



## IMMOBILIZATION, CHARACTERIZATION AND REMOVAL EFFICIENCY OF DYES POLLUTANTS USING LACCASE PRODUCED BY LOCAL ISOLATE *Pseudomonas aeruginosa* SR3

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### ABSTRACT

The present study was aimed for immobilization, characterization and application of laccase for removing the different pollutants using local isolate of *Pseudomonas aeruginosa* SR3. Immobilization of enzyme was conducted in two methods, entrapment by calcium alginate and agar-agar with maximum immobilization efficiency 49% and 32% respectively and covalent method by chitosan and gelatin with maximum immobilization efficiency 63% and 50% respectively. The immobilized laccase on chitosan was exhibited maximal activity at pH 5.0, and it was stable at pH 6.0. The optimal temperature for immobilized enzyme activity was 40°C. In the present study, laccase immobilization led to a significant stabilizing effect towards heat denaturation, the profiles of thermal denaturation at 55 and 65°C were markedly affected. The highest rate of enzyme specificity found with oxidation of O-tolidine, while gallic acid, vanillic acid are poor substrates for laccase mediated oxidation. Immobilized enzyme system maintained 56% of its efficiency even after 4 successive reaction cycles. The immobilized laccase maintained 50% of its activity after 10 days. The preliminary results by GC technique suggested that the degradation ratio of the hydrocarbons by crude laccase reached to 98.7%, 99% and 93.8% for catechol, anthracene and tannic acid after 1, 1.5 and 2 hr. respectively. Higher removal efficiency observed of crude enzyme to decolorization of yellow, red and black textile dyes reached 84%, 91.4%, and 88% through 3 hr respectively. However, approximately the same removal efficiencies were observed with purified laccase with hydrocarbons and decolorization of dyes. It has been concluded from the results to the possibility of using crude enzyme and purified to remove the pollutants from the environment.

**KEYWORDS:** Hydrocarbons, Laccase, Immobilization, characterization and Dye Removal.

### INTRODUCTION

Laccases are multicopper phenol oxidases which can oxidize large range of phenolic compounds and aromatic amines. One-electron oxidation, leading to generation cation radicals of the pollutants, is performed by the ligninolytic enzymes. Those radicals can initiate chemical reactions like cleavage of C-C bonds or hydroxylation, which can result in more hydrophilic derivatives<sup>[1]</sup>. In most cases laccases are monomeric glycoproteins contain around 500 amino acids and contain 15– 30% carbohydrate and have a molecular mass of 60–90 KDa. Laccases were widely distributed among plants, fungi<sup>[2]</sup> and bacteria<sup>[3]</sup>. Since laccases have been found in *Pseudomonas* sp.<sup>[4]</sup>, extracellular laccases are purified from (fermentation broth) of the selected organism. Typical purification protocol involves ultrafiltration, ion exchange, gel filtration, hydrophobic interaction, or other electrophoretic and chromatographic techniques. Immobilization is achieved by fixing enzymes to or within solid supports, laccase may be immobilized by a variety of methods (adsorption, entrapment, crosslinking and covalent bonding) mainly based on chemical and/or physical mechanisms<sup>[5]</sup>. The catalytic performance of immobilized

laccases is greatly influenced by their activity and stability at different pH and temperature conditions. Since the optimal pH of laccase activity around 4-6, when measured with phenolic substrates, immobilized laccase was unstable in neutral and alkaline pH<sup>[6]</sup>. In general, the optimum temperature of laccases activity usually ranges between 30-60 °C, and it was stable at 30-50°C and rapidly lose activity at temperatures above 60 °C<sup>[7]</sup>. Laccase has broad substrate specificity towards aromatic compounds, classical substrates of laccases include various lignin-derived phenols and aromatic amines as guaiacol, caffeic acid, gallic acid, and the commonly compounds used for the detection and measurement of laccase activities represented by syringaldazine, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,6-Dimethoxy phenol (2, 6-DMP), O-tolidine<sup>[5]</sup> and <sup>[8]</sup>. Laccases are applied in environmental pollutants detoxification, demand for removal of synthetic dyes from the textile industrial waste and have many possible applications in bioremediation it may be applied to degrade various substances such as undesirable contaminants, byproducts, or discarded materials<sup>[9]</sup>.

## MATERIALS & METHODS

### Microorganisms

The isolate *P. aeruginosa* SR3 was obtained from Biotechnology department in college science of Baghdad University, Iraq. The isolate is previously described as active degrader for aromatic hydrocarbons and laccase production.

### Chemicals

Nutrient agar, (o-tolidine), and all other reagent grade chemicals were purchased from Hi – Media and Sigma-Aldrich, India. Textile (black, yellow and red) dye was provided from Al Diwaniyah textile factory south of Baghdad.

### Production of laccase

The laccase production was conducted in optimum production media containing glucose 1g, yeast Extract 2g, NaCl 0.1g, CaCO<sub>3</sub> 0.02g, CuSO<sub>4</sub> 0.001, MgSO<sub>4</sub> 0.002g, FeSO<sub>4</sub> 0.01g, ZnSO<sub>4</sub> 0.009 and 0.04% p-ansidine as a hydrocarbon source. The pH of the medium was adjusted to 7 and incubated at 30 °C for 24hr, the cells were separated by centrifuge at 8000 rpm for 10 min. Then the filtrate was taken to estimate the enzyme activity and used as a crude extract for laccase enzyme for purification experiments.

### Laccase activity

Laccase enzyme activity was estimated according to the method described by Kalral [10] by using o-tolidine as a substrate, the oxidation of o-tolidine was detected by measuring the absorbance increase at 366 nm ( $366 = 27,600 \text{ M}^{-1} \text{ cm}^{-1}$ ) using a spectrophotometer.

### Immobilization of laccase enzyme

#### Immobilization of laccase by entrapment methods

##### Entrapment in agar-agar

Two ml of purified laccase (which previously purified by three steps includes concentration by sucrose, ion exchange and gel filtration with a purification fold 5.2 and yield reached to 17%) was mixed with 5 ml of sterile agar-agar solution (4%) at 40 °C, and shaken well for few minutes and poured into sterile petri dish and allowed to solidify, after solidification, cubes (1×1×1) cm were cut and washed with distilled water then the cubes of agar-bounded enzyme store in 0.1 M of sodium acetate buffer pH 5 at 4 °C, then immobilized enzyme activity was assayed<sup>[11]</sup>.

##### Entrapment in calcium alginate

Two ml of purified laccase was mixed with 5 ml of sterile sodium alginate solution (3%), stirred gently for 10 minutes, the mixture obtained was extruded drop wise through the sterile syringe (10 ml) into 0.2M of CaCl<sub>2</sub> solution to obtain small beads with 1 mm diameter and kept for 1 hr. The beads were washed with D.W to remove the non-immobilized enzyme. Then the beads of calcium alginate enzyme store in 0.5% (w/v) CaCl<sub>2</sub> at 4 °C. Then immobilized enzyme activity was determined<sup>[11]</sup>.

#### Immobilization of laccase by covalent methods

##### Covalent linkage by chitosan

One gm of chitosan was added to 10 ml of 2% glutaraldehyde solution, stirred gently and mixed for 2 hr. at 4 °C followed by an overnight incubation. The

glutaraldehyde bounded chitosan was washed extensively with water to remove the unbounded glutaraldehyde, then mixed with 10 ml of purified enzyme solution at 4°C over night for enzyme immobilization<sup>[12]</sup>. The resulted chitosan-glutaraldehyde - laccase conjugates were separated and washed, then Immobilized enzyme activity was determined

##### Covalent linkage by gelatin

Three ml of purified enzyme was added to 15 ml of gelatin solution, stirred gently for 10 minutes and then 0.6% of glutaraldehyde solution was add to the mixture in 28 °C, then poured into sterile petri dish and allowed to solidify at 18 hr in 4°C. After solidification, cubes (1×1×1) cm were cut and washed with 50 Mm tris-Hcl buffer<sup>[13]</sup>, then immobilized enzyme activity was determined

##### Determination the immobilized laccase activity

Immobilized laccase activity was estimated according to the method described by<sup>[10]</sup> by using 30 mg of immobilized enzyme instead of 0.7ml of enzyme.

#### Characterization of immobilized laccase

##### Effect of pH on immobilized laccase activity

The effect of pH on the activity of the immobilized laccase was determined by preparing o-tolidine as a substrate in different buffer solutions include 0.05M sodium acetate buffer (pH 4, 4.5, 5, 5.5, 6), 0.05 M sodium-phosphate buffer (pH 6.5, 7, 7.5) and 0.05 M Tris-base buffer (pH 8, 8.5, 9). Immobilized laccase activity was determined by using 30 mg of immobilized enzyme and the activity was measured according to the method described by<sup>[10]</sup>.

##### Effect of pH on immobilized laccase stability

Equal amount (w/v) from immobilized enzyme was mixed with the buffers at different pH (4-9) at a ratio of (1:1) and the mixture was incubated in a water bath for 15 min at 30°C, then the samples were transferred directly to ice bath, then the remaining activity % was estimated.

##### Optimum temperature on immobilized laccase activity

Immobilized laccase activity was estimated at different range of temperature include (25-70) °C with difference of 5 degrees between each temperature values.

##### Effect of temperature on immobilized laccase stability

Immobilized laccase was incubated at different temperatures ranged between 25-70°C for 15 min. followed by incubation in ice bath, remaining activity% of immobilized laccase was estimated.

##### Immobilized laccase Specificity

Solutions of o-tolidine, guaicol, catechol, tannic acid, gallic acid, purogallol and vanillic acid were used as substrates and prepared by dissolving (25mM) in sodium acetate buffer at pH 6. The effects of different substrates on immobilized laccase activity were examined separately.

##### Reusability test

The reusability of immobilized laccase enzyme was studied for 15 cycles, immobilized enzyme activity were measured for each cycle, the immobilized laccase was filtered, washed and placed into a new batch of o-tolidine solution for the second cycle. The experiment was repeated to test the reusability of next cycles<sup>[14]</sup>.

### Storage stability of laccase

The storage stability test was carried out to determine the leakage of immobilized laccase enzyme during the 30 days, immobilized laccase were stored in acetate buffer (pH 6) and sample of the buffer solution was collected every day from the first week then every five days, thereafter immobilized laccase activity was assayed [14].

### Application of laccase enzyme

#### Dyes decolorization

For decolorization experiments, the textile (yellow, red and black) dyes at concentration of 30 mg/l were used. The reaction mixture for the degradation of dyes contains 10 ml for each dyes and 1 ml (244 U/ml) of enzyme solution (crude and purified laccase) separately. The reaction mixture was incubated was shaken at 120 rpm in a shaker incubator at 40 °C in different time (0, 1, 2, 3 and 24) hr. Distilled water was used instead of enzyme in the control experiment. The percentage of removal efficiency for each dye was calculated by the absorbance at max according to Zhang [15].

Decolorization (%) =  $\frac{\text{Initial Absorbance} - \text{Final Absorbance}}{\text{Initial Absorbance}} \times 100$  Degradation poly aromatic hydrocarbons (PAHs)

Aromatic hydrocarbons catechol, anthracene and tannic acid were prepared at concentrations of 50 mg/l. The reaction mixture for samples consist of 10 ml of each hydrocarbons separately (1ml 244 U/ml) of enzyme (crude and purified laccase) separately. A blank contained 1ml of distilled water instead of enzyme. Both the sample and blank incubated at 40°C with shaken at 120 rpm for 1hr for catechol, 1.5 hr for naphthalene and 2 hr for tannic acid. The results determined by analysis performed using Gas chromatography (GC) technique under conditions [Column Reprosil 100, C18 dimension (25 x0.46) cm , mobile face (Acetonitrile b , -water) flow rate (1.2 ml/min.), column temperature (30 C°) and wave length (245nm)] [16]. Percent of degradation was calculated as follows:

Degradation (%) =  $\frac{\text{Initial Absorbance} - \text{Final Absorbance}}{\text{Initial Absorbance}} \times 100$

## RESULTS & DISCUSSION

### Immobilization of laccase enzyme

#### Immobilization of laccase by entrapment methods

The results illustrated in the fig. (1) showed that the calcium alginate was more suitable than agar-agar, since immobilization ratio of the calcium alginate entrapped laccase (49%), was higher than that of agar- agar, the decrease in immobilization ratio of laccase entrapped in agar-agar was due to the weakness of cohesion of agar-agar for entrapped enzyme, easy to dissolution, disintegration and high solubility of the gel during the immobilization the enzyme [17].

#### Immobilization of laccase by covalent methods

The results illustrated in the fig. (2) Showed that the covalent linkage by chitosan was more suitable than covalent linkage by gelatin, since immobilization ratio of the chitosan linkage laccase 63%, was higher than that of gelatin 50%. The immobilization of enzyme to chitosan carrier is commonly carried out via multipoint covalent attachment at high ionic strength, because it has been postulated that, in a first step, a salt-induced association between the protein and the support surface takes place [18].

### Characterization of immobilized laccase

#### Effect of pH on immobilized laccase

The effect of pH on the activity of the immobilized laccase on chitosan was investigated at different pH values varying from 4.0 to 9.0 (fig.3). The immobilized laccase exhibit maximal activity at pH 5.0. While free laccase has the best activity in pH ranged between (5.5 to 7), with maximum enzyme activity at pH 6.0 fig (4), the shift of optimum pH was attributed to the electrostatic interaction influenced by the carrier microenvironment [19].

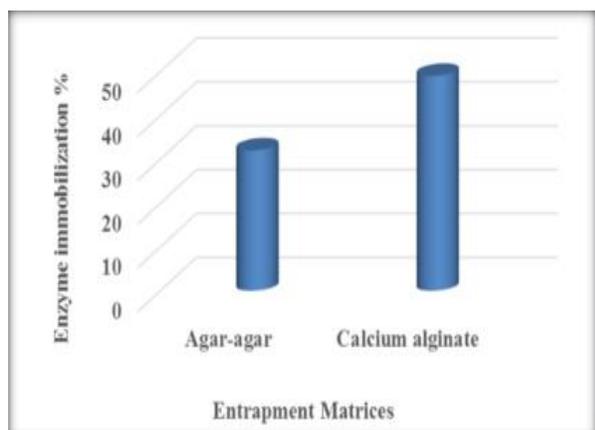


FIGURE 1: Enzyme immobilization % of *P. aeruginosa* SR3 laccase by entrapment methods (Agar-agar and Calcium-alginate)

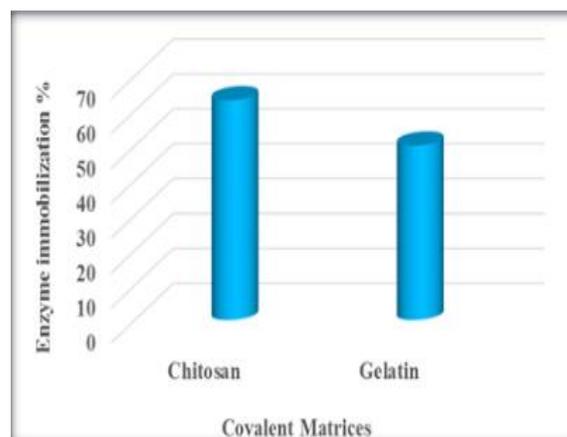
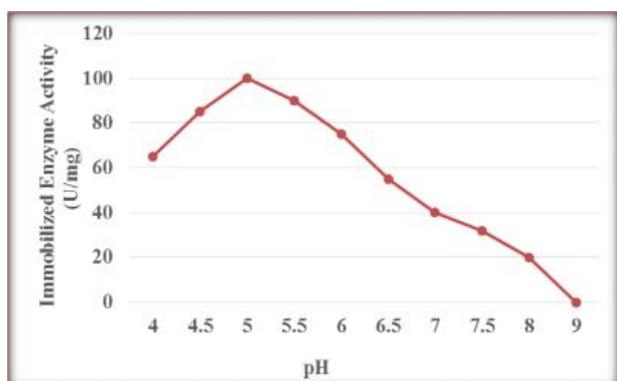
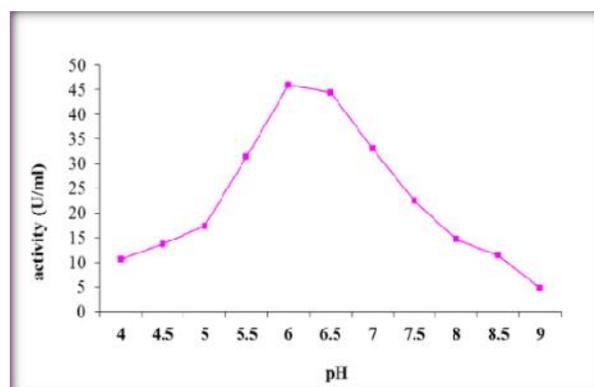


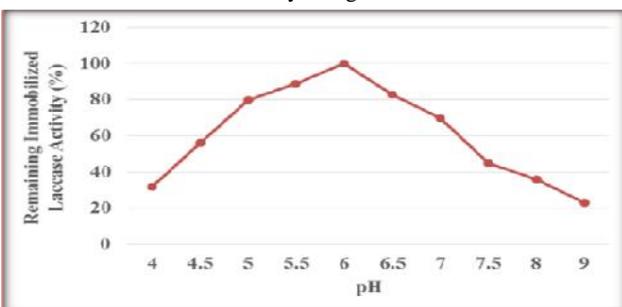
FIGURE 2: Enzyme immobilization % of *P. aeruginosa* SR3 laccase by covalent methods using Chitosan and Gelatin



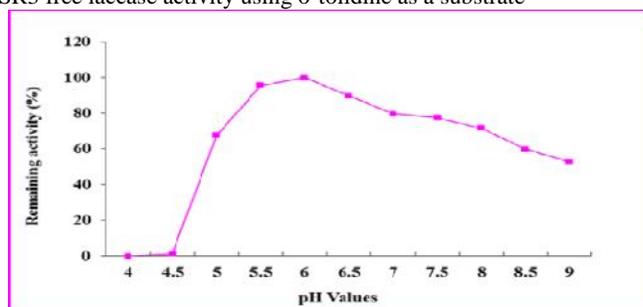
**FIGURE 3:** Effect of different pH values (4.0-9.0) on *P. aeruginosa* SR3 immobilized laccase activity using *O*-tolidine as a substrate



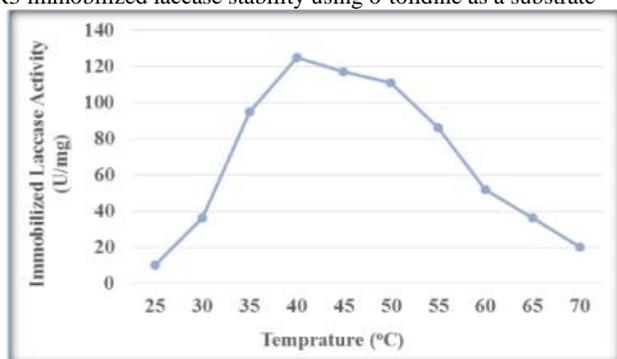
**FIGURE 4:** Effect of different pH values (4.0-9.0) on *P. aeruginosa* SR3 free laccase activity using *o*-tolidine as a substrate



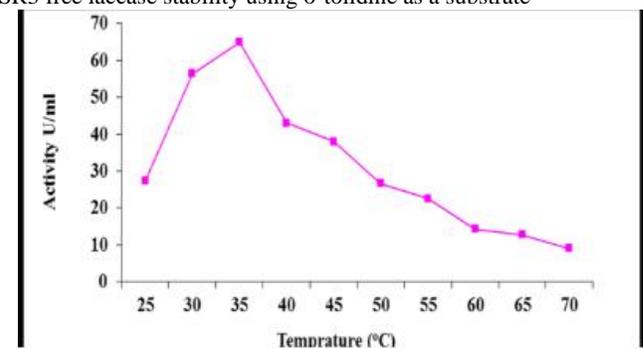
**FIGURE 5:** Effect of different pH values (4.0-9.0) on *P. aeruginosa* SR3 immobilized laccase stability using *o*-tolidine as a substrate



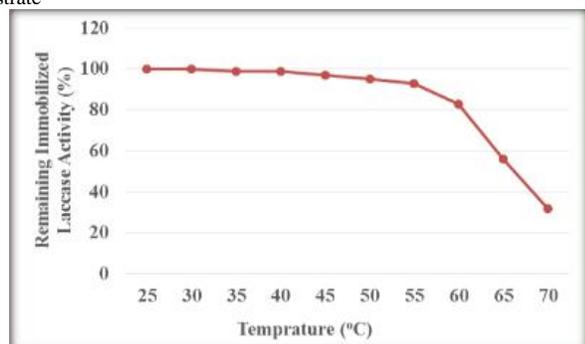
**FIGURE 6:** Effect of different pH values (4.0-9.0) in *P. aeruginosa* SR3 free laccase stability using *o*-tolidine as a substrate



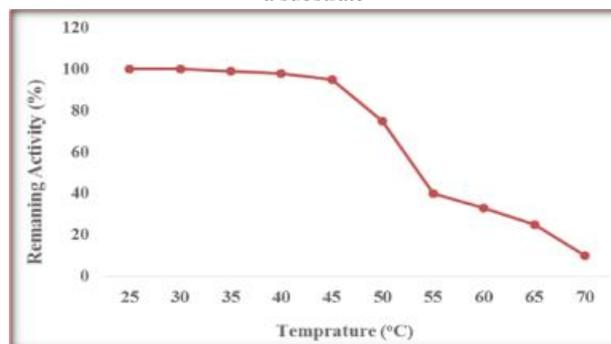
**FIGURE 7:** Effect of different range of temperatures (25-70) °C on *P. aeruginosa* SR3 immobilized laccase activity at pH 6.5 using *o*-tolidine as a substrate



**FIGURE 8:** Effect of different range of temperatures (25-70)°C on *P. aeruginosa* SR3 free laccase activity at pH 6.0 using *o*-tolidine as a substrate



**FIGURE 9:** Effect of different ranges of temperature (25-70) °C on immobilized laccase stability produced by *P. aeruginosa* SR3 at pH 6.5 using *o*-tolidine as a substrate.



**FIGURE 10:** Effect of different ranges of temperature (25-70)°C on *P. aeruginosa* SR3 free laccase stability at pH 6.5 using *o*-tolidine as a substrate

### Effect of pH on immobilized laccase stability

The immobilized laccase was stable in the pH range from 5 to 6; this indicated that the immobilization appreciably

improved the stability of laccase in the acidic region (fig. 5). In the pH range 5.0–6.5 immobilized laccase exhibited a comparable stability, whereas at more acidic pH values

immobilized laccase exhibited higher stability than the free counterpart fig. (6) [6].

**Effect of temperature on immobilized laccase activity**

The optimum temperature of the laccase immobilized in chitosan was found to be 40 C which was 5 C greater than those of the free enzyme (35 C) (fig.7). Free laccase activity was decreased too below 40 °C (fig.8). Such shift in optimum temperature of the immobilized laccase demonstrated that the chitosan matrix was effective in protecting the laccase activity under higher temperature conditions which may be due to enhancement of the conformational stability of the native form [20].

**Stability of immobilized laccase at different temperatures**

In the present study, laccase immobilization led to a significant stabilizing effect towards heat denaturation, the profiles of thermal denaturation at 65 and 70°C were remarkably affected (fig.9). Compared with free laccase it was maintained its activity at temperatures ranged between 25-45°C fig. (10), then the activity began to decrease with increasing temperature although at 50°C to 70°C. The decline in laccase activity at a temperature degrees more than 60°C belong to its sensitivity against high temperature, the enhanced thermal stability of laccase arising from immobilization could be an advantage for treating effluents at high temperatures.

**Substrates Specificity of immobilized laccase**

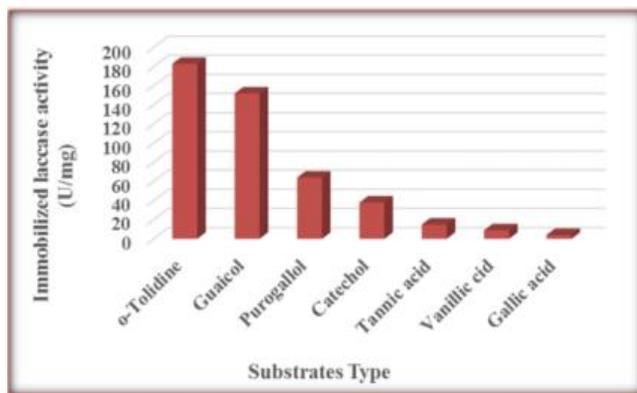


FIGURE 11: Effect of different substrates on immobilized laccase activity produced by *P. aeruginosa* SR3 at pH 5 and 40 °C

Several aromatic and phenolic compounds were studied as a possible substrates for immobilized laccase, the highest rate of specificity found for oxidation of o-tolidine (fig. 11). This study has revealed that gallic acid, vanillic acid are poor substrates for laccase mediated oxidation. The authors have correlated laccase substrate specificity with the respective substrate redox potential. It was demonstrated that the rate limiting step of the enzymatic reaction is in fact the transference of the first electron from phenol to the copper atom located at the enzyme active site [21].

**Reusability test**

After the fourth use, the residual activity for immobilized laccase on chitosan was found to be 56%, (fig.12). Decrease in the enzyme activity upon repeated usage is expected. Upon repeated uses, either blocking of some pores of support by substrate or product may take place, or enzyme may lose its activity and denature [22].

**Storage stability**

As depicted in fig. (13), downward trends were presented for the activities of immobilized laccase on storage for up to one month. Apparently, the decrease in activity of immobilized laccase was smaller in terms of both amplitude and rate. After 12 days, the remaining relative activity of immobilized laccase 65% and after 30 days, it was 19%. These results indicated that, in terms of storage stability, immobilized laccase is significantly superior to free laccase, which provides the possibility of long-term preservation for further use [23].

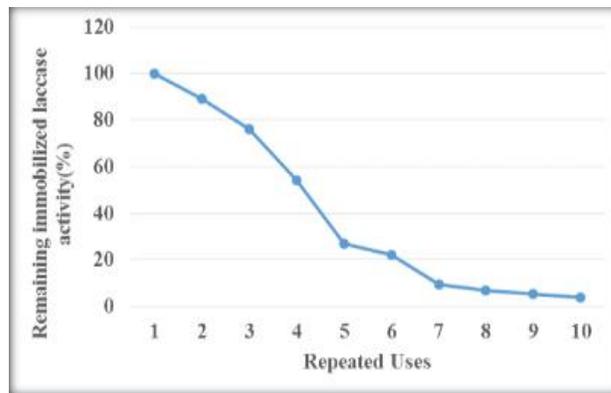


FIGURE 12: Effect of repeated uses of immobilized laccase enzyme produced by *P. aeruginosa* SR3

Table (1): Dyes degradation% by laccase enzyme (Crude & Purified) from

*P. aeruginosa* SR3 at 40 °C in pH 5.0 after 3 hr.

Dyes	Dyes degradation (%)	
	Crude enzyme	Pure enzymes
Textile Yellow	84.1	92
Textile Red	91.4	94.6
Textile Black	88.1	92

Table (2): Degradation efficiency (%) of PAHs compound by laccase (crude & purified) enzyme produced from *P. aeruginosa* SR3

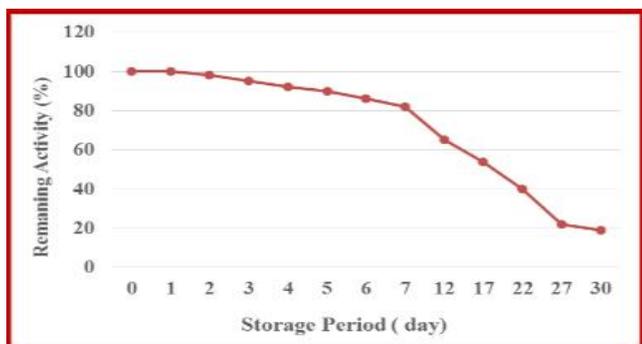
Hydrocarbons	Time (hr)	PAHs degradation efficiency %	
		Crude laccase	Purified laccase
Catechol	1	98.7	99.8
Naphthalene	1.5	99	99.8
Tannic acid	2	93.8	96

### Application of laccase enzyme

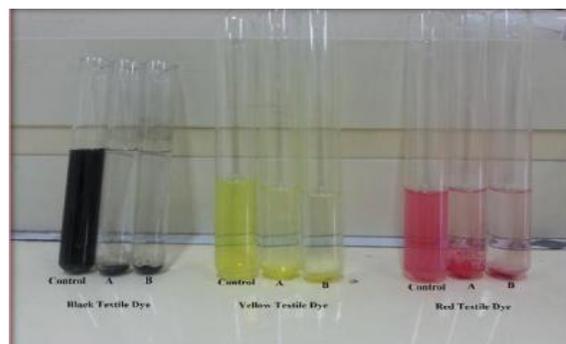
#### Dyes degradation

The various textile dyes (textile yellow, textile red and textile black) were used for degradation capability of laccase (crude and purified) at dyes concentration of (30 mg/l), during 3 hrs in pH 5.0 at 40°C. Absorbance of each dyes was recorded at suitable wave length for each one. Results in table (1) and fig. (14) showed that the value of each absorbance was decreased through the incubation time increases and stabilized after 3 hr and even after 24 hr, compared with absorbance of the control which didn't changed during 24 hrs. These results indicated that laccase have ability to degrade different dyes within 3 hrs these

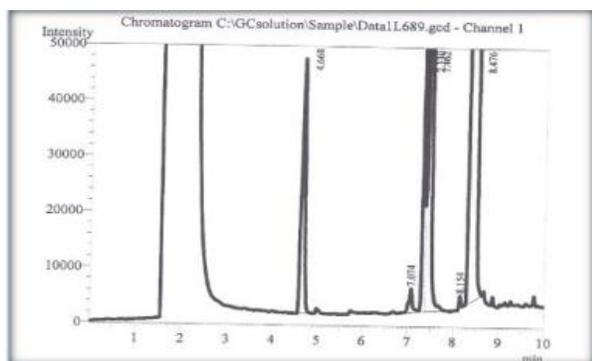
results are an indication of a significant differences in rate of degradation that is probably due to each dye have different structure which effects the degradation capability of laccase enzyme. Textile red exhibited higher degradation capacity with purified laccase and showed maximum removal extent of 94.6 % in 3 hrs with initial purified laccase activity of 344 U/ml, followed by textile yellow and textile black dye with removal efficiency of 92% for each one, (table 1). Whereas, crude laccase has proximately same efficient to degrade dyes compared with purified enzyme. Jiang<sup>[24]</sup> found that the dye decolorization capacity of purified laccase from *Coprinus comatus* was similar or even higher than those obtained with the crude in same conditions.



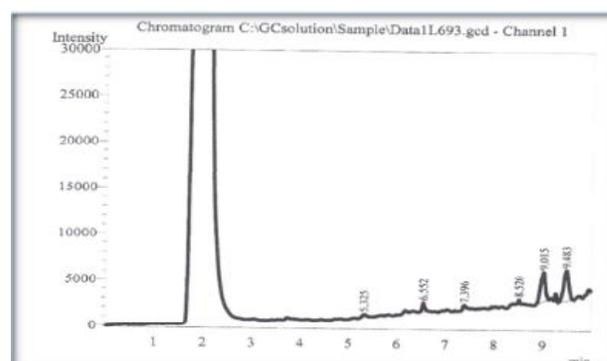
**FIGURE 13:** Effect of different periods of storage on the immobilized laccase activity produced by *P. aeruginosa* SR3



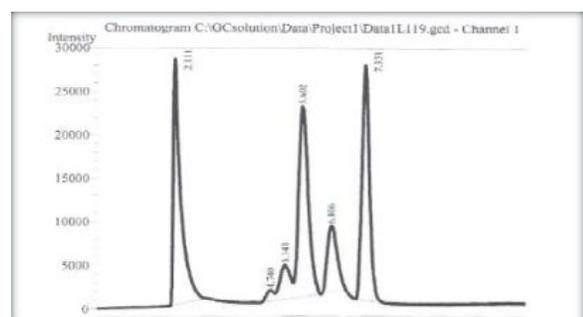
**FIGURE 14:** Dyes decolorization by purified laccase produced by *P. aeruginosa* SR3 in pH 5.0 and 40°C, at a concentration of 30 mg/l after 3 hr



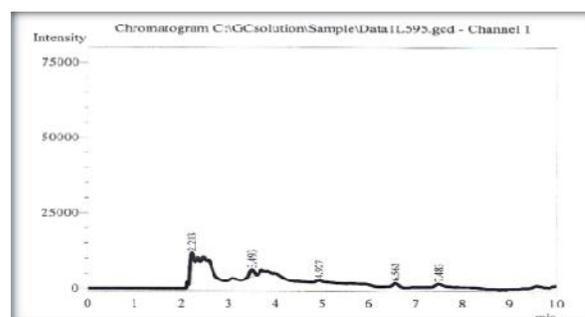
**FIGURE 15:** Chromatogram of catechol concentration at initial time of reaction with crude laccase enzyme



**FIGURE 16:** Chromatogram of catechol concentration after 1 hr. of reaction with crude laccase enzyme

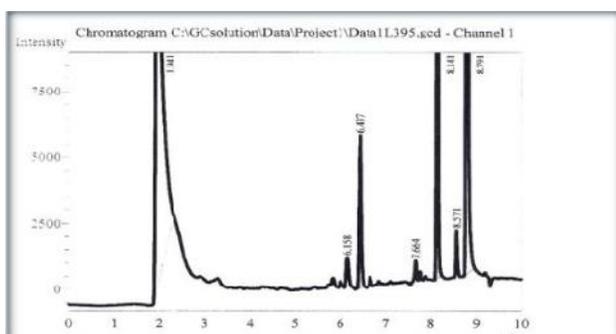


**FIGURE 17:** Chromatogram of catechol concentration at initial time of reaction with purified laccase enzyme

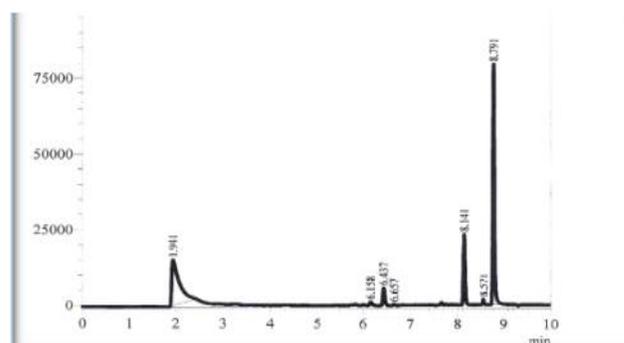


**FIGURE 18:** Chromatogram of catechol concentration after 1 hr. of reaction with purified laccase enzyme





**FIGURE 25:** Chromatogram of tannic acid concentration after 2 hr. of reaction with purified laccase enzyme



**FIGURE 26:** Chromatogram of tannic acid concentration after 2 hr. of reaction with purified laccase enzyme

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