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BIODEGRADATION OF SYNTHETIC TEXTILE DYES BY CHITOSAN BEADS CROSS-LINKED LACCASE FROM *Pleurotus ostreatus* IBL-02

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ABSTRACT

Laccase (EC.1.10.3.2) a blue copper protein is remarkable, ecologically well disposed and productive biocatalyst that can degrade the toxic contaminants. The utilization of laccase for the detoxification of toxic pollutants is extensively restricted by its vulnerability to changes in operational conditions and its poor reusability. To overcome this problem, indigenously produced laccase from *Pleurotus ostreatus* IBL-02 was immobilized using glutaraldehyde as cross-linker. Maximum immobilization efficiency (76.2%) was noticed with 3% Chitosan, cross-linked by 3% solution of glutaraldehyde. Cross-linking significantly altered the kinetic parameters K_m and V_{max} of laccase. The affinity of substrate towards biocatalyst improved (K_m decreased) consequently upgraded catalytic activity (increased V_{max}). Immobilization significantly raised the temperature optima while shifted the optimum pH towards slightly neutral from acidic. The presence of laccase in immobilized beads was also confirmed by Energy Dispersive X-ray (EDX) analysis. Immobilized laccase was used to decolorize five different synthetic textile dyes. Chitosan cross-linked laccase decolorized all the dyes more efficiently as compared to control.

Keywords: Laccase, Chittosan, Cross-linking, Immobilization, Biodegradation.

INTRODUCTION

Synthetic dyes are broadly utilized in industries for example, textile, leather, beauty care products, food and paper printing. These colored compounds are known as a standout amongst the major essential groups of pollutants, as they take part in the formation ofhighlycolored wastewater (Mittal *et al*., 2010). Typically, from 10-50% of the underlying dye load is existing in the dye bath effluent, offering ascend to an exceedingly colored effluent that can pollute and harm the aquatic environment (Moilanen*et al.,* 2010). Consequently, industrial effluents having toxic compounds must be treated before their release to the surroundings (Khlifia*et al.,* 2010). Removal of hazardous dyes from water bodies has been generally examined.Among different techniques such as chemical and photochemical strategies, and so on, biological treatments are known as the regular and across the board methods for dyes eradication (Gupta and Suhas, 2009). Decolorization of synthetic dyes by utilizing unique biocatalysts or enzymes has gotten extraordinary consideration in light of effective decolorization and co-production of nontoxic end products (Parshetti*et al*., 2010).

Laccases belong to the class oxidase having multiple copper atoms in their core hence called multi-copper oxidases (MCOs). MCOs constitute a large group of enzyme that has the ability to oxidize various kinds of phenolic and non-phenolic substrates with the expense of molecular oxygen (Brijwani*et al*., 2010; Canas and Camarero, 2010; Dwivedi*et al*., 2011). As they are nonspecific in nature so they can catalyzed various type of reactions ranging from degradation of polymeric compounds such as lignin to ring cleavage of xenobiotics like dyes and from cross-linking of phenolic compounds to herbicide removal (Coelho *et al*., 2010; Ke*et al*., 2011).

Enzymes are exceptionally particular biocatalysts, which may have their utilization boosted and enhanced by immobilization. The idea behind "enzyme immobilization" is basically to retain a fully active enzyme on such a support that cannot be solubilized and sustain even in extreme conditions (Minteer, 2011; Sassolas*et al*., 2012; Magner, 2013). Immobilization leads to a competent resurgence of the biocatalyst after enzymatic reaction, reusability for manifold catalytic cycles and minimized infectivity of reaction products by enzyme itself, crucial in pharmaceutical and food industries (Hanefeld*et al*., 2013). Furthermore, it lessens cost-effective use in uninterrupted fixed-bed operation, for instance (Sheldon and Pelt, 2013). Enhanced specificity, selectivity (Rodrigues *et al.,* 2013), storage and operational stability (Tran and Balkus, 2011) towards harsh environmental conditions like extreme pH, extremely high temperature and organic solvents are the salient features of an immobilized enzyme.

The most fascinating technique for laccase immobilization for industrial applications is covalent

binding. In this way, covalent binding has been the most generally utilized technique for laccase immobilization amid the most recent decade. During covalent binding, special groups present on the surface of immobilization support first activated by some cross-linking reagents then react with nucleophilic groups present on enzyme. Covalent interaction between enzymes and immobilization supports takes place due to the presence of side chain amino acids like arginine, aspartic acid, histidine of enzymes and level of reactivity in view of functional groups like imidazole, indolyl, phenolic hydroxyl, etc. (Singh, 2009).

The sustainable biopolymer, chitosan is considered as a perfect carrier to immobilize a protein. Chitosan is preferably used due to the presence of high amount of amino group that not only improve its linkage with enzyme, its solubility in slightly acidic solutions but also its insolubility more or less of its pKa value, mechanical strength and imperviousness to substance corruption (Cabana *et al*., 2011). Moreover, its generation is of minimal effort and environmentally fascinating (Delanoy*et al*., 2005). Furthermore, utilizing chitosan as immobilized support can diminish the defilement of debasement and improve decontamination prepare (Orrego*et al*., 2010). Utilizing glutaraldehyde as cross-linking reagent, the binding of biocatalyst on chitosan beads stays away from an immediate contact of the enzymes with the encompassing medium, it likewise make the reagents to reach the catalytically active site of enzyme (Valentina *et al*., 2011).

The study was conducted to improve the catalytic efficiency, thermostability and reusability of laccase by its immobilization on chitosan beads. Furthermore, dye decolorizing efficiency of immobilized laccase was investigated against different types of synthetic dyes.

MATERIALS AND METHODS

Production and Purification of Laccase: Pure culture of *Pleurotusostreatus*IBL-02 was obtained from Industrial Biotechnology Laboratory, Department of Biochemistry, University of Agriculture Faisalabad, Pakistan. Pure culture was maintained on Potato dextrose agar (PDA) slants at pH 4.5 and 28°C temperature. Extracellular laccase was produce from *P. ostreatus*IBL-02 using wheat straw as a growth substrate under pre-optimized solid state fermentation conditions. The laccase was isolated by filtration and centrifugation at 3,000xg. The enzyme was purified by a multiple steps i.e. ammonium sulfate, dialysis, ion-exchange chromatography and gel filtration.

Laccase enzyme assay: Extracellular laccase was assayed spectrophotometerically by monitoring 2, 2-azino-bis 3-ethylbenzthiazoline-6-sulphonate (ABTS) oxidation in sodium malonate buffer at 420 nm (Shin and Lee 2000). The specific activity of the enzyme was determined as described by Asgher*et al*. (2012).

Protein Estimation: Bradford micro assay (Bradford 1976) was followed for the determination of total protein contents in the enzyme extract before and after every purification step.

Immobilization of Laccase on chitosan beads:

Preparation of chitosan beads: It was carried out by the previously described method of Srivastava and Anand, (2014) with slight modifications. Chitosan solutions of different concentrations (2-4%) were prepared by dissolving chitosan powder in 1.5% acetic acid solution by mild heating with continuous stirring. The chitosan solution was filled in a syringe and allowed to fall drop wise into 1M of KOH solution kept for continuous stirring in order to get spherical size of beads. This solution was kept for stirring at a slow rate for 2 hrs at 30ºC to get ripened beads.

Activation of chitosan beads: The prepared beads were incubated with various concentrations of glutaraldehyde solution (1–3%, v/v) at 30ºC for 3 and 6 h. The activated beads were separated from the solution and thoroughly washed with Na acetate buffer (20 mM, pH 5.0) to remove unbound glutaraldehyde from the bead surface.

Immobilization of laccase onto activated chitosan beads: The activated chitosan beads were incubated with purified laccase (0.2–0.6 mg/ml) for 24 h at 4ºC for immobilization. Then, beads were washed gently for 2–3 times with Na acetate buffer (pH 5.0) in order to remove unbound enzyme molecules from the bead surface and directly used for measurement of laccase activity at 420 nm. The beads containing laccase were dried and stored at 4ºC for further studies. Immobilization efficiency (%) was evaluated for chitosan entrapped enzyme as follows:

$$\% \text{ Immobilization Efficiency} = \frac{\text{Total activity of immobilized enzyme}}{\text{Total activity of free enyme}} \times 100$$

Analysis of immobilized supports via Energy Dispersion X-ray (EDX): The control and immobilized laccase enzyme beads were subjected to Energy Dispersion X-ray (EDX) analysis for the confirmation of enzyme specific elements presence in beads.

Characterization of Free and Immobilized laccase: Effects of various kinetic parameters like temperature, pH and substrate concentration were studied on free andimmobilized laccase.

Effect of pH: In order to study the effect of pH optima on free and immobilized laccase, the reaction mixtures were incubated for 15min in buffers of varying pH i.e. 3-10. After incubation, the enzyme assay was performed

using standard assay protocol. The buffers used in this study were tartarate buffer, pH 3.0; sodium malonate buffer, pH 4.0; citrate phosphate, pH 5.0, pH 6.0; sodium phosphate, pH 7.0, pH 8.0, carbonate buffer, pH 9.0 and pH 10.0.

Effect of Temperature: To determine the optimum temperature for free and chitosan cross-linked laccase activities, the enzyme was incubated at different temperatures i.e. 20, 30, 40, 50, 60, 70, 80 and 90°C for 15min prior to carrying out standard enzyme assay.

Laccase thermal stability: Thermostability of laccase was assayed by incubating the free and immobilized laccases at optimum temperature for several times. Laccase efficiency was determined after every one hour following the respective protocol of enzyme assay.

Effect of Substrate Concentration: Determination of kinetic parameters: The effect of substrate concentration on free and immobilized laccase activity was examined by carrying out enzyme assay using varying concentrations of ABTS(0.1-1.0 mM) as substrate in 50mM sodium malonate buffer (pH 4.5).Lineweaver-Burk's reciprocal plots were constructed between 1/[S] and 1/[V_0] and kinetic parameters of Michaelis–Menten (K_m and V_{max}) were determined. Free and immobilized laccase were assayed at optimum pH and temperature.

Dye decolorization studies by free and immobilized laccase: Five different industrial (synthetic) dyes including Sandal-fix Turquoise blue GWWF (λmax: 664 nm), Sandal-fix Red C_4BLN (λmax: 540 nm), Sandal-fix Black CKF (λmax: 598 nm), Sandal-fix Golden yellow CRL (λmax: 414 nm) and Reactive T blue GWF (λmax: 660 nm) were used to investigate the decolorization capability of the free and immobilized laccase. The free and immobilized laccase were transferred to 250 mL Erlenmeyer flasks having 100 ml of individual dye solution (0.01g/100 mL) prepared in 50mM sodium malonate buffer of pH 4.5. The decolorization flasks were incubated at 30°C temperature on rotary shaker (120 rpm) for 3 days. Samples were taken after every 24h and centrifuged (8,000×g, 10 min) to eliminate the suspended particles. Residual dye concentrations were determined by measuring absorbence at corresponding wavelengths (λmax of original dye solution in 50mM sodium malonate buffer of pH 4.5). The following equation was used to estimate the percent dye decolorization.

$$D \quad (\%) = \frac{(A) - (A)}{(A)} \text{X } 100$$

Where *Ai* is the absorbance of untreated dye and *At*is the absorbance after treatment with free or immobilized laccase at incubation time t.

RESULTS AND DISCUSSION

Purification of Laccase: Locally isolated *P. ostreatus* IBL-02 was used to produce laccase under pre-optimized cultural conditions. The maximum production of laccase, (519.44U/mL) was observed in SSF medium under pre-optimized conditions. Laccase got fully saturated at 80% of ammonium sulfate to 1.26 fold purification with enzyme activity of 415 U/ml and specific activity of 253 U/mg. At the end of all the purification processes, enzyme activity and specific activity of laccase were observed as 268.05 U/mL and 687.31 U/mg, respectively with 3.4 purification fold (Table 1).

Asgher *et al*. (2012) reported that laccase produced by *Pleurotus ostreatus* was saturated by 80% of ammonium sulfate with 11% yield and 4.2-folds purification. Itoh *et al*. (2016) also observed that 80% saturation point was feasible for the purification of laccase produced from *Hericium coralloides* NBRC 7716. Conversely, Irshad *et al*. (2011) produced laccase from *Schyzophylum commune* IBL-06 and salted it out with 50% of ammonium sulfate saturation and observed the purity of laccase up to 3.95-fold at the end of purification procedure.

Table 1. Purification summary of laccase produced by *P. ostreatus* IBL-02 in solid state fermentationof wheat straw.

Purification steps	Total volume (mL)	Enzyme Activity (U/mL)	Protein contents (mg/mL)	Specific activity (U/mg)	% yield	Purification fold
Crude Enzyme	500	519.44	2.58	201.33	100	1.00
Precipitation	32	415	1.64	253	88	1.26
Dialysis	30	400.83	1.02	392.97	77	1.95
DEAE-Cellulose	13	273.33	0.59	463.27	53	2.30
Sephadex G-100	08	268.05	0.39	687.31	51	3.40

Laccase immobilization on Chitosan beads: Maximum immobilization (72%) of laccase was observed at 3% chitosan concentration (Fig. 1). 1 and 1.5%, solutions of Chitosan were dissipated in KOH solution with no bead formation, while beads formed by 2 and 2.5% of chitosan solution showed 38 and 53% immobilization efficiencies respectively. However the beads were fragile, discoid and vulnerable to damage while handling. On the other hand, at higher chitosan concentrations (above 3%) due to very viscous and thick solution that was difficult to cast. At

chitosan concentration of 3%, resulted beads were stable and uniform. Further increase chitosan concentration to 4%, beads formation was good but immobilization efficiency decreased 42%.

Maximum immobilization efficiency was observed after 3h of incubation in 1.5% glutaraldehyde solution (Fig. 2). At higher glutaraldehyde concentrations, the immobilization was decreased probably due to the binding of active aldehyde groups to chitosan, leading to multipoint binding of enzyme to the carrier that caused a spatial hindrance to inactivate the enzyme. As a matter of fact, the presence of NH_2 groups on the chitosan backbone greatly improves its potential during the cross-linking process. With lower concentrations of glutaraldehyde, lesser attachment points i.e., aldehyde groups, were generated which caused lower immobilization efficiency (Srivastava and Anand, 2014). In comparison to our findings, Jaiswal *et al*. (2016) got maximum immobilization yield (98%) of papaya laccase on chitosan beads of 2.5% concentration.

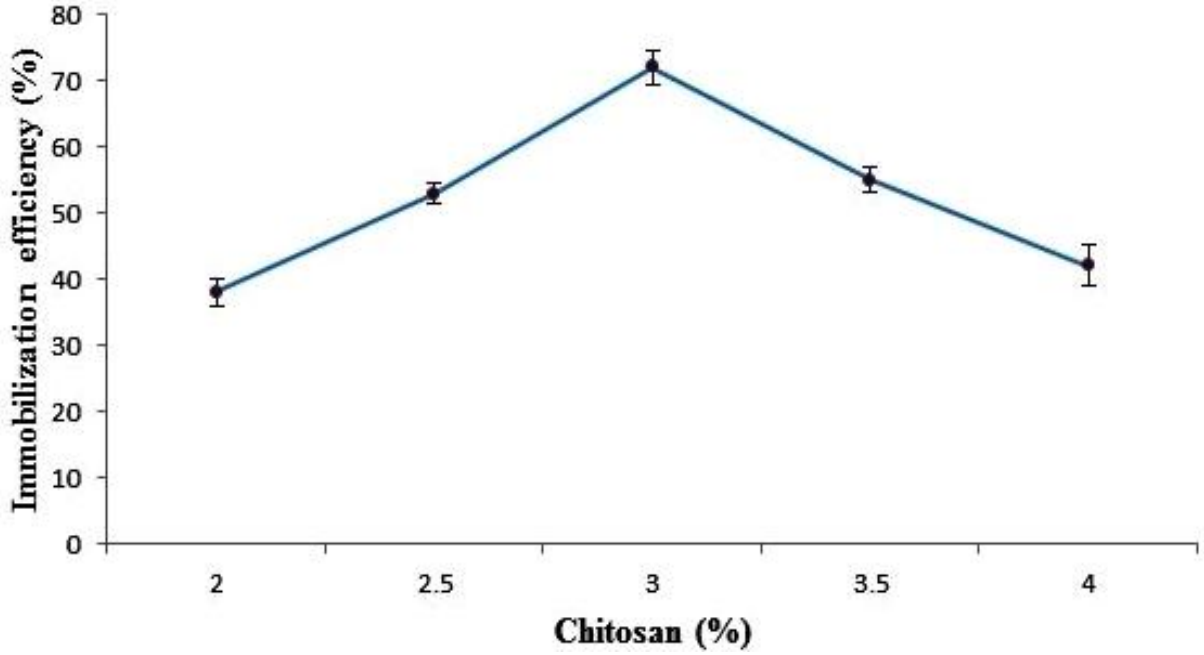

Fig. 1. Effect of different concentrations of Chitosan on laccase immobilization efficiency.

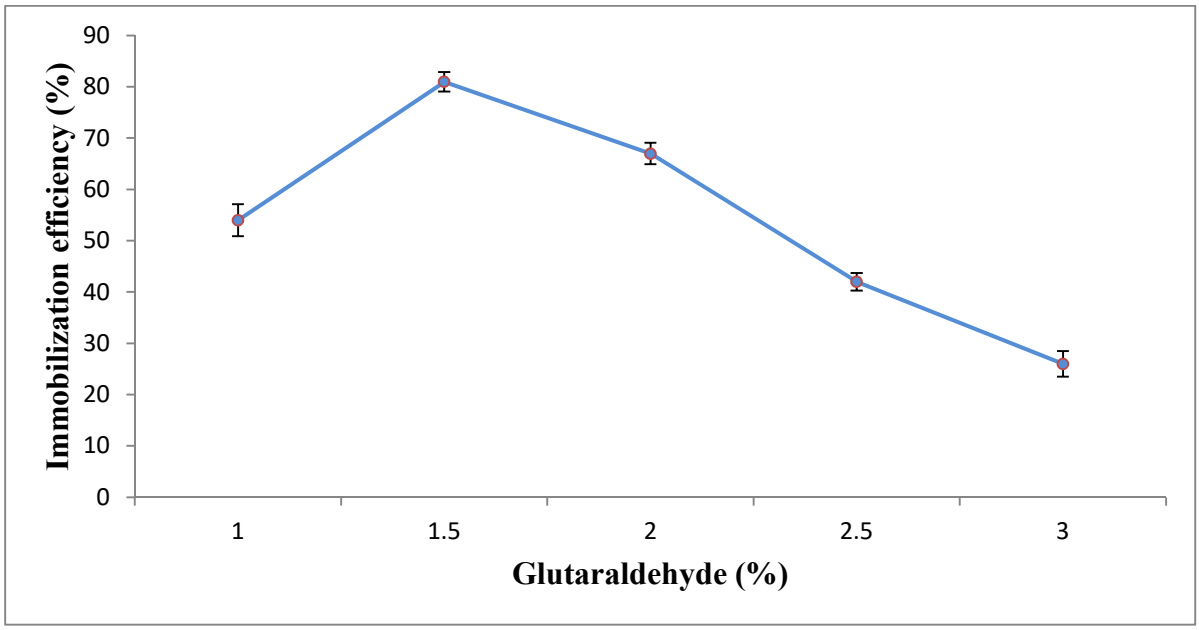

Fig. 2. Effect of different concentrationsof Glutaraldehyde on laccase immobilization efficiency.

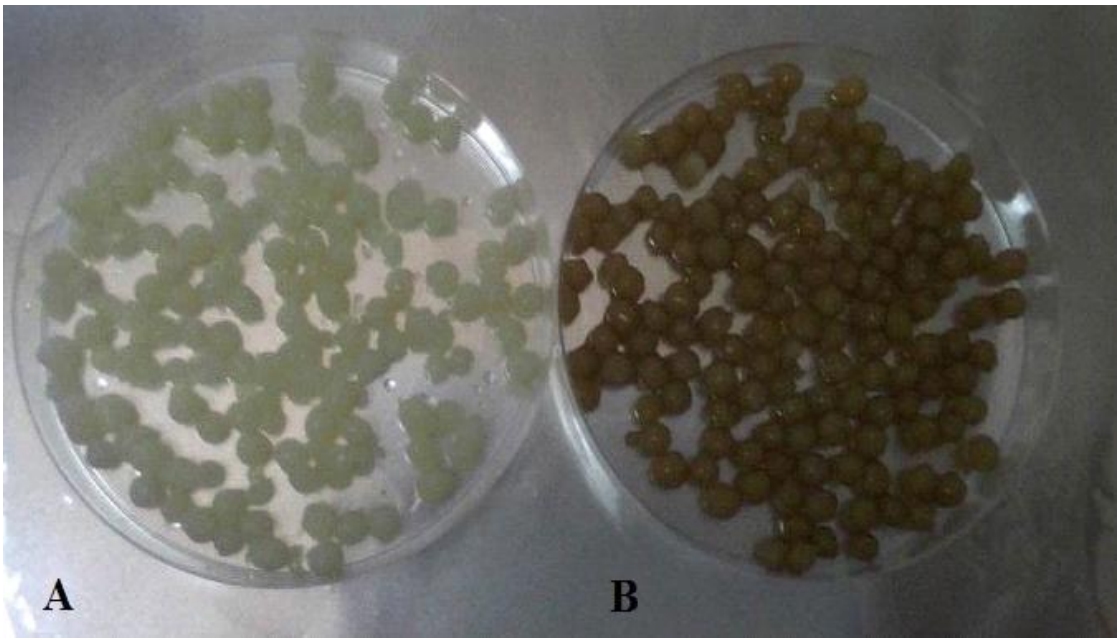

Fig. 3. (A) Chitosan beads without enzyme (B) Chitosanbeads with immobilized Laccase

Energy Dispersive X-ray (EDX): Energy dispersive X-ray (EDX) was used for the confirmation of presence of copper atoms in laccase immobilized beads. As copper is the characteristic of laccase, a blue copper oxidase. EDX of laccase immobilized beads revealed that copper was present in the beads hence confirming the immobilization of laccase, while copper was absent in beads without enzymes (Table 2 & Fig. 4).

Table 2. Elements detection by EDX for confirmation of Copper containing laccase enzyme presence in immobilization support.

Beads		**Elements detected by EDX**						
Chitosan Beads	**Standard**	C K	O K	Na K	__	__	__	KK
	Laccase	C K	O K	Na K	Cl K	Ca K	Cu K	KK

*C, carbon; K, potasium; O, oxygen; Na, sodium; P, phosphorus; Cl. chloride; Ca, calcium; Cu, copper

Effect of pH on activities of free and immobilized Laccase: The immobilized laccase was found to be catalytically active over an extensive variety of pH when contrasted native enzyme. Results demonstrated that optimum pH of the enzyme shifted from 5.0 to 6.0 (Fig. 5). Bozic *et al*. (2012) reported that Laccase exhibited high catalytic activity over a broad range of pH and temperature showing highest activity at pH 4.5 and the activity decreased with increasing pH. Contrary to our results, Asgher *et al*. (2012) and Noreen *et al*. (2016) reported the shift of optimum pH of laccase towards acidic range when immobilized on Ca-alginate beads and in sol gels respectively. These shifts in pH optima are might be due to the proton absorption capability of carriers.

Effect of temperature on activities of free and immobilized Laccases: The temperature-activity description of free laccase showed maximum activity at 35°C whereas chitosan bound Laccase showed optimum activitiy at 60°C (Fig. 6). Immobilization brought an enhancement in thermal behavior of the enzymes due to stabilization of their three dimensional structures. Chitosan beads immobilized laccase showed a slow decline in its activity above 60°C. The arrangement of covalent bonds amongst laccase and chitosan significantly enhanced thermo-stability of the enzymes contrasted with the adsorption of this protein on chitosan (Cabana *et al*., 2011).

Similar to our results, Lin *et al*. (2015) observed maximal activity at 35°C which decreased gradually as the temperature increased and was almost completely lost at 65°C. On the other hand, in contrast with free laccase, modified laccase apprehended a higher activity at a high temperature (65°C) and more or less 30.8% of its novel activity was retained. In contrast to our results, Jaiswal *et al*. (2014) found an increase in optimum temperature of papaya lacasse after immobilization on chitosan beads from 70°C to 80°C. Such shift in optimum temperature of the immobilized laccase exhibited that the chitosan lattice was compelling in ensuring the laccase activity in harsh

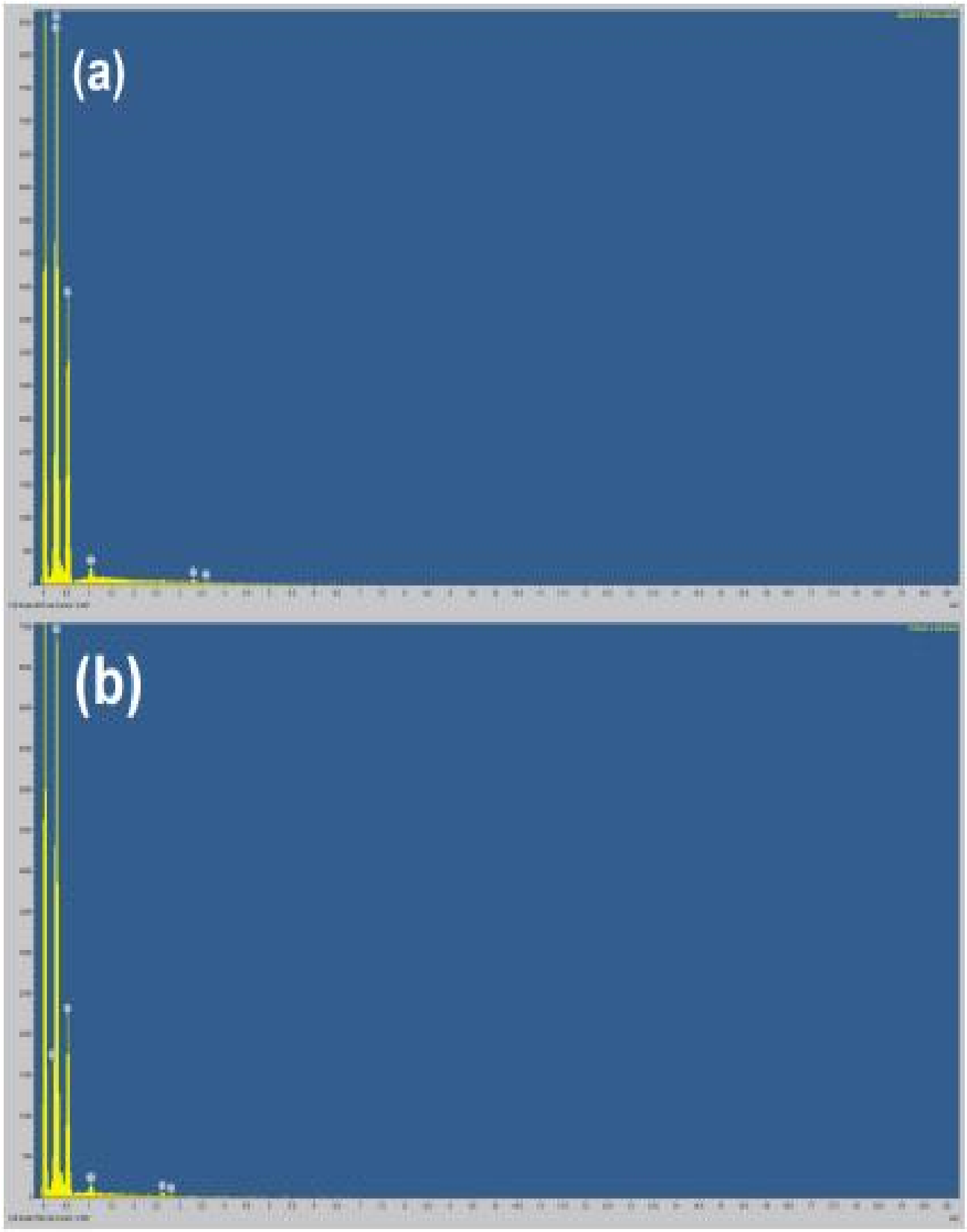

Fig. 4. EDX image of (A) standard chitosan beads (B) chitosan beads immobilized with laccase enzyme from *P. ostreatus* IBL-02.

environment of pH and temperature which might be because of upgrade of the conformational soundness of the local frame.

Thermal Stability: Free and immobilized laccase showed different results when incubated at 60 ºC for several hours (fig. 7). After 5 h of incubation immobilized laccase retained its 37.8% of its activity, while free laccase only retained 6.6% of its activity at similar conditions. It indicates that immobilization of laccase on chitosan beads via cross-linking not only enhanced its catalytic activity but makes it thermostable against high temperature. Due to immobilization, increased thermo-stability of laccase emerges from covalent limits connected with the enzyme interacted with activated chitosan beads, which improves enzyme conformational firmness and hence enhanced its thermal stability contrasted with native enzyme.

The long-term operating stability of the immobilized enzyme plays a key role in its important industrial applications for reducing processing costs (Liu *et al*., 2012). Correspondingly, high thermal stability reported by Mogharabi *et al*. (2012) on account of crystal violet decolorization by immobilized laccase in alginate–gelatin mixed gel, which ascribed to the defensive part of the readied gel for keeping up catalyst action.

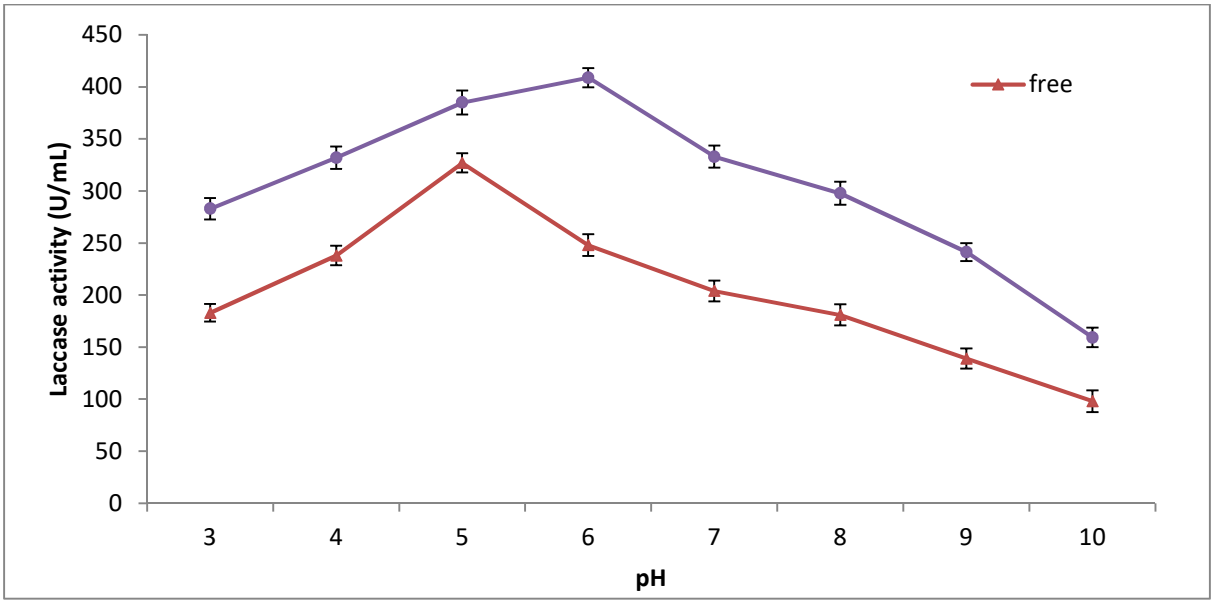

Fig. 5. Effect of varying pH on the activities of free and immobilized Laccase

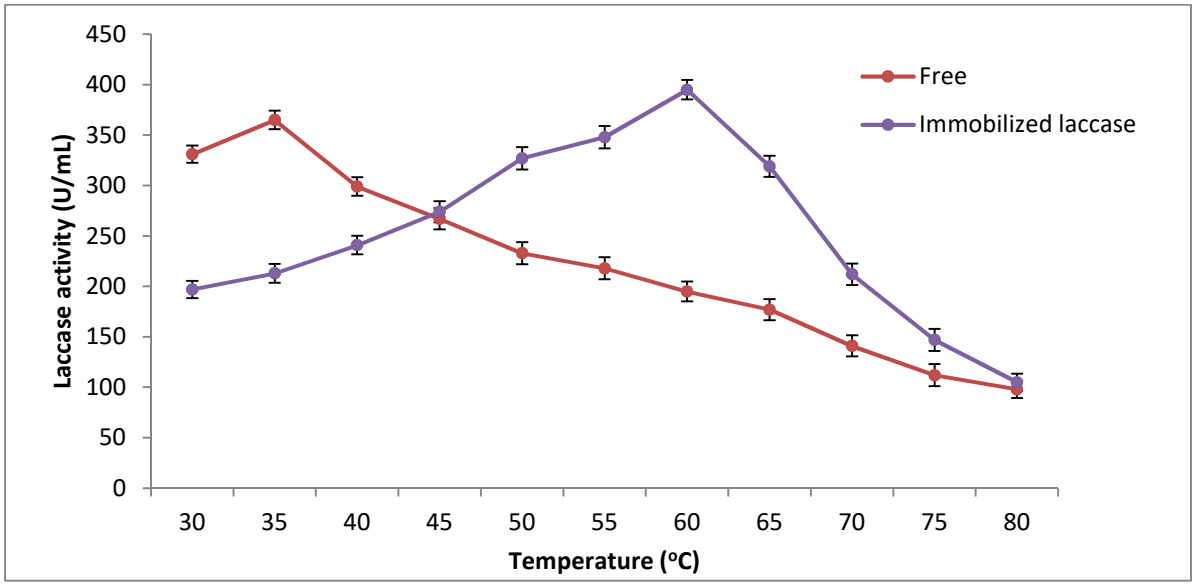

Fig. 6. Effect of varying temperature on the activities of free and immobilized Laccase

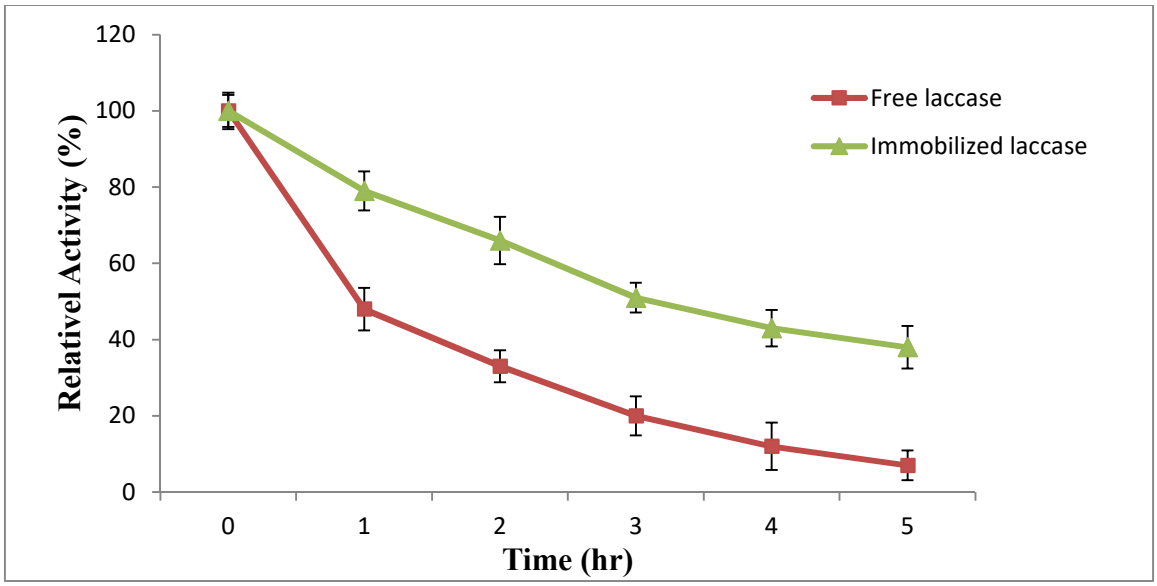

Fig. 7. Thermal stability of free and chitosan linked laccase at 60 ºC.

Effect of substrate concentration: Determination of K_M and V_{max}: The values of K_M and V_{max} were estimated by intercepting the line on the X-axis and Y-axis of the reciprocal plot, respectively utilizing ABTS as substrate (Fig. 8). The maximum catalytical activity (V_{max}, 130µM/min) was observed at 1.0 mM of ABTS in case of free enzyme with K_M of 21.01μM was obtained. Chitosan attached laccase had slightly lower K_M values (16.71μM) but their V_{max} (166.7 µM/min) were significantly improved.

Immobilization process brings about alteration of a portion of the functional groups exhibit on catalyticaaly active site and in addition on the surface of ligninolytic enzymes bringing about a lower K_M, better catalytic competence and amplified thermo-stability. In previous studies, a number of scientists have reported that immobilized laccase had bring down K_M and higher V_{max} than the free counterparts, and immobilized laccase demonstrated higher catalytic efficiency, reusability and operational stability due to high V_{max} (Makas *et al*., 2010; Jaiswal *et al*., 2014). In contrast, Jaiswal *et al*. (2016) observed an increase in K_M value as compared to free laccase, when immobilized in chitosan in case of ABTS as substrate, whereas reduce in case of hydroquinone. It was also observed that the V_{max} decline after immobilization by both the substrates.

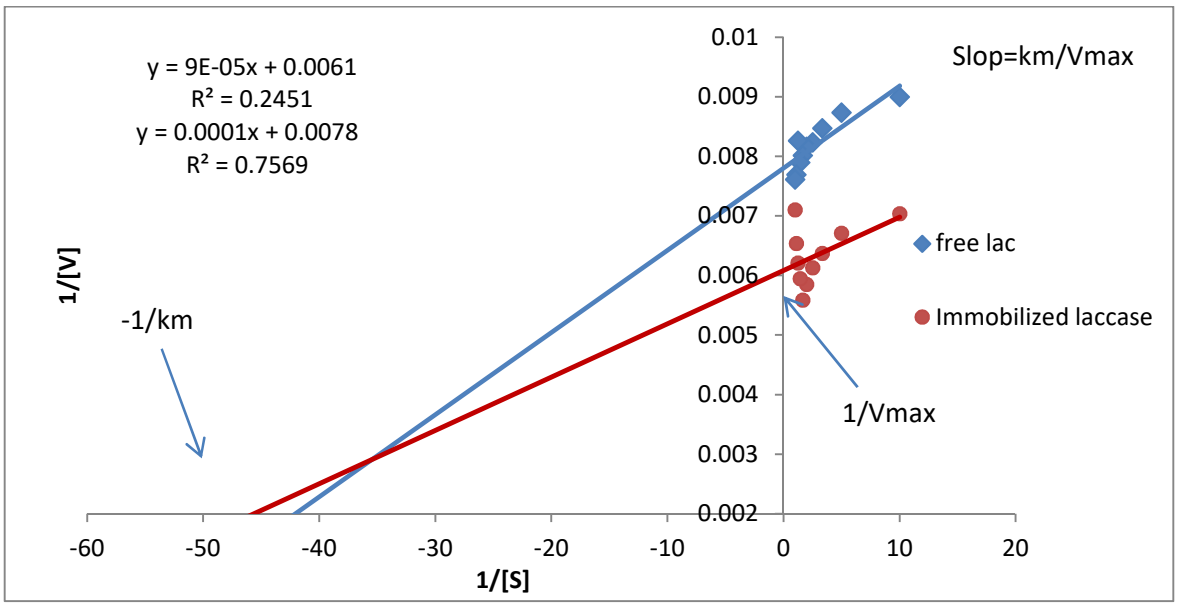

Fig. 8. Determination of K_M and V_{max} by Lineweaverburk plot for free and immobilized laccase with ABTS as substrate

Decolorization of Synthetic Textile dyes by Immobilized Laccases: Due to exclusive characteristics of immobilized laccases, like high stability, non-specificity for substrate and reusibility make them attractive for use in bioremediation of dyes. Dyes are extensively utilized in textile, paper, cosmetic and pharmaceutical. Laccase can decolorize various dyes like azo, anthraquinone and triphenylmethane, through free radical mechanism by creating phenolic compounds.

Free and immobilized Laccases were utilized for the decolorization of five textile dyes (S.F. T.Blue FBN, S.F. Red C4BLN, S.F. Turq Blue GWWF, S.F. Golden Yellow CRL and S.F. Black CKF). Immobilized laccase from *P. ostreatus* IBL-02 demonstrated diverse decolorization profiles for various dye-stuffs (Table 3). Immobilized laccase from *P. ostreatus* IBL-02 color stuffs Significant dye decolorization was shown by the immobilized enzyme. But the best decolorization result (90±4.9%) was observed for S.F. Golden Yellow CRL. This is mainly due to the more available catalytic sites of the enzymes on higher surface area available on the immobilization supports (Ramírez-Montoya *et al*., 2015; Maneerung *et al.,* 2016).

In literature, many reports are available on dye decolorization utilizing laccase immobilized on various matrices. Lu *et al*. (2007) observed almost 66% degradation of Alizarin Red dye in the presence of ABTS as mediator after one hr. Alginate-gelatin gel was prepared to immobilize laccase and used for decocorization (87%) of amido black (Mogharabi*et al*., 2012).Asgher*et al*. (2012) reported 100% decolorization of a textile dye, Drimarin Blue after 5h of treatment with *Pleurotusostreatus*laccase immobilized by hydrophobic sol-gel entrapment. In the same way, decolorization of 76% and 64% for Acid blue and Acid orange, respectively, was found after 65 min incubation using the laccase of *Paracniothyriumvariabile*immobilized on porous silica beads (Mirzadeh*et al*., 2014).Previously, Lin *et al*. (2015) also reported 49% and 83.3% decolorization of methyl red by free and immobilized laccase, respectively. While higher decolorization was achieved in case of Orange II dye using immobilized laccase as compared to free laccase. It is important to note that free enzymes are difficult to be reutilized in repeated industrial batch operations where, as the immobilized enzymes can be recycled without significantactivity loss (Zheng *et al*., 2012).

Table 3.Maximum absorbance and synthetic dyes removal by laccase immobilized on chitosan beads.

Dyes	Classification	λmax	Dye removal by immobilized laccase (%)	
			Free laccase	Chitosan beads
S.F. T. Blue FBN	Reactive	605	46±3.3	74±5.2
S.F. Red C4BLN	Reactive	540	39±2.3	89±4.1
S.F. T. Blue GWWF	Reactive	662	67±5.3	88±4.2
S.F. Yellow CRL	Reactive	439	27±4.3	90±4.9
S.F. Black CKF	Reactive	584	36±1.8	86±3.2

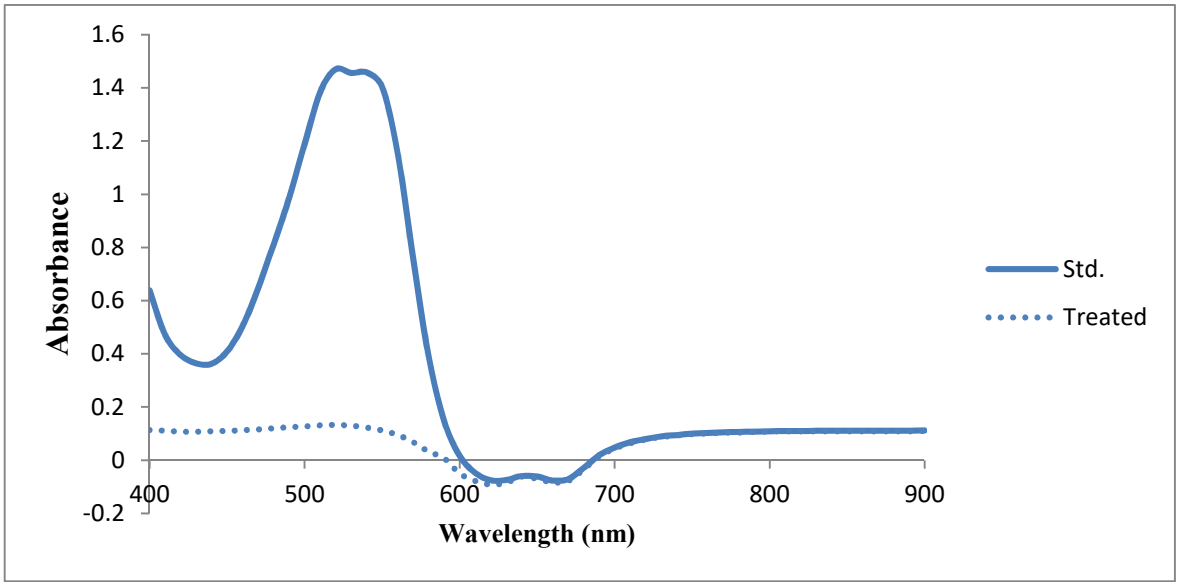

Fig. 9.UV-vis absorption spectra of S.F. Red C4BLNdye after treatment with chitosan linked laccasefrom*P. ostreatus* IBL-02

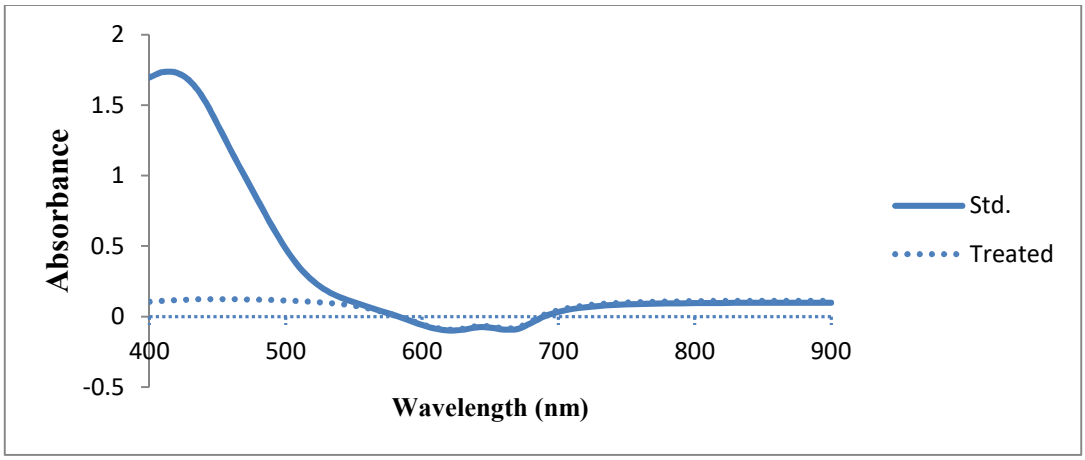

Fig. 10.UV-vis absorption spectra of S.F GoldenYellow CRL dye after treatment with chitosan linked laccase from*P. ostreatus* IBL-02

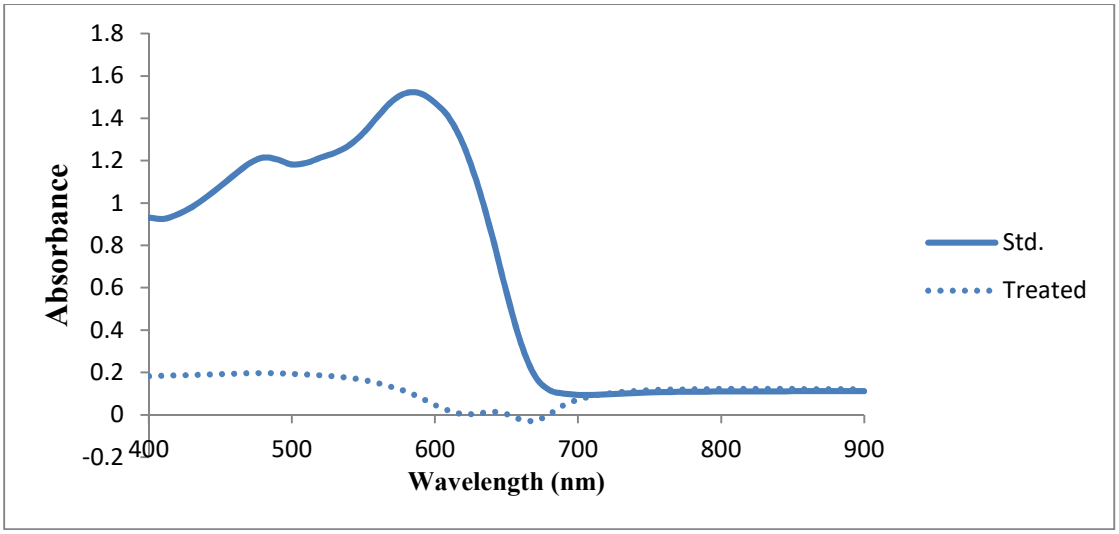

Fig. 11.UV-vis absorption spectra of S.F. Black CKF dye after treatment with chitosan linked laccase from*P. ostreatus* IBL-02

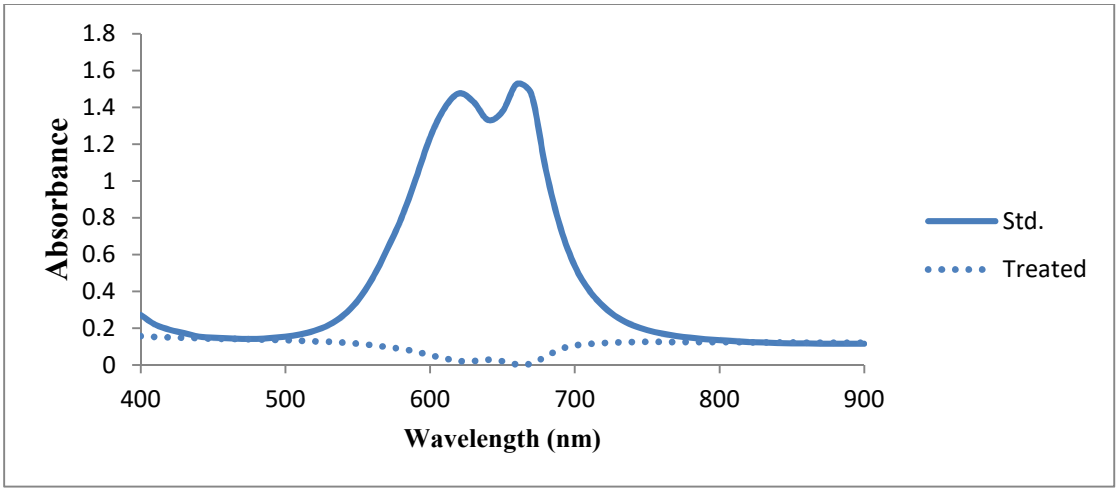

Fig. 12.UV-vis absorption spectra of S.F. Turq Blue GWWF dye after treatment with chitosan linked laccase from*P. ostreatus* IBL-02

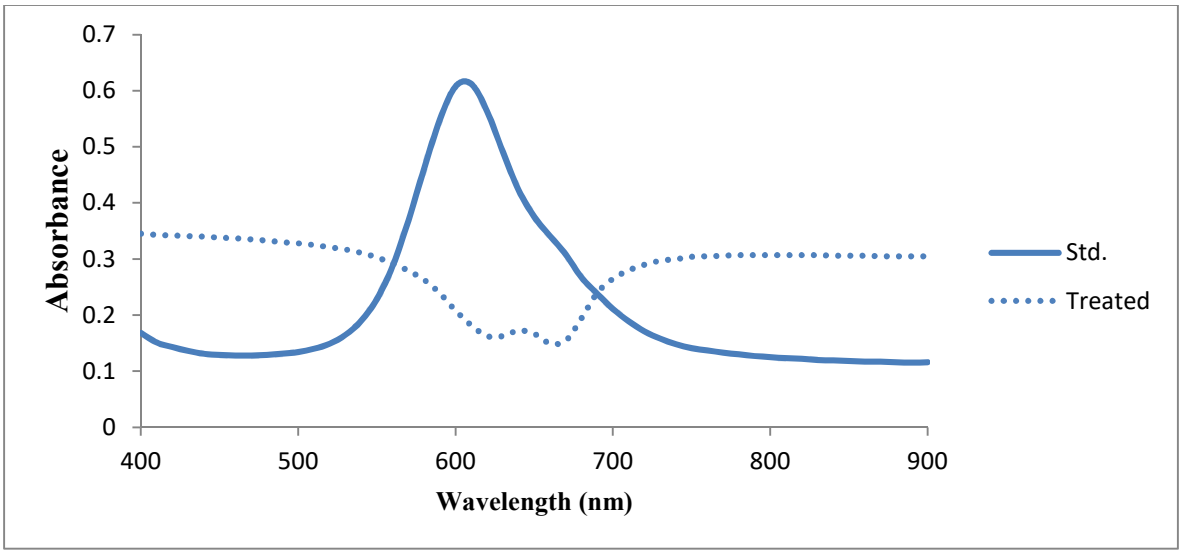

Fig. 13. UV-vis absorption spectra of S.F. T. Blue FBNdye after treatment with chitosan linked laccases from *P. ostreatus* IBL-02.

Conclusion: Laccase produced from *P. ostreatus* IBL-02 after cross-linking with chitosan showed better temperature and pH optima and kinetic properties as well. It means laccase cross-linking on activated chitosan beads not only enhanced its catalytic activity but stability in harsh conditions too. It was also noticed that the immobilized laccase has a great potential to decolorize dyes as compared to free enzyme. So it is suggested that this immobilized enzyme can be used to degrade dye based textile effluent as it proved to be more effective, robust and stable dye decolorizer.

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