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Characterization of *Aspergillus flavus* NG 85 Laccase and Its Dye Decolorization Efficiency.

N. M. Khalil*, M. I. A. Ali, S. A. Ouf and M. N. Abd El-Ghany.

Department of Botany and Microbiology, Faculty of Science, Cairo University, Giza 12613, Egypt.

ABSTRACT

The purified laccase of *Aspergillus flavus* NG85 isolated from Saint Catherine Protectorate, showed a molecular weight of 68.5 kDa. Its optimum activity obtained at enzyme concentration of 0.15 mg, substrate concentration of 10 mg/ml, temperature of 47.5 ^{o}C, pH of 5 and K_m of 9.09 mg/ml. The enzyme retained 85% of its activity after 4 hours of incubation at 50 ^{o}C and 88% of its activity after 4 hours of incubation at pH 5. Copper sulfate exhibited the highest significant promotive effect on laccase activity, while sodium azide caused complete inhibition. The maximum and rapid decolorization of the purified laccase was achieved with malachite green. Among seven metallic ions tested, copper ion was the best in decolorization efficiency. Furthermore, decolorization of the real textile effluent by purified laccase predominantly occurred within the first 4 days. The putative gene for laccase was isolated, sequenced and recorded in Gene bank under accession number KM522917.2.

Keywords: laccase, optimization, synthetic dyes, decolorization.

Corresponding author

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INTRODUCTION

Laccases (Lac, EC1.10.3.2), (benzenediol: oxygen oxidoreductases) are blue copper oxidases that catalyze the one-electron oxidation of phenolics, aromatic amines and other electron-rich substrates [1]. They transfer electrons to dioxygen yielding water, with the oxidation of a wide range of reducing substrates including phenolic compounds and aromatic amines [2]. The oxidation of a reducing substrate by laccase typically involves the loss of a single electron and the formation of a free (cation) radical. The radical is in general unstable and may undergo further laccase-catalyzed oxidation (e.g. to form quinone from phenol) or nonenzymatic reactions (e.g., hydration, disproportion or polymerization) [3]. Laccases are similar to other phenol-oxidizing enzymes, which preferably polymerize lignin by coupling of the phenoxy radicals produced from oxidation of lignin phenolic groups. However, the substrate range of laccase can be extended to non-phenolic subunits of lignin by the inclusion of a mediator such as 2, 2/-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) [4]. On SDS-PAGE, most laccases show mobilities corresponding to molecular weight of 60-100 kDa, of which 10-50% may be attributed to glycosylation [3, 5].

Laccases have many biotechnological applications because of their oxidation ability towards a broad range of phenolic and non-phenolic compounds [6]. Other applications of laccase include the cleaning of the industrial effluents, mostly from industries like paper industry, pulp, textile & petrochemical industries. Laccases also used in the medical diagnostics and for cleaning herbicides, pesticides and some explosives in soil. Laccase has many applications in agricultural, medicinal and industrial areas [7].

Synthetic dyes are widely used in industries as textile, leather, cosmetics, food and paper printing [8]. Reactive dyes are colored molecules used to dye cellulose fibers [9]. These dyes result in the production of large amounts of high-colored wastewater. A special problem found in the application of synthetic dyes that they are resistant to biodegradation [10]. Wastewaters from textile dying process are usually treated by physical or chemical processes, which include physical–chemical processes electrokinetic coagulation, electrochemical destruction, irradiation, precipitation, ozonation, or the Katox method that involves the use of active carbon and the mixture of certain gases as in air [11]. However, due to the chemical nature, molecular size and structure of the reactive dyes these classical processes can cause a problem in the environment and better treatments can be obtained using bioprocesses [9]. Recently, enzymatic treatments have attracted much interest in the decolourization/degradation of textile dyes in wastewater as an alternative strategy to conventional chemical and physical treatments, which present serious limitations [12].

The present work aimed to purify and investigate laccase from the fungus *Aspergillus flavus* NG85. The aim was extended to survey the decolorization efficiency towards seven dyes. Moreover, the industrial application in terms of textile wastewater treatment was considered.

MATERIALS AND METHODS

Laccase production by *Aspergillus flavus* NG85

Aspergillus flavus NG85 was previously isolated from Saint Catherine Protectorate soil at South Sinai, Egypt **[13]**. The fungus strain was grown on the optimized medium for laccase production with the following components (g/L): glucose, 24; peptone, 2.51; K_2HPO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01. The culture was incubated at 36.7 ^{o}C for 10 days.

Purification of *Aspergillus flavus* NG85 extracellular laccase

Laccase was precipitated by adding 80 % NH_4SO_4 to the fermentation broth and then resuspended in 0.1 M acetate buffer (pH 5). It was dialyzed against sucrose to elute excess of water inside the bag and concentrate the protein. The protein content and laccase activity were determined and the specific activity was calculated. After dialysis, the sample was applied to DEAE-Sephadex column pre-equilibrated with 0.1 M acetate buffer (pH 5). The column was eluted with a linear gradient of 0–0.5 M NaCl in 0.1 M acetate buffer (pH 5). Elution was monitored at 280 nm for detection of protein and fractions (5 ml) were collected at a flow rate of 15 ml / hour. Protein content and laccase activity were assessed and the specific activity was calculated.

The active fractions were pooled and stored at 4°C. The pooled fractions of highest laccase activity obtained from the previous column were applied in a small amount of phosphate buffer pH 8 to Sephadex G-100 column (1.3 x 90 cm), which had been previously equilibrated with the same buffer. Elution was performed at a flow rate of 12 ml / hour and 5 ml fractions were obtained. The protein content and laccase activity were determined. The fractions which showed highest laccase activity were pooled for further investigation. The purity of the enzyme was checked by SDS-PAGE. The molecular weight of the purified laccase was estimated using Thermo Scientific™ PageRuler™ Prestained Protein marker [14].

Enzyme assay

Laccase activity was assayed according to Olga et al. [15], with guaiacol as substrate. The reaction mixture contained 3.9 ml acetate buffer (10 mmol/L, pH 5.0), 1 ml guaiacol (2 mmol/L) and 0.1ml properly diluted enzyme solution was incubated at 35°C for 30 min. Absorbance was read at 470 nm (εmax = 6,740 Mol-1 cm-1). In the blank, guaiacol was replaced with acetate buffer.

Effect of enzyme concentration

In this experiment, the effect of laccase enzyme, with different protein concentrations of 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16 and 0.18 mg protein/ ml the reaction mixture, on laccase activity was investigated. The reaction, as described before, was performed at 35° C for 30 minutes incubation period. In each case, the blank sample contained the same protein concentration previously boiled to stop the enzyme activity.

Effect of substrate concentration

The effect of substrate concentration on the purified laccase activity was studied using different guaiacol concentrations of 0.5, 1, 2, 4, 6, 8, 10, 12 and 14 mg / ml reaction mixture. The blanks were prepared using the same substrate concentrations previously boiled before the enzyme reaction. The enzyme activity was determined as described before.

Determination of the kinetic constant (k_m) for the purified *Aspergillus flavus* laccase using Lineweaver-Burk plot

The results of the previous experiment were used to calculate the value of the kinetic parameter (k_m) (Michaelis constant). This is done by plotting 1/S versus 1/V according to the Lineweaver-Burk plot [16],

Where, S is substrate concentration (mg/ml) and V is reaction velocity (laccase units/ml/ hour). The substrate concentrations tested were from 0.5-4 mg/ml in the reaction mixture.

Effects of pH and temperature on laccase activity and stability

To determine the optimum pH, laccase activity was measured in buffers ranging between pH 2.0 to 8.0 The following buffer solutions were used: 50 mM acetate buffer solution for pHs 2, 3, 4 and 5; 50 mM citrate buffer solution for pHs 4 and 5; and 50 mM phosphate buffer solution for pHs 6, 7 and 8, each at a concentration of 0.1 M. The reaction mixture was carried out with the optimum enzyme and substrate concentration. Incubation was conducted at 35 °C for 30 minutes. The blanks were treated in the same way in each case except for boiling before the enzyme reaction. The laccase activity was measured as previously described.

The stability of the enzyme to pH was studied by incubating optimum concentration of laccase enzyme for 1, 2, 3 and 4 hours at 47.5 °C and pH values of 4, 5 or 6. The residual laccase activity was quantified at the optimum pH and temperature.

For the optimum temperature, laccase activity was measured at the optimum pH, optimum concentration of enzyme and substrate and various temperatures. These mixtures were incubated at the following temperatures: 15, 25, 35, 45, 55, 65 and 75 °C for 30 minutes. Blanks were made for the different temperatures but boiled before the enzyme reaction. laccase activity was assayed as previously mentioned.

Thermostability was analyzed by incubating the enzyme solutions in water baths at 50, 60 and 70 ^{o} C for 1, 2, 3 and 4 hours. Optimum substrate concentration was added and incubation was at optimum temperature and pH for 30 minutes. All experiments were repeated three times with three replicates each time.

Effects of metal ions and inhibitors on laccase activity

Metal salts were tested at a concentration of 10 mM. These were: $FeSO_4.7H_2O$, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, $MnSO_4.H_2O$, NaCl, KCl or $CuSO_4.5H_2O$. The effects of inhibitors were tested at concentrations of 1, 5 or 10 mM/L. These were sodium azide, ethylene diamine tetraacetic acid (EDTA), thiourea, ethanol, sodium dodecyl sulfate (SDS). Individual effect or was incorporated in the standard enzyme activity assay, and the activity was determined at the optimal temperature and pH. The enzyme activity in the absence of an effector was set to 100%.

Removal of some dyes by purified laccase

The purified laccase enzyme was used to decolorize seven synthetic dyes. The assays were carried out at 47.5^{o}C. The reaction mixture in a total volume of 0.2 mL contained (final concentration): acetate buffer (50 mM, pH 5.0), dyes: 25, 50 or 100 mg/L (Bromothymol blue, Congo red, malachite green, eosin, crystal violet, azure B, coomassie blue) and 100 µL purified laccase. The decolorization of dye, expressed as decolorization (%), was calculated as the formula: decolorization (%) = [(Ai− At)/Ai] × 100, where, Ai: initial absorbance of the dye, At: absorbance of the dye along the time [17].

Effect of some metal ions and organic solvents on decolorization of malachite green

The assays were carried out at 47.5°C. The reaction mixture in a total volume of 0.2 mL contained (final concentration): acetate buffer (50 mM, pH 5.0), dyes (malachite green: 50 mg/L), different metal ions (100 mM) or different organic solvents (25%) and100 µL purified laccase (0.1 U). Decolorization was monitored and calculated by the method described above.

Laccase mediated decolorization of a real textile effluent

The purified laccase enzyme was used to decolorize real textile effluent (diluted 1:10). Purified laccase was added to the sample at 100 µL and the mixture was incubated at 47.5 ^{o}C and 150 rpm for 6 days. Decolorization of the effluent was monitored every 24 hours by measuring absorbance between 325 and 800 nm. All experiments were conducted in triplicate.

Isolation and sequencing of putative laccase gene

DNA was extracted using Thermo Scientific Gene JET Plant Genomic DNA Purification Mini Kit #K0791. The concentration and purity of DNA samples was measured spectrophotometerically by adding 10 µl DNA to 990 µl de-ionized water in 1-ml quartz cuvette and optical density was recorded at 260 and 280 nm. The ratio of OD_{260}: OD_{280} should be more than 1.9 [18]. Forward and reverse primers were designed for amplification of putative laccase gene using PCR, they were designed by the aid of NCBI database multiple sequences alignment. Laccase coding sequence was PCR amplified using primers,

Forward primer: TCTTCCCGGGTCCGACTATT.
Reverse primer CGTTGGCCTGGGTAAAGGAT.

The PCR mixture contained: 10x reaction buffer, 1 µl; forward primer, 1µl; reverse primer, 1µl; 2.5 mM dNTPs, 2 µl; 25 mM $MgCl_2$, 4 µl; DNA, 2 µl and Dream *Taq* polymerase (Thermo Scientific) , 0.5U per reaction; up to 50 µl with sterile de-ionized water. The PCR was carried out as an initial denaturation for 10 min at 94°C followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min., and extension at 72°C for 1 min, then final extension at 72°C for 15 min. The PCR product was applied to agarose gel electrophoresis.

Sequencing of the PCR product was performed in forward and reverse direction on GATC company by using ABI 3730xl DNA sequencer by combining the traditional Sanger technology with the new 454

technology, primers used in PCR were used also in the process of sequencing. The sequence has been submitted to GenBank and an accession number was obtained. The deduced amino acid sequence was done according to website http://web.expasy.org/translate.

RESULTS AND DISCUSSION

Production and purification of laccase

Laccases have been discovered and studied since the nineteenth century, they were reported to be isolated from bacteria, fungi, higher plants and insects [1, 17, 19, 20], but the majority is found in higher fungi. Laccases produced by white-rot fungi have been widely used in various detoxification processes since they oxidize aromatic pollutants such as chlorophenols, PAHs or dyes [21, 22, 23, 24]. The isolate, *Aspergillus flavus* NG85, used in this work was obtained from a previous study [13] from Saint Catherine Protectorate soil (South Sinai).

Laccase production by *Aspergillus flavus* NG85 was conducted in optimized cultural conditions. After $(NH_4)_2SO_4$ precipitation, anion exchange and hydrophobic interaction chromatography, laccase was purified 1.83-fold with 44.99% yield from the fermentation broth. The specific activity was 21.02 units/mg after ammonium sulphate precipitation while specific activity was 40.61 and 76.61 units/mg after DEAE-Sephadex and Sephadex G-100, respectively as shown in Table 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that the purified laccase is homogenous, as seen by a single band of protein, and has an apparent molecular weight of 68.5 kDa (Figure 1). The reached results confirm that recently recorded by Rezaei *et al*. [8], regarding the fungal laccase isolated from *Paraconiothyrium variabile*. They stated that the enzyme is homologous, purified 11.99 folds using ammonium sulfate precipitation showed single protein band and has a molecular weight of 68.5 kDa. Suwannawong *et al*. [25] reported that partially purified laccase from the crude extract of a solid state culture of *Lentinus polychrous* by salt fractionation, Sephacryl S-300 and DEAE-cellulose columns in succession. They detected the laccase activity as a single peak (fractions 43-60), with molecular weight 45 kDa which was lower than the most commonly reported fungal laccases, between 50-130 kDa [26, 27].

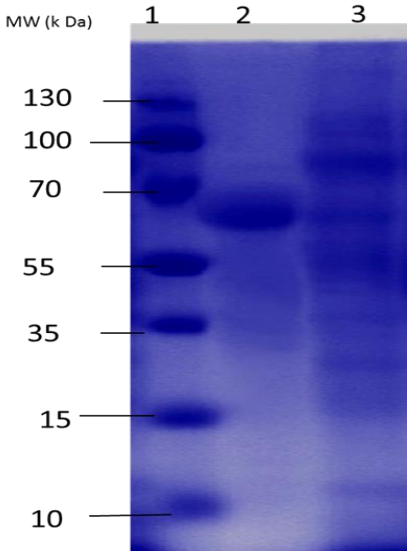

Figure 1: SDS-polyacrylamide gel electrophoresis of *Aspergillus flavus* NG85 laccase. Lane 1: protein marker (The Thermo Scientific™ PageRuler™). Lane 2: purified laccase. Lane 3: crude laccase extract.

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Table 1: A summary of the purification steps of laccase from *Aspergillus flavus* NG85

Purification Step	Total protein (mg ± SE)	Total laccase activity (units ± SE)	Specific activity (units/mg)	Purification fold	Recovery (%)
Crude extract	396.5±3.12	250.5±2.6	11.50	1.00	100.00
Ammonium sulfate	90.98 ±1.91	912.33±1.16	21.02	1.82	44.99
Dialysis	89.57±0.53	899.87±1.23	21.21	1.84	44.70
DEAE- Sephadex	38.87±0.53	1578.51±1.71	40.61	3.53	23.67
Sephadex G-100	19.57±0.53	1499.17±1.45	76.61	6.66	35.27

* Recovery (%) = laccase units as % from total laccase activity in crude extract, SE: standard error.

Characterization of purified laccase

Characterization of the purified *Aspergillus flavus* NG85 laccase in the present study, with the aid of regression analysis, revealed that the optimum protein concentration was 0.15 mg enzyme protein/ml in the reaction mixture. No significant increase in enzyme activity above the optimum concentration (Figure 2-a). The optimum substrate (guiacol) concentration was 10 mg/ml and no significant change in enzyme activity was observed above this optimum concentration (Figure 2-b). According to the Lineweaver-Burk plot, the k_m value was found to be 9.09 mg/ml.The Km values of laccases from different source organisms have different substrate preferences [28]. Laccases in general combine high affinity for ABTS and syringaldazine with high catalyst constant,whereas the oxidation of guiacol and DMP is considerably slower. The k_m values of purified laccase towards guiacol differ according to *Aspergillus* species where it measured 7.2 mg/ml for *Aspergillus nidulans* CASVK3 [29] and 2.84 mg/ml for *Aspergillus flavus* [29].

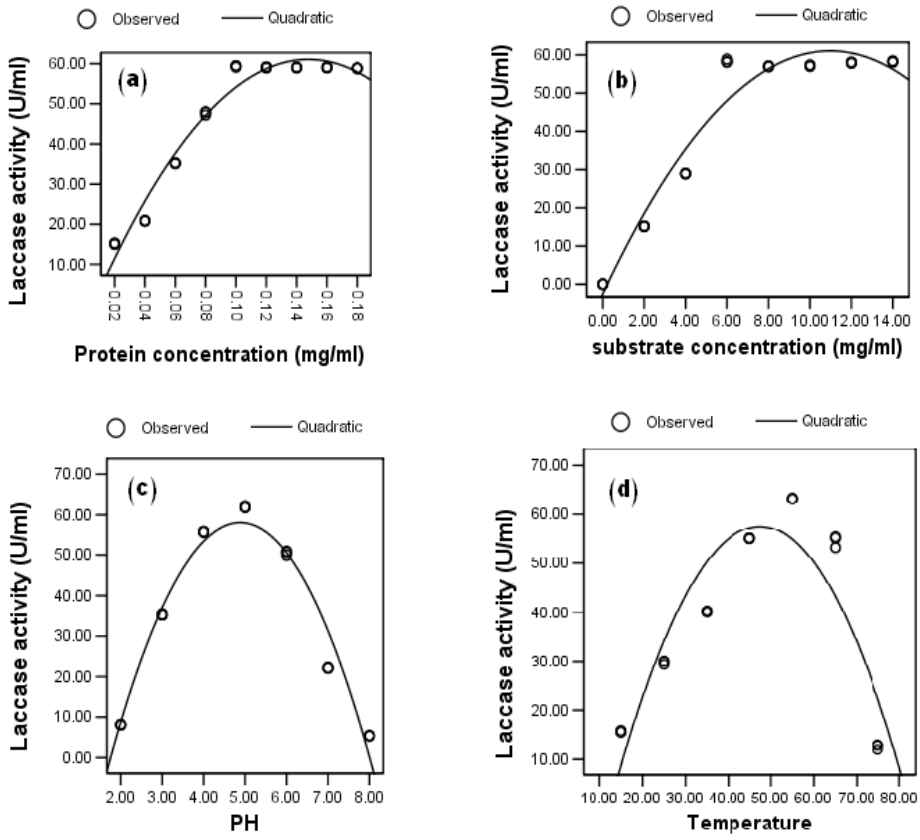

Figure 2: Effect of different factors laccas activity from Aspergillus flavus NG85 (a) protein concentration, (b) substrate concentration, (c) pH, (d) temperature.

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In this study, the *Aspergillus flavus* NG85 laccase exhibited maximum activity at pH 5 (Figure 2-c). The enzyme was stable exhibiting 80, 88 and 85 % of its activity at pHs 4, 5 and 6, respectively (Figure 3-a). The instability of the enzyme at higher pH is mostly due to the inhibition of the activity by hydroxide ion that generally prevents catalysis of the substrate [28]. The optimum temperature for enzyme activity was 47.5^{o}C (Figure 2-d). The enzyme was thermostable and it retained 85, 75 and 63 % of its activity after 4 hours of incubation at 50, 60 and 70 ^{o}C, respectively (Figure 3-b). Gomaa and Momtaz [30] stated that laccase isolated from *Aspergillus flavus* showed optimum temperature of 50 ^{o}C and optimum pH was 3.

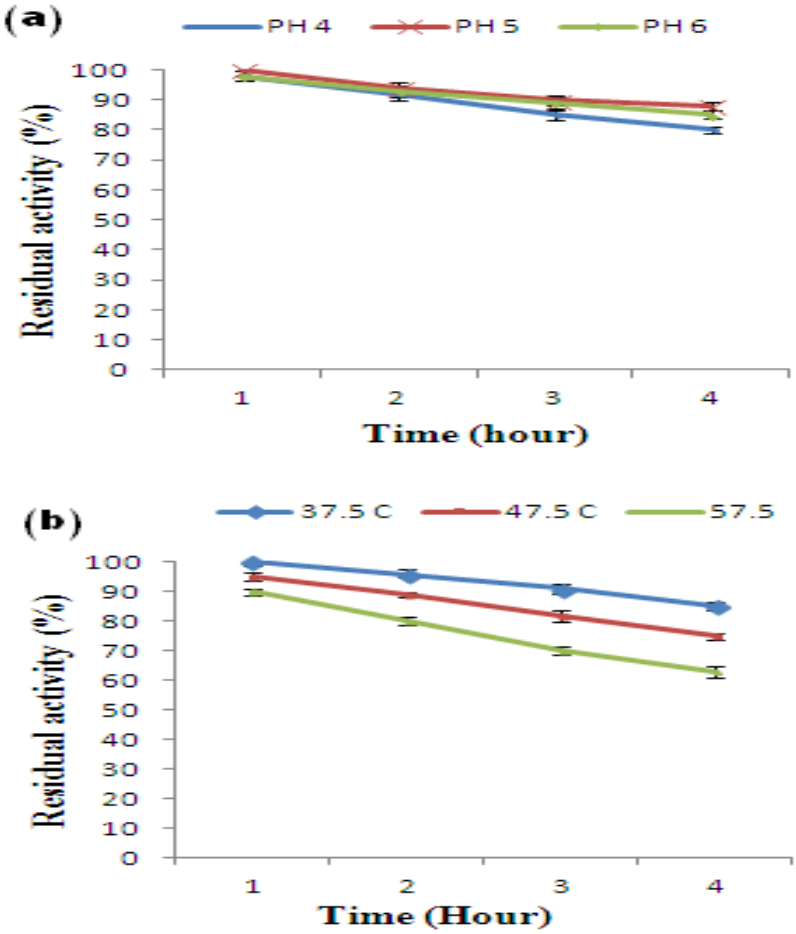

Figure 3: Stability of *Aspergillus flavus* NG85 laccase towards pH (a), temperature (b).

Effects of metal ions and inhibitors

Laccase was highly tolerant to metal salts and organic solvents. There was a significant inhibition in laccase induction exhibited by ferrous sulfate, magnesium sulfate or calcium chloride at a concentration of 10 mM. These compounds may cause chelating the copper atoms of laccase, modify the amino acids residues of the proteinic part, may induce a change in configuration of the glycoprotein [20]. A statistically non-significant change was noticed for potassium chloride or sodium chloride at a concentration of 10 mM. As shown in (figure 4-a), an enhancement in laccase production was caused by manganese sulfate at a concentration 10 mM. However, the highest significant promotive effect was due to copper sulfate addition at a concentration of 10 mM with relative activity percentage of 143.2 %. The addition of copper into the culture medium has been found to cause laccase folding properly due to the more efficient transport of cupper to the golgi components [31,32]. Zou *et al*. [33] reported that Mg^{2+} and Al^{3+} ions significantly increased the activity of laccase isolated from *Hericium coralloides* at concentrations of 12.5-100 mM, whereas Fe^{2+} and Hg^{2+} ions significantly inhibited the laccase activity at 12.5-100 mM. Ca^{2+}, Mn^{2+}, Cd^{2+}, Co^{2+} and K^{+} ions inhibited enzyme activity at 100 mM but increased activity at relatively lower concentration ranging from 12.5 to 50 mM. Ding *et*

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al. [34] found that laccase activity was greatly enhanced by most metal ions and that an increase of 488.51% was obtained by adding 0.1 mM Cu^{2+} as copper sulfate. The laccase activity was completely inhibited by all concentrations of Fe^{2+} applied as ferrous sulfate.

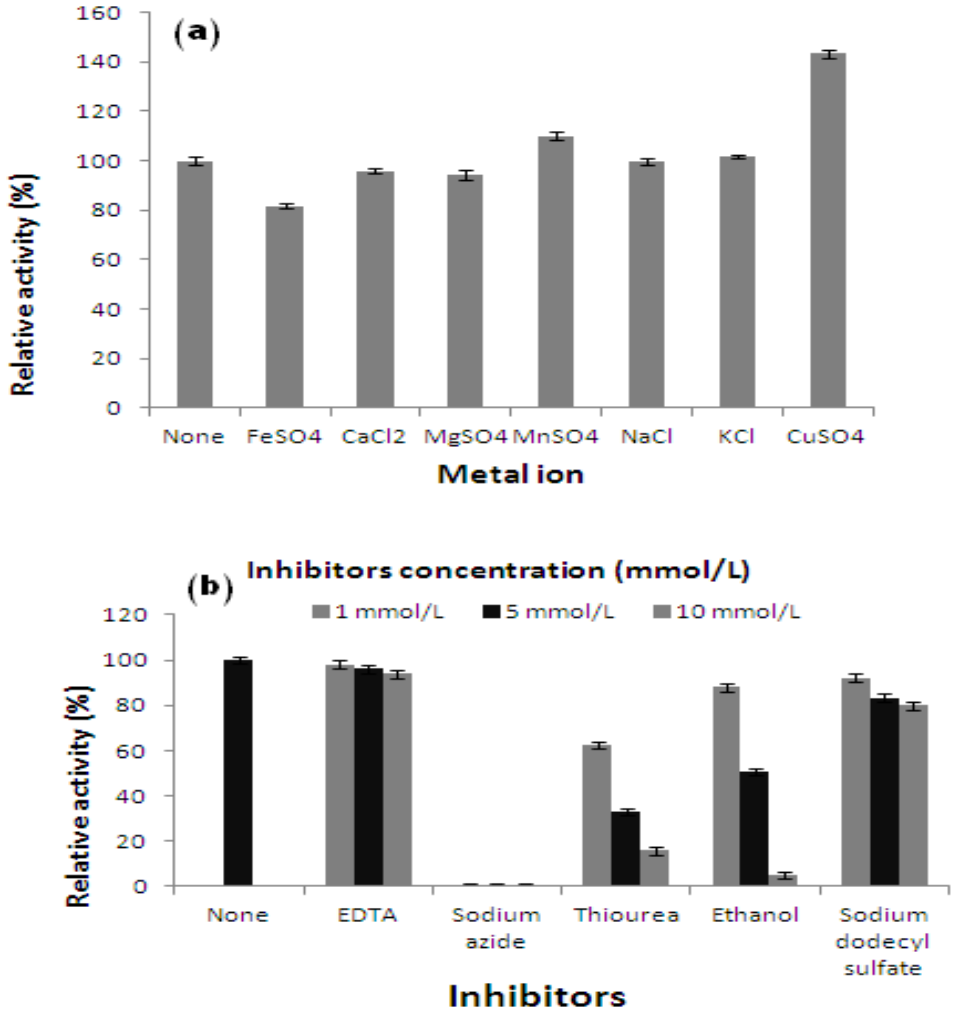

Figure 4: Effect of Effect of different factors laccas activity from Aspergillus flavus NG85 (a) metal ions, (b) inhibitors, on *Aspergillus flavus* NG85 laccase activity.

On the other hand when testing the effect of some inhibitors in the present work (Figure 4-b), the enzyme activity was completely inhibited by sodium azide at all tested concentrations (1, 5, 10 mmol/L). This inhibition may be due to the binding of the azide anion to the laccase copper causing disruption in the electron transfere system resulting in enzyme inhibition [20]. At 1mmol/L concentration, thiourea, ethanol and sodium dodecyl sulphate caused 62.3%, 88.12% and 92.11% relative activity, respectively. Whereas the metal ion chelator EDTA was not an efficient inhibitor of laccase production at 1mmol/L. Even higher concentration (10 mmol/L) of EDTA did not inhibit guiacol oxidation by a laccase produced by *Aspergillus flavus* NG85. Ramírez-Cavazos [5] reported that the purified laccase from from *Pycnoporus sanguineus* was strongly inhibited by $NaN_3$and NaF even at lower concentrations while it showed high tolerance to urea, Na_2SO_4, NaCl, mild tolerance to acetonitrile, Dimethylformamide , acetone, ethanol and dimethyl sulfoxide, and EDTA.

Removal of some dyes by purified *Aspergillus flavus* NG85 laccase

Nozaki *et al*. [35] stated that the speculated high redox potential and low substrate specificity of laccase could be beneficial for its practical application, which was corroborated by laccase-catalyzed breakdown of structurally different dyes. In this study, the decolorizing capability of purified laccase on synthetic dyes was evaluated without the help of small redox mediators. Purified laccase was able to

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effectively decolorize dyes of different classes, some of which were notoriously recalcitrant to biodegradation, e.g., bromothymol blue, congo red, malachite green, eosin, crystal violet, azure B, or coomassie blue.

As shown in figure 5 (a-g), purified laccase could efficiently decolorize different synthetic dyes in the absence of any redox mediator. Decolorization efficiency showed the same trend at the three tested concentrations (25, 50 and 100 mg/L). However, increasing the dye concentration was concomitant with somehow less decolorization. The dyes; malachite green, bromothymol blue, crystal violet, coomassie blue, eosin, congo red or azure B applied at 100 mg/L could be respectively decolorized up to 84.1%, 80%, 78%, 80.4%, 65%, 60.9% or 30.9%, respectively within 17 hours. The highest decolorization potency exhibited against malachite green was attained within only 8 hours. Mogharabi *et al*. [24] in their work with laccase from *Paraconiothyrium variabile*, reported dye removal of crystal violet, coomassie blue G-250, bromothymol blue, amido black 10B, methyl red, eosin, methyl green and malachite green with maximum decolorization of 86.9% and minimum of 32.8% obtained in the case of amido black 10B and eosin, respectively. Yang *et al.* [36] investigated decolorization capacity of the laccase from *Cerrena sp.* HYB07 on six different dyes and found that the maximum decolorization was achieved by malachite green (94.1%), followed by bromothymol blue (73.7%).

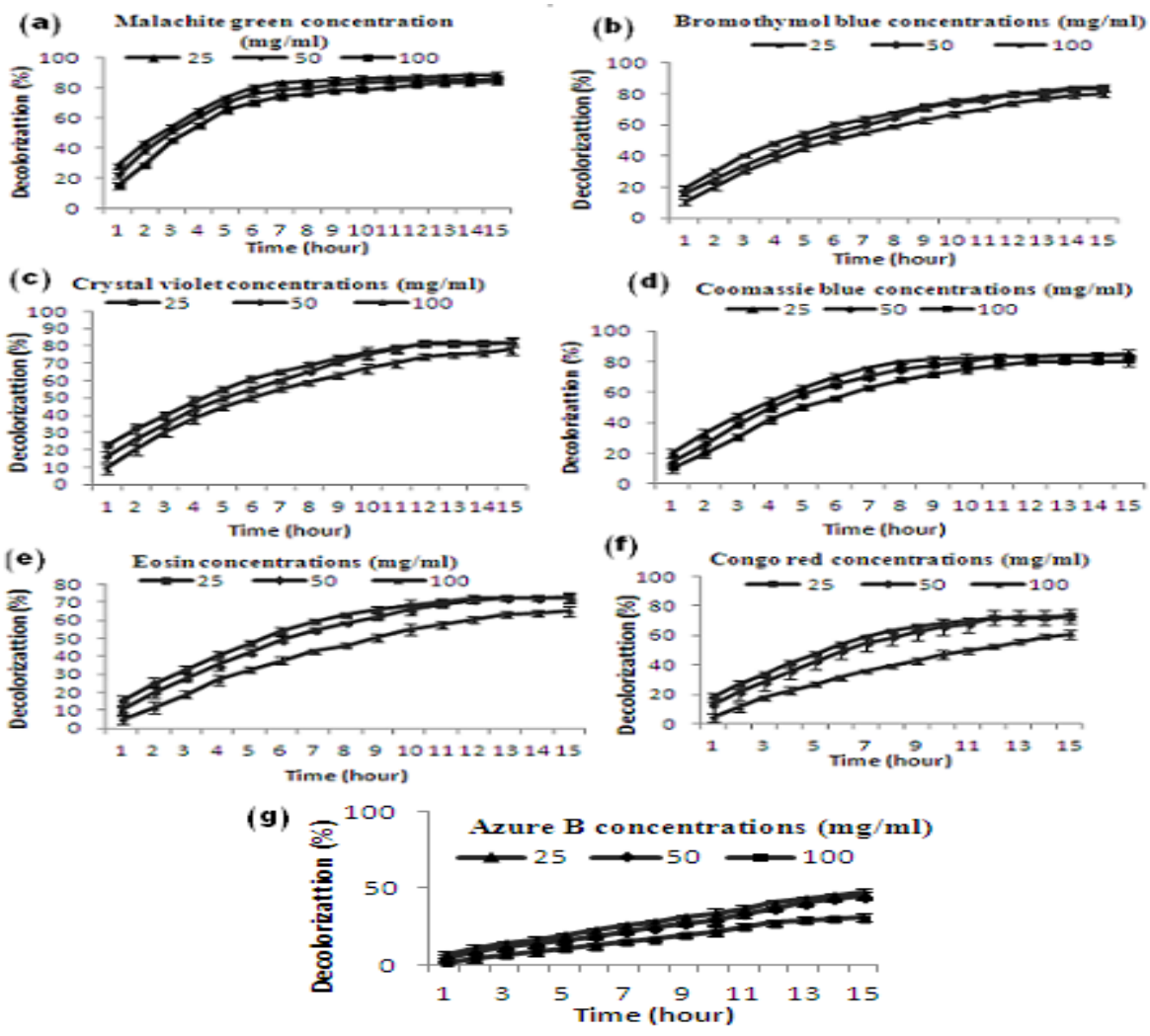

Figure 5: Decolorization of different dyes due to laccase from *Aspergillus flavus* NG85. (a) malachite green, (b) bromothymol blue, (c)crystal violet, (d) coomassie blue, (e) eosin, (f) congo red or (g) azure B.

Decolorization efficiency in presence of some metal ions or solvents

In order to evaluate the ability of purified laccase to decolorize a synthetic dye in presence of high concentrations of metal ions, purified laccase was further used to decolorize malachite green. As shown in (Figure 6-a), the enzyme caused 86.3% decolorization in absence of any metal ions. Purified laccase had a

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strong capacity for decolorizing malachite green with the coexistence of high concentrations of different metal ions. Malachite green (50 mg/L) decolorization reached up to 85.7%, 89.7 and 97.4 in the case of coexistence of $CaCl_2.2H_2O$, $MnSO_4.H_2O$ and $CuSO_4.5H_2O$, respectively, within 17 h by purified laccase.

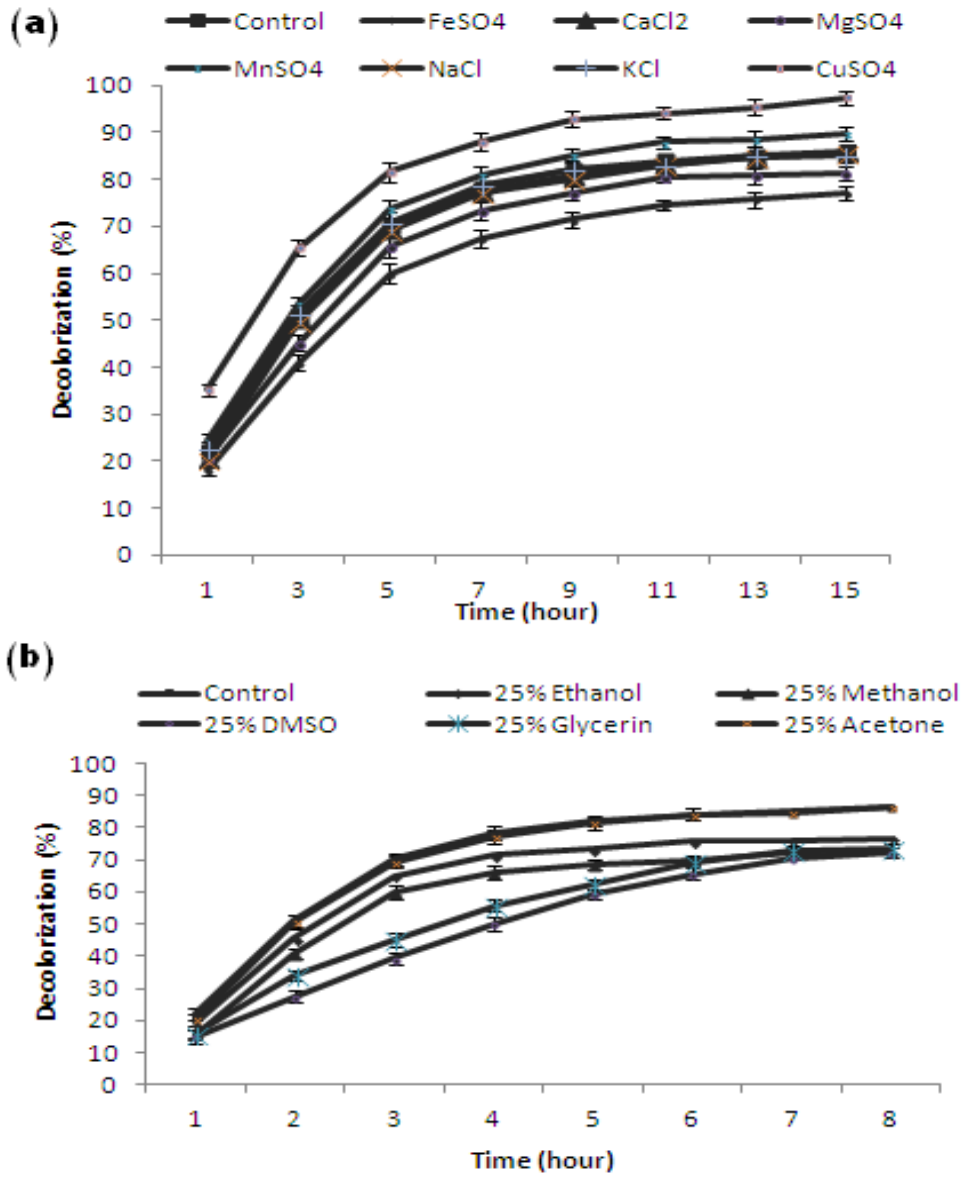

Figure 6: Decolorization of malachite green with coexistence of metal ion (a) or organic solvents (b) by *Aspergillus flavus* NG 85 laccase , control is conducted without addition of any metal compounds.

The ability of purified laccase to decolorize malachite green in presence of some organic solvents was tested. Figure (6–b) shows that the enzyme decolorized 86.3% malachite green in absence of any organic solvents. Purified enzyme had strong capacity for decolorizing malachite green with the coexistence of different solvents. Malachite green (50 mg/L) with the coexistence of ethanol , methanol , Dimethyl sulfoxide (DMSO) , glycerin or acetone, each at 25% could be decolorized up to 76.9%, 76.8%, 72.4%, 72.1%, 73.4%, 86.1% , respectively within 17 hours by purified laccase.

Zhuo *et al*. [17] studied the capacity for decolorizing malachite green with the coexistence of high concentrations of different metal ions. Malachite green (50 mg/L) with the coexistence of $CuSO_4$, $MnSO_4$, $MgSO_4$, $MgCl_2$,$LiCl_2$,KCl , $CaCl_2$, $AlCl_3$,$CdCl_2$, $NiCl_2$ (final concentration: 100 mM) could be decolorized with maximum value of 95.2% with $MnSO_4$ and minimum value of 51.1% with $CdCl_2$,respectively, within 20 h by

laccase enzyme. Decolorization efficiency within 20 h for malachite green (50 mg/L) with the coexistence of methanol, ethanol, methyl cyanide, DMSO, propylene glycol, glycerin, ethylene glycol, acetone, isopropyl alcohol (final concentration: 25%) could reach 95.2%, 95.1%, 95.5%,80.0%, 50.4%, 86.8%, 83.0%, 94.6%, 95.6%, respectively.

Real textile effluent decolorization by purified laccase

Besides degrading a variety of dyestuffs, purified laccase also decolorized a real textile effluent. Hence, laccase holds great promise for applications in biodegradation and bioremediation, especially treatment of dye effluents. In this work, decolorization of the real textile effluent by purified laccase predominantly occurred within the first 4 days, accompanied by flattening of the absorption spectrum (Figure 7-a). A negative control was conducted in parallel, and there was little decrease in the absorbance in the absence of enzyme (Figure 7- b). Yang *et al*. [33] stated that decolorization of the real textile effluent by purified laccase from *Cerrena sp*. predominantly occurred within the first 3 days, accompanied by flattening of the absorption spectrum.

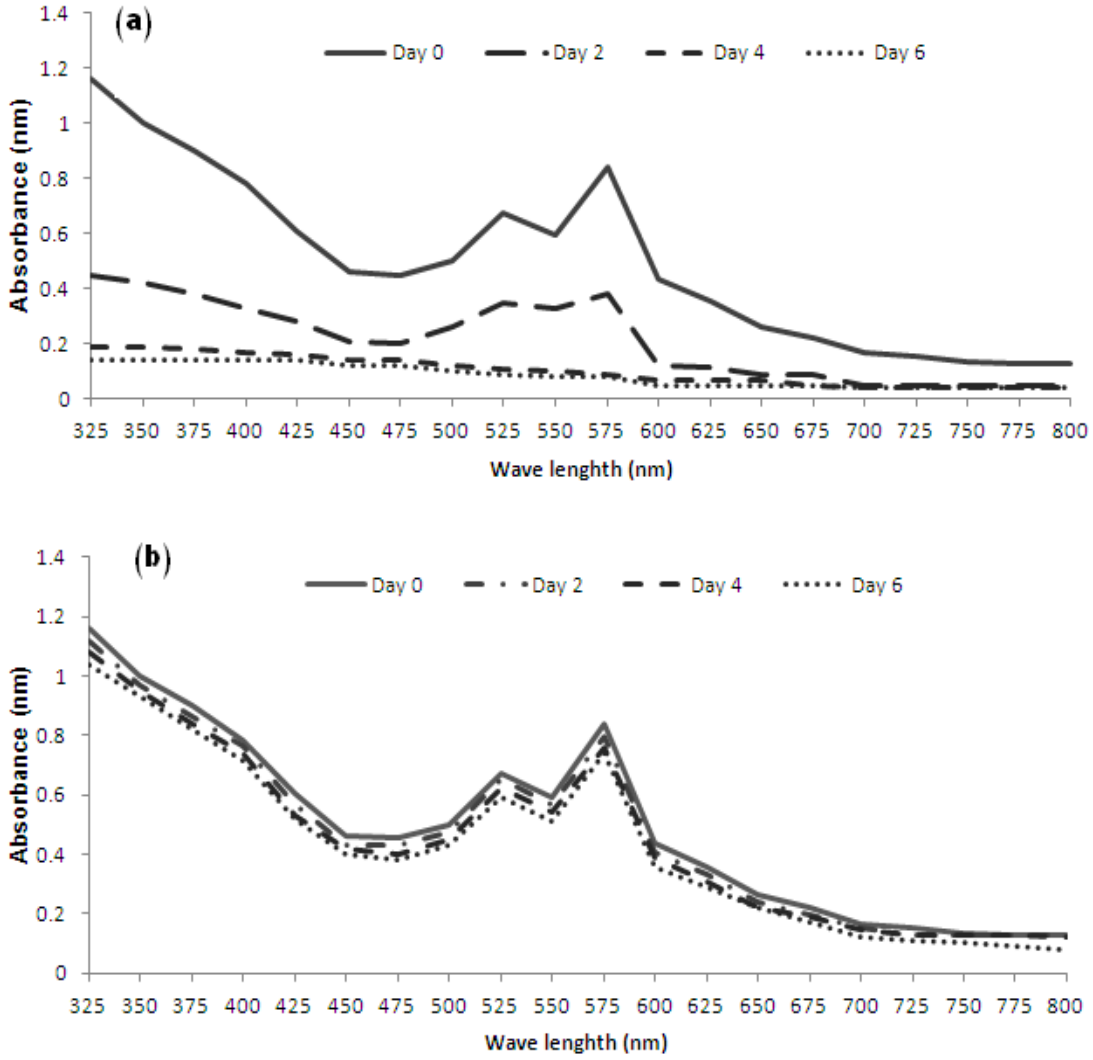

Figure 7: Decolorization of real textile effluent by *Aspergillus flavus* NG 85 laccase (a) or without laccase (b)

Molecular isolation, sequencing of laccase gene from *Aspergillus flavus*

After contig and assembly of the forward and the reverse sequences, the nucleotide sequence of laccase was found to contain 808 bp, coding for a putative polypeptide of 218 amino acid residues as shown in Figure 8. The sequence was deposited in the NCBI GenBank and an accession number,

KM522917.2, was given. Blast analysis using blastx revealed a high degree of similarity of the deduced amino acids sequence with laccase of *Aspergillus oryzae* RIB40. This supports the idea that the deduced sequence of amino acids from the partial sequence of laccase in *Aspergillus flavus* NG85 is similar with the multicopper oxidase especially with laccase in *Aspergillus flavus* with the highest degree of similarity. The calculated molecular weight of the deduced polypeptide is 23.7055 kDa, and the estimated isoelectric point is 4.82. The deduced amino acids sequence revealed that the most abundant amino acids were glycine and Leucine (9.6%), whereas the least abundant amino acids were lysine (0.5%) followed by cystine (1.4%). Vasina *et al*. [37] showed that multiple alignment of the translated amino acid sequences for laccases of *Trametes hirsuta* 072 with other laccases from *Trametes* species have the characteristic laccase copper-binding conserved domains.

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tattgaggcctgttctggcgataccctttaatcaatgtcacaaatgcactacaaggtgaa   60
          I  E  A  C  S  G  D  T  L  L  I  N  V  T  N  A  L  Q  G  E
ccaatctctatccactggcatgggcttcacgtccatagtaagtcattcttctcaacatat   120
          P  I  S  I  H  W  H  G  L  H  V  H  S  K  S  F  F  S  T  Y
gcctaggatgatcataagactgacgttctagatacaatggatggtgttcccggagtgaca   180
          A  -  D  D  H  K  T  D  V  L  D  T  M  D  G  V  P  G  V  T
cagaatgccatcccacctggatcgaccttcatgtacaacctgactattccccaagaccaa   240
          Q  N  A  I  P  P  G  S  T  F  M  Y  N  L  T  I  P  Q  D  Q
agtggcacctttttggtaccatggccataccggaacctcgagggcggatggcctatacggc   300
          S  G  T  F  W  Y  H  G  H  T  G  T  S  R  A  D  G  L  Y  G
ggctttgttgtgcatgcgccttcttcccggcctaccgttcgcggactcatggcccgtgac   360
          G  F  V  V  H  A  P  S  S  R  P  T  V  R  G  L  M  A  R  D
agcgccgagtccctgcagtacgggtacgaaagagagttcctactgttgatcggggattgg   420
          S  A  E  S  L  Q  Y  G  Y  E  R  E  F  L  L  L  I  G  D  W
taccatcagcctggtgctcaggtcttagcctggtacatgagcatcgcatctttcggaaat   480
          Y  H  Q  P  G  A  Q  V  L  A  W  Y  M  S  I  A  S  F  G  N
gaggtatgttagtatgcatgtcaccatgcgaatcagcactaatacagtgtagccagtgcc   540
          E  V  C  -  Y  A  C  H  H  A  N  Q  H  -  Y  S  V  A  S  A
tgactcgctgctaatcaacggggcaggaagcttcgactgctctatggccgtgcctgctag   600
          W  L  A  A  N  Q  R  G  R  K  L  R  L  L  Y  G  R  A  C  -
acccgtggattgcattgaacaacaggccaacctctcgtacctatcagacatagacacatc   660
          T  R  G  L  H  W  T  T  G  Q  P  L  V  P  I  R  H  R  H  I
ctttaggttgcgcgtggtcaacaccgggtactacctccatcacacttcaaagtaaccaaa   720
          L  -  V  A  R  G  Q  H  R  V  L  P  P  S  H  F  K  V  T  K
agctaacacccccacagttcccttgcaggattcaccctatcattcgaaaacaaacccccta   780
          S  -  H  P  H  S  S  L  A  G  F  T  L  S  F  E  N  K  P  L
accctaatccaagtagacagcaccgaa                                     808
                T  L  I  Q  V  D  S  T  E
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Figure 8*:* Nucleotide and deduced amino acid sequences of *Aspergillus flavus* NG85 laccase

CONCLUSIONS

Laccase produced by *Aspergillus flavus* NG85 isolated from Saint Catherine protectorate, was purified to homogeneity. The enzyme was biochemically characterized. It showed thermal as well as pH stabilities. The decolorization efficiency against synthetic dyes was remarkable especially in presence of copper ions. Besides degrading a variety of dyestuffs, purified laccase also decolorized a real textile effluent. Hence, laccase holds great promise for applications in biodegradation and bioremediation, especially treatment of dye effluents.

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