) vs 1/T were made to determine the activation energy. To determine the thermostability, the purified laccase was incubated at temperatures ranging from 30, 40 and 60°C for various times (10–50 min). The residual activity was determined with ABTS as the substrate.

## Results

### *Purification of laccase by ion exchange chromatography*

The laccase enzyme was purified extracellularly from *Pleurotus ostreatus* in two steps, using chromatographic DEAE-Sephacryl 6B and Sephacryl S-200 gel filtration chromatography Table (1). All purification procedures were performed at 25°C. The laccase is purified from a cell-free culture medium (250 ml) after being filtrated and centrifuged at a rate of 10,000 rpm for 30 min. The concentration of the filtrate is one tenth of the volume at 4°C with an Amicon ultrafiltration system which equipped with a filter type PTGC (pore size 10,000 normal molecular weight limit (NMWL)). The centrifugations of solution at 10,000 rpm for 60 minutes and the supernatant was collected and exchange of extensive buffer was performed using stirred ultrafiltration cell (Amicon 8200, YM-30 membrane) with 0.01 M acetate buffer (pH = 6.0). The clarified cell extract (25 ml) with specific activity 55.9 U/mg was loaded onto a DEAE-Sephacryl 6B column (0.5 x 10 cm), pre-treated with polyvinyl alcohol (1 g %) and equilibrated in 0.01 M acetate buffer (pH = 6.0). At this step, specific activity increased to 202.3 U/mg. The end

step, the enzyme fractions obtained from the ion exchange column were concentrated and pooled and this sample was applied to the Sephacryl S-200 column (0.5 x 10 cm) equilibrated with 10 mM Tris-HCl 0.15 M NaCl (pH 8). At this step, a single peak was obtained, and activity of the enzyme appeared in fractions (5-9) as shown in (fig.1) while fraction (7) gave a specific activity of 348.8U/mg. The bound protein was then eluted with the same buffer. Active fractions were stored at -4°C. At the end of the process, laccase was purified 6-fold.

### SDS Polyacrylamide gel electrophoresis

Fraction 7 which gave the highest activity of laccase enzyme (348.8 U/mg), was subjected to SDS polyacrylamide gel electrophoresis as illustrated in Fig. (1).

The pure laccase produces one band on an SDS-PAGE gel protein was about 75 kDa as determined by SDS-PAGE Fig. (2).

### Effect of temperature on activity of the purified laccase and its stability

The effect of temperature on the activity of the purified laccase was assayed in 50 M sodium acetate buffer (pH 4.5). The optimal temperature for the enzyme activity was 35°C as shown in Fig. (3).

During the examination of thermal stability, the enzyme must be incubated for one hour at temperatures 20, 35, 40, and 60°C. The purified enzyme retained 70% at 35°C of its beginning activity after 1h Fig. (4).

Temperature effect on enzyme activity of enzyme was assayed in 50 mM of sodium acetate buffer (pH 4.5)

### Determination of the energy activation

The activation energy ( $E_{act}$ ) of the purified laccase between 20°C and 55°C at its optimum pH (pH = 4.5) was 16.3 kJmol<sup>-1</sup> as shown in Fig. (5). This is higher than 3.9 kJmol<sup>-1</sup> of *Pleurotus florida* laccase [10] and 12 kJmol<sup>-1</sup> of *P. sajor-caju* laccase [11].

### The pH effect on activity of the purified laccase and its stability

The pH effect on laccase activity and its stability using ABTS as a substrate was studied; the activity of laccase was investigated in the pH range of 3.7–8. The optimal PH of the enzyme was determined to be 5 and it became less active at level of pH 7 and 8 as shown in Fig. (6).

Effect of pH on the laccase was measured in 0.1 M sodium acetate buffer (pH 3.7, 4, 4.6, 5 and 5.5) and 0.1 M phosphate citrate buffer (pH 6, 6.5, 7 and 8). To analyze the pH stability, the purified laccase was incubated at 25°C for ½ h at the previous pH range.

A study of the enzyme stability showed that the enzyme at PH value above 7 was unstable, but the purified laccase was very stable at pH values from 3.7 to 5. Laccase retained 99% of its initial activity after ½ h at pH 4.6 Fig. (6), and 97.8%, 96.7%, 94.3 % of its retained initial activity after 1/2 h at pH 3.7,4,5 respectively. At pH 7, 8 laccase was less active as it retained only 34% and 20% of its initial activity respectively.

### Kinetic parameters of the Purified Laccase

As shown in Fig. (7) relation between substrate concentration ABTS and enzyme activity ( $\mu$ M) and  $\Delta$  absorbance min<sup>-1</sup>. Activity was measured at different ABTS concentrations (100, 200, 300, 400, 500, 600, 700 and 800  $\mu$ M) in 50 mM of sodium acetate buffer (pH 5) at 35°C.

The data in Table (3) summarized kinetic parameters of the purified laccase enzyme

**Table (1): Steps in purifying to homogeneity the laccase enzyme detected in the supernatant of *Pleurotus ostreatus* ATCC-56270**

| Purification Step | Total volume (ml) | Enzyme Activity (U/ml) | Total Activity (Units) | Protein (mg) | Specific Activity (U/mg) | Purification Fold |
|-------------------|-------------------|------------------------|------------------------|--------------|--------------------------|-------------------|
| Crude extract     | 250               | 28.57                  | 7142.5                 | 127.8        | 55.9                     | 1                 |
| DEAE-sepharose    | 25                | 83.33                  | 2083.25                | 10.3         | 202.3                    | 3.62              |
| Sephacryl-HR      | 5                 | 30.0                   | 150.0                  | 0.43         | 348.8                    | 6.24              |

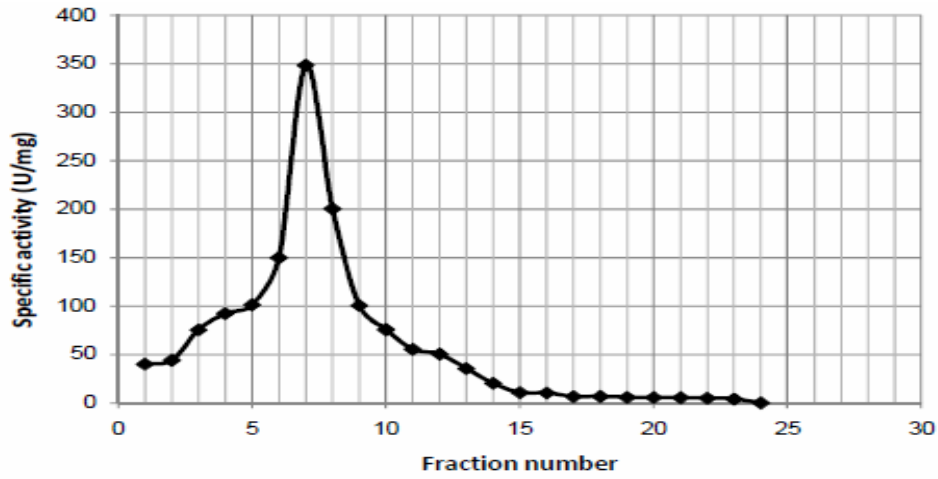


Fig. (1): Active fractions of purified laccase from *Pleurotus ostreatus* ATCC 56270

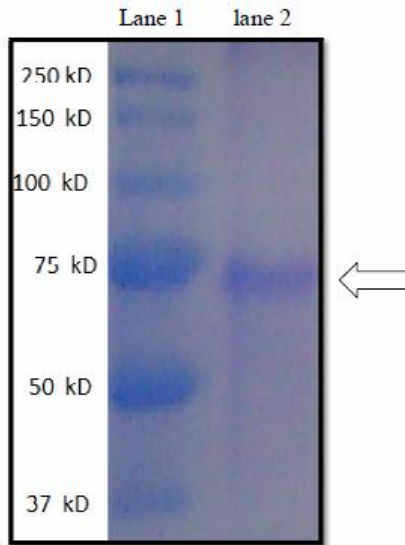


Fig. (2): SDS polyacrylamide gel electrophoresis of standard proteins (Bangalore Genei) (lane 1), purified laccase from *Pleurotus ostreatus* ATCC-56270 (Lane 2)

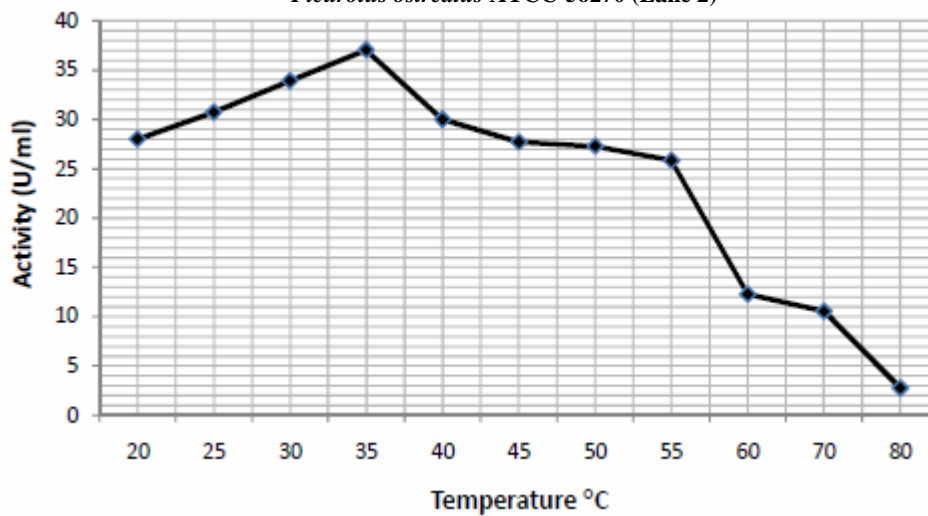


Fig. (3): temperature effect on activity of purified laccase of *Pleurotus ostreatus* ATCC 56270

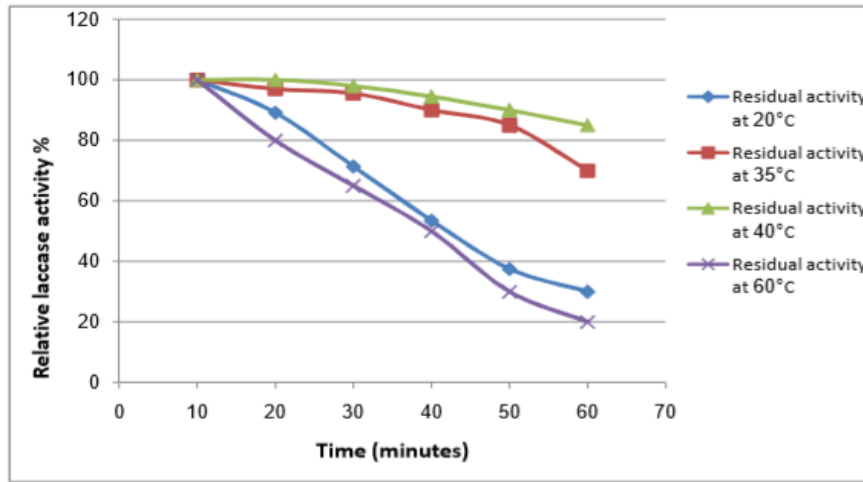


Fig. (4): Thermal stability of laccase purified of *pleurotus ostreatus* ATCC 56270 represented as % relative activity vs temperature after 1h incubation at the indicated temperatures

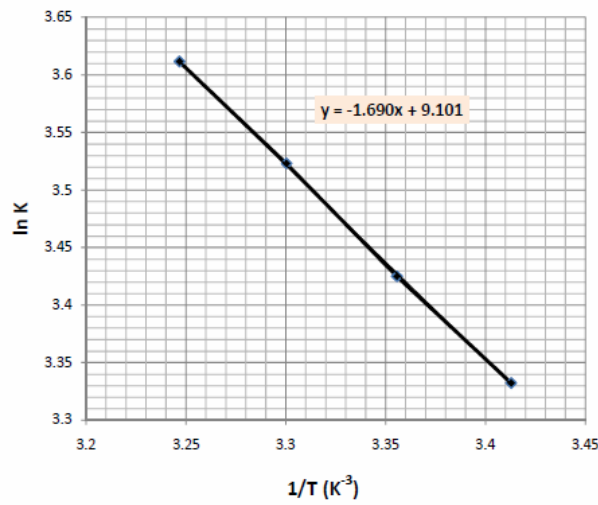


Fig. (5): Plots of the log of the reaction rate vs (1/T kalvin) were made to determine the energy of activation

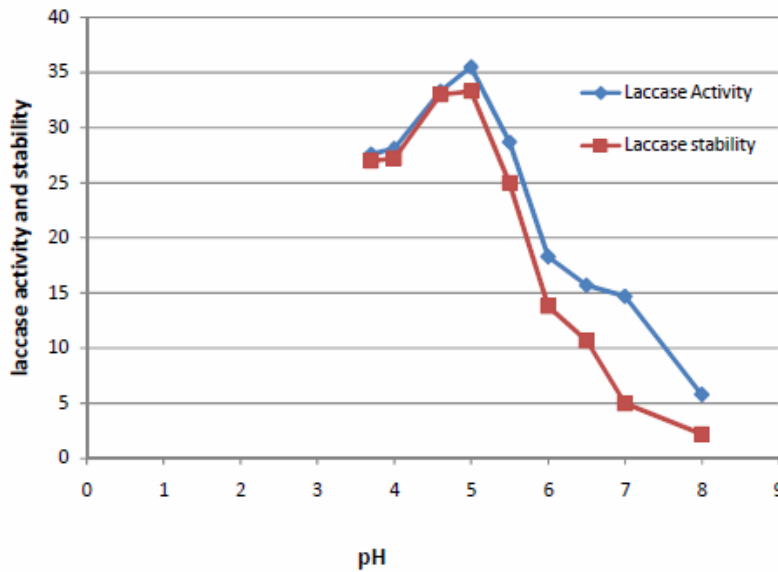


Fig. (6): Effect of The PH effect the stability and activity of the purified laccase of *pleurotus ostreatus* ATCC 56270

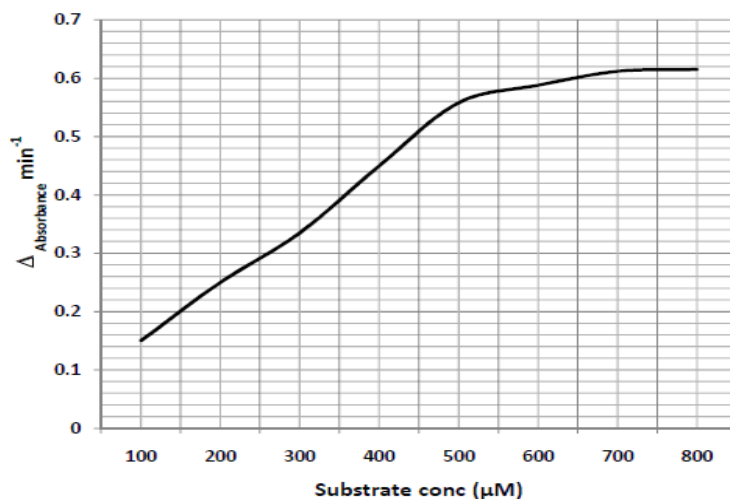


Fig. (7): Relation between substrate concentration ( $\mu\text{M}$ ) and  $\Delta_{\text{absorbance}} \text{min}^{-1}$ . Activity

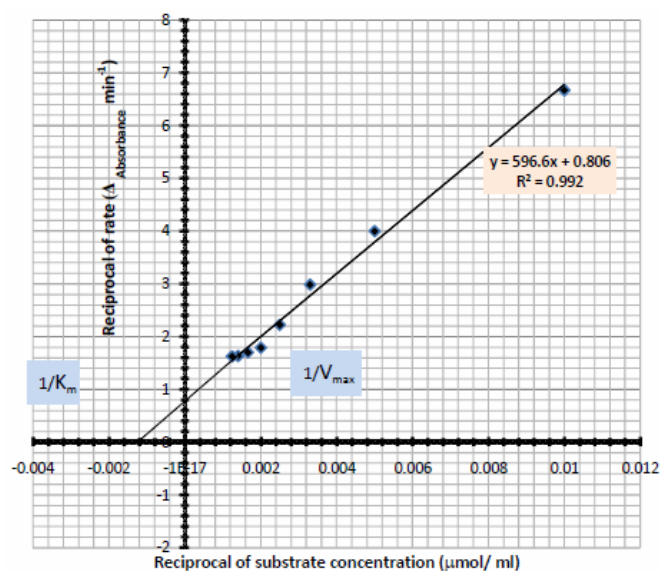
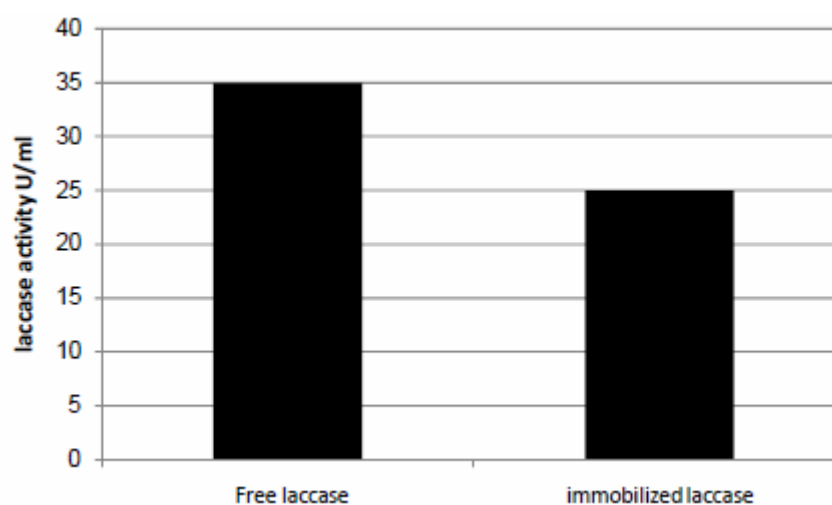


Fig. (8): Lineweaver-Burk double reciprocal plot of reaction rate ( $\Delta_{\text{absorbance}} \text{min}^{-1}$ ) versus the reciprocal of substrate concentration for the purified laccase

Table (2): Kinetic parameters of the purified laccase

| Substrate | $K_m$<br>(mMol/l) | $V_{max}$<br>( $\mu\text{mol L}^{-1}\text{min}^{-1}$ ) | $K_{cat}$<br>( $\text{min}^{-1}$ ) | $K_{cat}/K_m$<br>( $\text{min}^{-1}\mu\text{M}^{-1}$ ) |
|-----------|-------------------|--------------------------------------------------------|------------------------------------|--------------------------------------------------------|
| ABTS      | 1.4               | 1.2                                                    | 3450                               | 2464                                                   |



**Fig. (9): laccase activity of the free and the immobilized enzyme on alginate**

#### *Encapsulation of the purified laccase*

A natural polymer as alginate can easily be converted into hydrogels via divalent cations cross-linking. Encapsulation of laccase and Alcohol dehydrogenase in alginate beads was studied according to Wang et al. [12]. The immobilized laccase beads (2-3mm) were formed by entrapping the enzyme in alginate beads, laccase from *S. psammoticus* partially purified laccase was immobilized by entrapment physically in alginate beads.

The activities of the free and the immobilized laccase were assayed at temperatures 30°C in a thermostatically controlled water bath under the standard assay conditions. The results were depicted in Fig. (9) which shows that free laccase (35U/ml) has a higher activity than the encapsulated laccase (24.3 U/ml).

#### **DISCUSSION**

In the present study, the laccase enzyme produced by *Pleurotus ostreatus* ATCC 56270 was subjected for purification steps. The cell free filtrate was concentrated using Amicon Ultra filtration system giving 55.9 U/mg activity, then loaded on DEAE-sepharose 6B column pretreated with polyvinyl alcohol (1g %) and equilibrated in 0.01M acetate buffer (pH= 6.0) to increase the activity up to 202.3 U/mg with 3.62 purification fold. Then a second column of Sephacryl S-200 column (0.5 x 10 cm) was equilibrated with 10 mM Tris-HCl,

0.15 M NaCl (pH 8) to obtain a single peak with specific activity 348.8U/mg for fraction (7) with purification fold 6.24. Findings of other investigators have shown that the production of crude extracellular laccase by *Anoxybacillus gonensis* P39 using DEAE-celulose ion and the laccase was purified utilizing exchange chromatography and Sephacryl S200 gel filtration chromatography techniques resulting in 4.16, 11.69, and 83.4 purification folds [13]. The purified laccase gave one band on SDS-PAGE gel protein with 75 KDa [14]. The purified laccase from *Melanocarpus albomyces* gave 80 kDa [15].

As for the effect of temperature on the purified laccase produced by *Pleurotus ostreatus* ATCC 56270, the optimal temperature was found to be 35°C, while Wang and Hsu [16] reported the optimal temperature to be 30°C by endophytic fungus *Monotospora* sp. and Irshad et al. [17] reported it as 40°C from *Schizophyllum commune* IBL-60. Laccase Low thermostability was observed as it retained only 20% of its initial activity after 1h incubation. Thermal stability varied with different temperatures, this depends on microorganisms sources. For example, after 1 h incubation at 55°C [15], the laccase from *T. harzianum* retained 70% of its starting activity, whereas the laccase produced from *Daedalea quercina* was more stable below this temperature. Laccase was most stable at 40°C as it retained 100% of its activity after 20 minutes incubation,

85% of its activity after 1h incubation at the same temperature. At 60°C laccase was less stable as it retained only 20% of its initial activity after 1h incubation. However, the activity decreased at higher temperatures [18-19].

The activation energy (Eact) of the purified laccase between 20°C and 45°C at its optimum pH (pH = 4.5) was 16.3 kJmol<sup>-1</sup> as shown in Fig. (18). This is higher than 3.9 kJmol<sup>-1</sup> as reported by *Pleurotus florida* laccase [10] and 12 kJmol<sup>-1</sup> as reported by *P. sajor-caju* laccase [11]. On the other hand, it is lower than 60 kJmol<sup>-1</sup> of *Coriolus versicolor* (19). The effect of the pH and pH stability on the laccases which were produced from *C. hirsutus*, *T. atroviride*, and *Chalara* (syn. *Thielaviopsis*) *paradoxa* CH32 was optimal at pH range of 4–6 [15]. The activity of many laccases decreases rapidly beyond the optimum pH [20-22], but this laccase showed a high relative activity over a broad pH range from 3.7 to 5. This could be a very useful characteristic for various industrial applications. Various substrates with a decrease in oxidation rate was observed at a higher pH, which may be due to ionization of critical amino acids (Asp and Glu), this indicates that the enzyme was inactive at a higher pH [23].

To improve the catalytic properties of laccase, such as the high affinity for its substrate and relatively high turnover numbers, especially for the oxidation of ABTS, this enzyme that proved to be highly competitive for this industrial application was used. The apparent Km value of the enzyme for ABTS as determined from the Line weaver-Burk plot Fig. (6) was estimated to be 1.4 mM under standard assay conditions. V max is the maximum begging rate in the Michaelis-Menten equation (1.2 μmol L<sup>-1</sup> min<sup>-1</sup>). The kcat value for ABTS, 3450 min<sup>-1</sup>, was higher than that of the laccase of *C. subvermispota* which was 101 s<sup>-1</sup>, *T. villosa* (2700 s<sup>-1</sup>), *P. cinnabarinus* (920 s<sup>-1</sup>), *Myceliophthora thermophila* (440 s<sup>-1</sup>), and *Botrytis cinerea* that was 23 s<sup>-1</sup>[24]. The previous result suggested that the ABTS might be an effective mediator of this enzyme. For the laccase mediator to be effective the laccase structure should be modified by increasing its kcat so as to improve its stability against free radical attack.

Laccase was immobilized by entrapment into alginate. After entrapping of the enzyme, the immobilized laccase beads (2-3 mm) were formed. In the present study, the preparation of calcium alginate and copper alginate beads from two

different cations such as calcium and copper respectively. Calcium alginate is the most commonly used for enzyme immobilization support while copper alginate gels are used for laccases specifically [25]. There is a different parameter where the formation and the mechanical and structural properties of alginate beads depend on the alginate concentration, and concentration of the cations and nature of the cations [26].

### Conclusion

The process of laccase purification has been successfully performed by anion exchange chromatography using DEAE sepharose and sephacryl HR. The molecular weight of laccase was 75 KDa determined by SDS- PAGE. The optimum pH value for the activity was 5, and for the stability was 6.5-7.5, the optimum temperature for the activity was 35°C and the stability of the enzyme was 40°C. The enzyme retained 100% of its activity after 20 minutes incubation, 85% of its activity after 1 h incubation at the same temperature. The free laccase (35 U/ml) has a higher activity than encapsulated laccase (24.3 U/ml).

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