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CHITOSAN BEADS IMMOBILIZED *SCHIZOPHYLLUM COMMUNE* IBL-06 LIGNIN PEROXIDASE WITH NOVEL THERMO STABILITY, CATALYTIC AND DYE REMOVAL PROPERTIES

P. Sofia[1*], M. Asgher[1], M. Shahid[1] and M. A. Randhawa[2]

[1]Department of Biochemistry, [2]National Institute of Food Science & Technology, University of Agriculture, Faisalabad
[*]Corresponding Author e-mail: sjaries86@yahoo.com

ABSTRACT

The lignin mineralizing enzymes system of white rot fungi has tremendous catalytic prospect for oxidative bioremediation of a number of toxic pollutants and many other industrial uses. Immobilization enables the reuse of enzymes and making them industrially relevant and economical biocatalysts. *Schizophyllum commune* IBL-06 was cultivated for the secretion of lignin peroxidase (LiP) in pre-optimized solid state fermentation medium of corn stover. A high yield of lignin peroxidase (1347.3 U/mL) was recorded in crude culture supernatant. LiP was purified (5.65 folds) by a pre-standardized four step protocol comprising ammonium sulphate fractionation, dialysis, DEAE cellulose ion exchange and Sephadex G-100 column chromatography. The 38 kDa single polypeptide *S. commune* IBL-06 LiP migrating as a single clear band on both native and SDS-PAGE gels. The purified enzyme was then immobilized on chitosan beads activated with gluteraldehyde (cross-linker).Scanning electron microscopy (SEM) was conducted for the confirmation of LiP attachment on chitosan beads. The highest textile dye decolorization (95.45%) potential was observed with chitosan-immobilized enzyme at 30°C without hemolytic toxicity. The chitosan beads-LiP retained approximately more than 70% activity after three repeated runs that gradually decreased to 35 % after 7th cycle of reusability. The immobilized LiP was found to show superior dye removal properties as compared to free LiP. Higher thermo stability, lower K*m*and high V_{max} features of chitosan beads immobilizedLiP suggested its suitibility for variousbiotechnological and industrial applictaions.

Keywords: *Schizophyllum commune* IBL-06, lignin peroxidase, chitosan beads immobilization, characterization, dye decolorization.

INTRODUCTION

The enzymes are nitrogenous complex protein molecules with specific catalytic functions which are produced by living cells to catalyze biochemical reactions required for life (Alam *et al*., 2009). Enzymes have excellent properties, like catalytic activity, high selectivity and specificity. Despite all these characteristics, they also have some disadvantages. As solubility in reaction media, instability, thermo stability, so that it is difficult to retrieve enzymes from the reaction mixture (Chen *et al*., 2012). Because of this problem, some properties of the enzymes should be enhanced before their implementation at industrial scale in order to reduce the cost of a chemical process. The operational stability, reusability and recovery of enzymes applied in the industrial process can be improved by the use of mutagenic effects, genetic engineering, and the immobilization of enzymes or process alterations (Asgher *et al*., 2013a).

Enzyme characteristics such as catalytic efficiency, specificity and multiple uses in industrial processes are increasingly replacing the conventional chemical methods in laboratories as well as in industries. However, the marketing of enzymes is at a slower pace due to their high cost and problems of storage (Asgher *et al*., 2013a; Shi *et al*., 2003). Immobilization strategies help to develop stable, recoverable and reusable enzymes for multi-purpose industrial and environmental applications resulting in significant financial benefits (Asgher *et al*., 2008b). Immobilized enzyme formulations tend to be influenced by the structural properties of enzyme as well as the support matrix (Wang *et al*., 2012). The specific interactions between carrier material and the enzyme provide modified enzymes with different biochemical, kinetic, mechanical and chemical properties. Most frequently employed protein (enzyme) immobilization methods are surface binding, adsorption and entrapment (Thakur *et al*., 2015). Whereas, entrapment or encapsulation is selected over surface binding because it is easier, cheaper, and convenient and the structure of enzyme remains protected (Asgher *et al*., 2012c). A number of methods have been reported for the treatment of textile industrial effluents and dye degradation which are not economically and environmentally suitable. These facts certainly require the development of an effective, cost-efficient and environmentally-friendly technology for the detoxification and degradation of industrial effluents containing dyes. Currently the primary methods applied

for the treatment of textile effluents based on physical or chemical procedures, which are incredibly expensive and the accumulation of concentrated sludge results in environmental disasters (Alam *et al*., 2009; Parshetti *et al*., 2012). Conversely, bio-logical processes offer affordable and eco-friendly remediation technologies and they just don't produce big quantities of sludge (Bilal *et al*., 2015).The curiosity about new biocatalyst practice has increased during the last two decades because of the increasing applications of xenobiotics, the degradation of these chemicals are not effective and competent with conventional chemical processes (Missau *et al*., 2014).The basidiomycetes; white rot fungi (WRF) are the micro-organisms which widely studied for industrial dye-degradation. Without any assert to completeness, the ligninolytic enzymes (LiP, MnP, Lac) of WRF involved in the process of dye decolorization (Cheng *et al*., 2007; Maciel *et al*., 2010). LiP has great Redox potential and broad range of applications in various industrial techniques. Purified LiP from *Kocuria rosea* revealed a higher catalytic activity to decolorize reactive dyes through various groups, indicating that it is an extremely versatile peroxidase (Qiu *et al*., 2009). Technically the immobilized enzymes can easily be separated from the reaction mixture and recycled in repeated industrial batch operations as compare to soluble enzyme thus avoiding the laborious steps. Additional benefit is the stabilization against the hardness of reaction conditions which are harmful to the soluble enzyme. On the other hand, the reuse of enzymes provides the benefits of cost which are often an essential pre-condition for the establishment of a continuous batch process at industrial scale (Bhushan *et al*., 2015; Thakur *et al*., 2015).

Chitosan is a naturally occurring polymer having very good biocompatibility, low toxicity, chemically inertness and high hydrophobicity properties that render it suitable for developing high performance biocatalysts. In literature reported that, Chitosan in the form of microspheres or beads have twofold ability to entrap the amount of enzymes (Chang *et al*., 2007; Datta *et al*., 2013). This manuscript reports the immobilization of LiP from *Schizophyllum commune*IBL-06 on chitosan beads using glutaraldehyde as cross-linking reagent to improve its thermo stability and to explore practical capability of immobilized LiP for decolorization of reactive synthetic textile dyes.

MATERIALS AND METHODS

Production of LiP: *Schizophyllum commune* IBL-06 was cultured in SSF medium of corn stover that was pre-dried and ground to powder form (40 mm mesh size particle). Seed culture associated with *Schizophyllum commune* IBL-06 (10^6 spores/mL) obtained from Industrial Biotechnology laboratory, Department of Biochemistry, University of Agriculture, Faisalabad was prepared by rising the pure culture of fungus for 3 days in Kirk's basal growth medium at pH 4.5 and 30ºC temperature. Production flasks (500 mL) contained 5g corn stover moistened with growth (Kirk's) basal salts medium of pH 4.5 and other pre-optimized nutrients (moisture contents, 69%; lignocellulosic material, 5.0g; pH, 4.5; Temperature, 30°C; carbon additive, 1% glucose; nitrogen supplement, 0.2% yeast extract; C: N ratio, 30:1; inoculum, 5 mL and incubation time, eight days). The flasks were sterilized (121ºC) in laboratory scale autoclave (Sanyo MLS-3020U, Japan) for fifteen minutes and inoculated with 5 mL seed culture. The experimental flasks were kept in still culture incubator (Sanyo MIR-254, Japan) at 30 ºC for 8 days. LiP was extracted by adding 100 mL distilled water and shaking in orbital shaker (Gallen kamp, England) for half an hour at 150 rpm. After that the bio mass of the flasks were filtered and filtrate was centrifuged and stored at (4ºC) in refrigerated (Dawlance, signature). The crude supernatant was assayed for LiP.

LiP Purification: Purification of LiP was carried out by ammonium sulfate saturation, DEAE ion exchange chromatography and Sephadex-G100 gel filtration chromatography. Different concentrations of ammonium sulfate were added and after dissolving the samples were kept at 4ºC for 12 hr in refrigerator and then centrifuged at 10,000 rpm for 15 min (Yasmeen *et al*., 2013; Bailey and Robins, 1972). LiP was completely precipitated at 80% saturation. After dialysis, the collected positive fractions were pooled and loaded on diethylaminoethyl cellulose (DEAE-Cellulose) column for enzyme purification on the bases of ionic binding affinity. Sample was poured on the surface of column and the elution rate was adjusted at 0.5 mL/min. fifty positive fractions containing 1 mL of the eluted sample were pooled together. The high peak fractions were pooled and examined for LiP. After resolving through ion exchange column the pooled LiP active fractions were run on Sephadex-G100 (Pharmacia) gel filtration (Batool *et al*., 2013). By applying 1.5 mL of the sample, the eluted fractions were assayed for LiP and protein content.

SDS-PAGE: The molecular mass of Lignin peroxidase was determined by running the purified enzyme on Native and Sodium dodecyl sulphate poly-acrylamide gel electrophoresis. SDS-PAGE was carried out on stacking and resolving gel as described in the method of (Laemmli, 1970) by means of Mini-gel electrophoresis apparatus. The protein bands of sample and standard were stained with Coomassie Brilliant Blue G-250 and molecular mass of LiP was measured in contrast to bands for molecular weight of standard protein markers (Thermo scientific, USA).

Immobilization of Lignin peroxidase using Chitosan beads: LiP was immobilized on chitosan beads using the

method illustrated by (Krajewska, 1990) with minor alterations. Chitosan (1.0-3.5%) was prepared in 1% acetic solution by heating at 45ºC with continuous shaking. The solution was added drop wise to 1M KOH solution to obtain beads of uniform shape and size (diameter 5 mm) and the resulting beads were kept at room temperature for hardening, filtered, washed and stored in 100mM tartarate buffer of pH 3.0 at 4ºC. Chitosan beads were activated by treating with 0.5-2.5% (v/v) glutaraldehyde overnight, at room temperature (25ºC). Activated beads were washed five times with buffer to remove any unattached glutaraldehyde. Varying concentrations of LiP were added to the beads and incubated overnight at 4ºC. Chitosan-LiP beads were stored at 4ºC in 100mM tartarate buffer of pH 3.0 until used. Immobilization efficiency (%) was calculated by applying the following expression:

$$\text{Immobilization efficiency (IE)} = \frac{I}{A-B} \times 100$$

Whereas I = immobilized enzyme (U/g of bead) A = added enzyme (U/g of bead); B = unbound enzyme (U/g of bead)

Scanning Electron Microscopy (SEM): Surface characteristics of chitosan beads with or without LiP were studied with the help of SEM imaging. Chitosan beads were dried on a filter paper and slice with a scalpel to obtain the cross section of beads. After that, the beads were placed on a stand and the cross section photograph was obtained by Scanning Electron Microscopy.

Characterization of Free and Immobilized LiP

Effect of temperature on enzyme activities: The temperature –activity profiles of free and immobilized LiPs were studied by incubating the enzymes at temperatures varying between 25-70ºC for 15min. Enzyme assay was carried for each temperature incubated LiP (Asgher *et al*., 2012d).

Effect of pH on enzyme activities: In order to study the pH optima for soluble and Chitosan beads immobilized LiP (C-LiP), reaction mixture was incubated for 15min in buffers of varying pH such as: potassium chloride buffer, pH 2.0; sodium tartarate buffer, pH 3.0; sodium melonate buffer, pH 4.0; citrate phosphate buffer, pH 5.0; potassium phosphate buffer pH 6.0; sodium phosphate buffer pH 7.0 & pH 8.0; and potassium carbonate buffer, pH 9.0 & pH 10 (Asgher *et al*., 2012e). After incubation, the enzyme assay was performed using standard assay protocol.

Effect of substrate concentration on enzyme activities: Varying concentrations of veratryl alcohol (0.1-1.0 mM) were used as substrate in the assay mixture. The LiP activities were assayed for each concentration to study the effect of enzyme activities at different substrate concentrations. The K_m and V_{max} values for free and C-LiP were measured by Lineweaver-Burk reciprocal plot transformation of Michaelis-Menton equation.

Dye decolorization: Immobilized and free LiP was used for decolorization of reactive dyes solutions (0.01g/100mL) prepared in 100 mM tartarate buffer of pH 3.0. The dyes were Sandal-fix Foron blue E_2BLN, Sandal-fix Turq blue GWWF , Sandal-fix Red C_4BLN, Sandal-fix Black CKF, Sandal-fix Golden yellow CRL and Reactive T blue GWF. After incubation at 30ºC temperature on rotary shaker (150 rpm), the contents of flasks were filtered and filtrates were centrifuged (10,000 rpm). The decolorization was assessed by determined the absorbance of supernatants at specific wavelength (λ_{max}) of the respective dyes. The percent decolorization was determined by the following formula:

$$\text{Decolorization (\%)} = \frac{(Ai) - (At)}{Ai} \text{ X } 100$$

Where A*t* is the absorbance, A*i* is the initial absorbance and t is the incubation time.

Storage stability: Storage stability of chitosan-LiP was studied for a period of forty days at 4ºC and the residual activity was checked from time to time, with veratryl alcohol as substrate by the previously described standard assay protocol.

Lignin Peroxidase Assay: At the end of each purification, immobilization and characterization step the LiP activity was measured according to the method of (Tien and Kirk, 1988) by monitoring the oxidation of veratryl alcohol to veratraldehyde in 100 mM tartarate buffer (pH 3.0) in the presence of hydrogen peroxide as mediator at 25°C. The reading of each sample was taken after every 10 minutes time interval at 310 nm (ε_{270} = 9300 M cm-).

Estimation of Protein Contents: Total protein contents in the crude extract before and after each step of purification was measured by using BSA as standard by the previously described method of (Bradford, 1976).

Statistical study: All the experiments were carried out in triplicate and the data were statistically evaluated. The means and standard error of means were computed for every treatment and SE values have been shown in graphs as Y-error bars (Varalakishami *et al*., 2004).

RESULTS AND DISCUSSION

Production of Lignin peroxidase: A certified culture of *Schizophyllum commune* IBL-06 was cultivated under pre-optimized conditions (Yasmeen *et al*., 201;Munir *et al*., 2015) for LiP production in SSF of corn stover yielding 1347.3 U/mL LiP activity with specific activity of 391.71U/mg in crude extract when the SSF culture flasks were inoculated with 5mL homogenous spore suspension of *S. commune* and supplemented with 1%

glucose and 0.2% yeast extract as carbon and nitrogen additives in 30:1 ratio (C: N); 1mM veratryl alcohol as mediator, 1mM $CuSO_4$ as metal ion and incubated at 30ºC for 8 days.

Lignin peroxidase Purification: The four steps purification protocol comprising ammonium sulfate precipitation, dialysis, DEAE-cellulose ion exchange chromatography and Sephadex-G100 gel filtration resulted in 5.65~fold LiP purification. LiP was completely salted out at 80% ammonium sulfate $(NH_4)_2SO_4$ saturation. The activity of selected fractions was 840.86 U/mL with increase in specific activity and decrease in protein content which indicated that unwanted proteins were removed as shown in Table 1. Purified LiP from *Loweporus lividuswas* has been reported with a purification factor of 3.47 along with 3.8% activity yield after DEAE-cellulose anion exchange chromatography (Yang *et al*., 2005; Kumar *et al*., 2014).

Molecular mass determination through SDS-PAGE: The purified LiP resolved on native and sodium dodecyl sulphate poly acrylamide gels. A single band of 38kDa was found on both native and SDS–PAGE which was an evident that purified LiP from *S. commune* is a monomeric protein (Fig.1). A number of LiP isozymes produced and purified from *Phanerocheate chryososporium* and *Pleurotus sajor caju* have molecular weight in the range of 38-46kDa have previously been reported (Yadav *et al*, 2009a;Yadav*et al*., 2009b).

Immobilization of Lignin peroxidase: The total weight of enzyme beads obtained from chitosan glutaraldehye solution was 42g and LiP immobilization efficiency of chitosan beads was found to be 89.5%. The magnified photograph of LiP cross linked on chitosan beads taken with digital camera is shown in Fig.2 Chitosan beads immobilized LiP showed maximum immobilization efficiency with 2.5 % chitosan concentration. At lower concentrations of chitosan i.e. 1% and 1.5%, the beads showed less enzyme activity as the beads were not formed properly and also leakage of LiP occurred due to the large pore size of the less tightly cross linked delicate beads. On the other hand at higher chitosan levels, the enzyme activity was also low due to stickiness of LiP linked beads, which reduced the pore size and thus delayed the access of the substrate into the beads (Qiu *et al*., 2009; Yasmeen *et al*., 2013).

Optimization of Glutaraldehyde concentration: Further, chitosan beads were incubated with varying concentrations of glutaraldehyde (0.5-2.5%) solution for activation, beads activated with 1.5% glutaraldehyde showed upper most immobilization efficiency (80.24%). Some aldehyde groups were created on the surface of chitosan beads treated with glutaraldehyde (used as cross-linking agent) that may form Schiff's base with amino group of the enzyme leading to enhanced immobilization efficiency. The presence of $-NH_2$ groups on the chitosan backbone greatly improves its potential during the entrapment process. Lower concentrations of glutaraldehyde produce lesser attachment points which caused lower immobilization potential (Dwevedi and Kayastha, 2009).

Effect of Bead size on immobilization Efficiency: Chitosan beads were prepared of different sizes for the entrapment of LiP. The size of the beads was deliberated by Scanning Electron Microscope at different angles for each bead size. The highest rate of vertaryl alcohol oxidation with maximum immobilization efficiency (84.41%) was obtained with 2.5 mm bead size bounded LiP. Immobilization efficiency of LiP cross linked with the chitosan support matrix reduced as the bead size enlarged due to the outflow of enzyme from beads surface. This is because of insufficient packing and weak cross linking between enzyme and the beads. The rate of reaction is affected by the size of the final lattice formed by the bead. The substrate has to diffuse into the beads for the enzymatic reaction to take place in the immobilized enzyme system. Previous studies have reported that the bead size of 2.5 mm diameter caused minor substrate and product diffusion hindrance as compared to the larger bead sizes (Srivastava and Anand , 2014;Wentworth*et al*., 2004).

Scanning Electron Microscopy (SEM) of free and LiP immobilized Chitosan beads: The free and C-LiP beads were subjected to Scanning Electron Microscopy (SEM) to study the surface properties that confirmed effective LiP immobilization on beads (Fig.3) due to high surface area provided by beads. The roughness of LiP immobilized beads surface significantly increased which confirmed cross-linking of enzyme protein particles with chitosan microspheres.

Characterization of free and Chitosan beads immobilized LiP

Effect of immobilization on pH-activity profile: The free and immobilized LiP was incubated in buffers of varying pH, ranging from 2.0-10.0 before carrying out enzyme assay. It was noted that optimum pH was 3.0 and 4.0 for soluble and immobilized enzymes, respectively (Fig.4). By immobilization on chitosan beads the optimum pH for LiP activity shifted toward neutral values. A number of studies have described that the surface area of beads where enzyme is confined has a cationic or anionic characteristics. This charged surface area of beads along with cross-linked enzyme yields a charged microenvironment which eventually has an effect on enzyme protein and modifies the actual pH of the immobilized enzyme (Asgher *et al*., 2012e). The pH relative activity profile of chitosan beads immobilized LiP was wider in the pH range of 4.0-6.0 and it was stable up to 94.63 % of the optimal. Previously, reported

that immobilization on chitosan support matrix results in broadening of pH optima (Chang and Juang, 2007; Wentworth *et al*., 2004). It has also observed that immobilization is likely to result in a conformational change of the enzyme, which may lead to activity enhancement or enzyme denaturation (Asgher *et al*., 2012d).

Effect of immobilization on Temperature-activity profile of LiP: The maximum activity of free and immobilized LiP was observed at 35ºC and 55ºC, respectively (Fig.5). Free enzyme lost its activity at temperatures beyond 35ºC while immobilized enzyme was entirely active at 55ºC. There is a variety of previously citing reports indicating an increase in temperature optima upon enzyme immobilization. Thermo stability of enzyme improves after immobilization because the enzyme molecules are bonded in the beads which obstructed the over-all movement of proteins. Therefore, the temperature array of enzyme relative activity in contrast to the soluble enzyme becomes broader as a result of immobilization (Asgher *et al*., 2012e).

Thermal Stability: The resistance of LiP towards temperature at certain time is surely a vital improvement for industrial applications of this enzyme. Thermo-stability of LiP was improved upon immobilization on chitosan support matrix. The relative activity was about 30% for the free enzyme after 4 hr but it was 50% for C-LiP after 8 hr temperature experience (Fig.6). Enzyme immobilization using glutaraldehyde as cross linker is an efficient mean because of its thermo stabilization due to insertion of extra linkages to limit the free motion of enzyme at higher temperatures. As a result, amide linkages specific to enzyme are protected from disruption resulting in stability of enzyme on higher temperature ranges (Kahraman *et al*., 2007; Zhan *et al*., 2013). Normally, upon enzyme immobilization there is increase in hydro-phobic interactions of support matrix and enzyme which leading to enhance the thermostability of enzymes (Dwevedi and Kayastha, 2009). The LiP immobilized on chitosan beads has better thermostability suggesting its suitability for industrial applications.

Effect of immobilization on kinetic characteristic: K_m& V_{max} values: K_m and V_{max} for soluble and chitoan cross linked LiP was determined by incubating the enzyme with varying concentrations of its substrate (0.1-1.0 mM) at optimum pH and temperature. Activities of both free and C-LiP showed first order kinetics but became constant (V_{max} achieved) at a certain substrate concentration due to saturation effect and became zero order. As illustrated in Fig.7, the Lineweaver-Burk double reciprocal plot was drawn in order to calculate more accurate values of K_m and V_{max} for free and immobilized enzyme. The values of K_m and V_{max} for free LiP were 61.57µM and 845.1U/mL, Whereas, for chitosan beads immobilized LiP the corresponding values for K_m and V_{max} were 50.31µM and 903.6 U/mL respectively. The increase in V_{max} and decrease in K_m values shows that immobilization enhanced the affinity of the enzyme for its substrate resulting in improved catalytic efficiency. Likewise, sol-gel immobilized LiP had lower K_m and higher V_{max} values as compare to its free counterparts (Asgher *et al*., 2008b). Immobilization of a variety of enzymes through different strategies has been found to increase enzyme substrate interactions which endorse higher catalytic properties (Zhan *et al*., 2013; Cabana *et al*., 2007.

Dye decolorization by free and immobilized lignin peroxidase: In order to investigate the dye decolorization potential of free and C- LiPs, six reactive textile dyes were used. It was noted that the immobilized LiP has higher decolorization efficiency towards reactive dyes as evaluated to its free counterpart. The dyes name, wavelength, CI numbers and percent color removal for all dyes is shown in Table 2. The free and modified LiP was transferred to 250 mL Erlenmeyer flasks containing dye (0.01g) in (100mL) buffer solution. The decolorization flasks were incubated at 30°C on rotary shaker (150 rpm).The negative control of soluble LiP was prepared by the addition of heat-killed LiP in to the flasks having solution of various dyes and incubating the experimental flasks as described earlier. Similarly for immobilized LiP, activated chitosan beads lacking LiP used as negative control. It was noted that the immobilized LiP possess higher decolorization ability as compared to free enzyme. The purified *Schizophyllum commune* IBL-06 LiP decolorized S. F. Foron Blue E_2BLN (89.71%), S. F. Red C_4BLN (87.54%), S. F. Turq Blue GWWF (95.43%), S. F. Golden Yellow CRL (83.87%), S. F. Black CKF (63.76%) and Reactive T Blue GWF (69.86%). The highest decolorization (95.43%) was observed after 6 hrs of incubation with chitosan-immobilized enzyme at 30°C. In our piece of work, purified and immobilized LiP after incubation decolorized all six textile dyes, but the heat-deactivated LiP did not decolorize the experimental dyes. The wavelength of maximum absorbance (λ_{max}) and the optical density (OD) of the dye samples were monitored with a double beam UV-Vis spectrophotometer (Fig.8) (Dynamica, Halo BD-20, UV-Vis spectrophotometer, Australia).

The absorption spectra of six control dye solutions are presented in Fig.8 showing different peaks for dye degradation products. These findings designated that ligninolytic enzymes are implicated in decolorization of dyes also confirmed through recent studies (Nasir *et al*., 2013;Bilal and Asgher, 2015).It was noted that by increasing the incubation time, the dye decolorization was enhanced progressively. In a previous study LiP was the major enzyme secreted by *S. commune* IBL-06 for

decolorization of Solar brilliant red 80 which showed maximum dye decolorization efficiency (87.70%) after 6 days of incubation (Bilal and Asgher, 2015). LiP from *K. rosea* and *Schizophyllum* sp. F17 decolorized a number of dyes to various degrees including azo, tri-phenylmethane, hetero-cyclic, metal complexes at neutral pH, strongly demonstrating that the enzyme added a crucial role in dyes degradation (Asgher *et al*., 2013).

Storage Stability and Reusability: The immobilized LiP retained 40% of its activity after 35 days when preserved in buffer at 4ºC, while for the free enzyme; the retained activity was less than 30% after ten days (Fig.9). Enhanced storage stability via immobilization has also been accounted by a range of reported studies (Chang and Juang, 2007; Shi*et al*., 2003).The operational stability of modified LiP was studied by recycling the same beads for consecutive cycles. The chitosan coupled enzyme could be easily separated from the dyes solution and reused in seven repeated cycles (after washing with respective buffer) for decolorization of six different textile dyes. The sample mixture of dye solution of same composition was used for the repetitive catalytic runs. The chitosan bound enzyme retained significantly decolorization potential (66.42 %), (64.3%), (69.87%) (50.03%) (34.7%) and (48.5%) for S. F. Foron Blue E_2BLN, S. F. Red C_4BLN, S. F. Turq Blue GWWF, S. F. Golden Yellow CRL, S. F. Black CKF and Reactive T Blue GWF respectively even after three cycles that gradually decreased to 20-25 % after 7th cycle (Fig.10).The loss in activity may be due to decease in strength of binding between support matrix and enzyme. On further repeated use, the enzyme may seep out from the immobilization matrix (Asgher *et al*., 2013a).

Toxicity Assay: Haemolytic Activity: The rate of haemolysis is an indication of the toxicity on erythrocytes using various concentrations of the synthetic chemical substance. In this study, the hemolysis caused by free and immobilized LiP treated dye samples was studied to evaluate the toxicity reduction by enzymatic treatment. Triton-X 100 (0.1%) was used as positive control which showed 97.84±1.04 % haemolysis, whereas Phosphate Load Saline-PBS was a negative control with zero percent haemolysis. Fig.11 shows a comparison of lysis of human erythrocytes with free and immobilized LiP treated textile dye samples. The results showed that the dye solutions treated with immobilized LiP were less toxic as compared to those treated with free LiP suggesting that chitosan beads immobilized Lip was more efficient dye degrader as compared to its free counterpart. LiP is a copper containing enzyme used to decolorize azo dyes by producing phenolic compounds through a highly non-specific free radical mechanism. These phenolic compounds avoided the formation of toxic aromatic amines. This considerable decrease in toxicity of effluent treated with free and immobilized ligninolytic enzyme depicted the considerable alleviation of cytotoxicity (Bilal *et al*., 2016). *Leptosphaerulina sp.* was also used to decolorized Novacron Red, Remazol Black and Turquoise Blue, without production of toxic compounds (Placido *et al*., 2016).

Table 1. Purification summary for LiP produced from *Schizophyllum commune* IBL-06 in solid state fermentation

Sr. No	Purification steps	Total volume (mL)	Enzyme Activity (U/mL)	Protein contents (mg/mL)	Specific activity (U/mg)	Purification fold.
1	Crude Enzyme	300	1347.3	2.74	391.71	1
2	Precipitation	25	1256.97	1.59	790.54	2.02
3	Dialysis	21	1058.06	1.27	833.12	2.12
4	DEAE-cellulose	19	894.62	0.75	1192.83	3.05
5	Sephadex G-100	15	840.86	0.38	2212.79	5.65

Table 2. Decolorization of six textile dyes using chitosan immobilized Lignin peroxidase

Name of dye	Classification	λ_{max}	Color Index No. (C.I. No.)	Decolorization (%)
Sandal Fix Foron Blue E_2BLN	Reactive dye	560	A30	89.71±0.69
Sandal Fix Red C_4BLN	Reactive dye	540	195A	87.55±2.09
Sandal Fix Turq Blue GWWF 165%	Reactive dye	664	21	95.43±1.26
Sandal Fix Golden Yellow CRL	Reactive dye	414	145A	83.87±2.33
Sandal Fix Black CKF	Reactive dye	598	Mixture	63.76±1.08
Reactive T Blue GWF	Reactive dye	660	21	69.86±1.11

*****incubation time, 5days

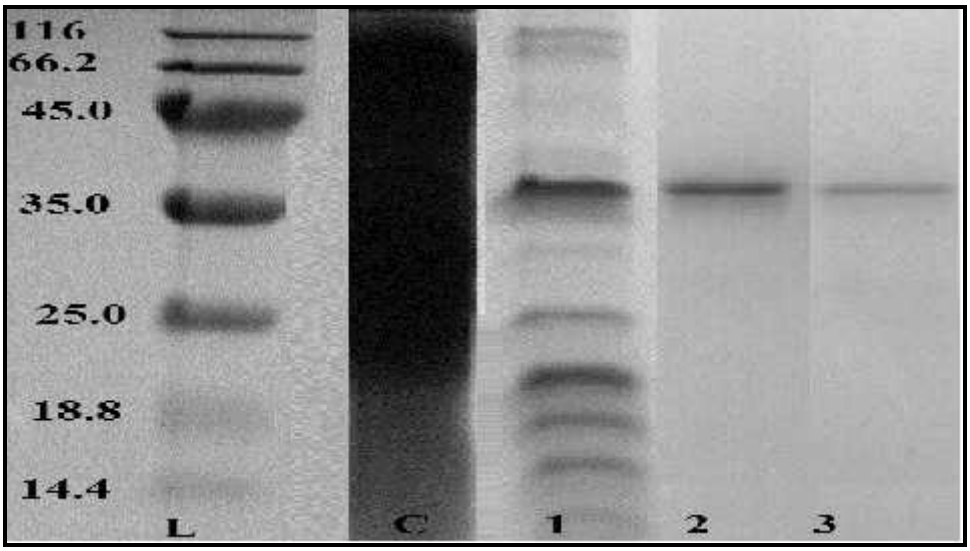
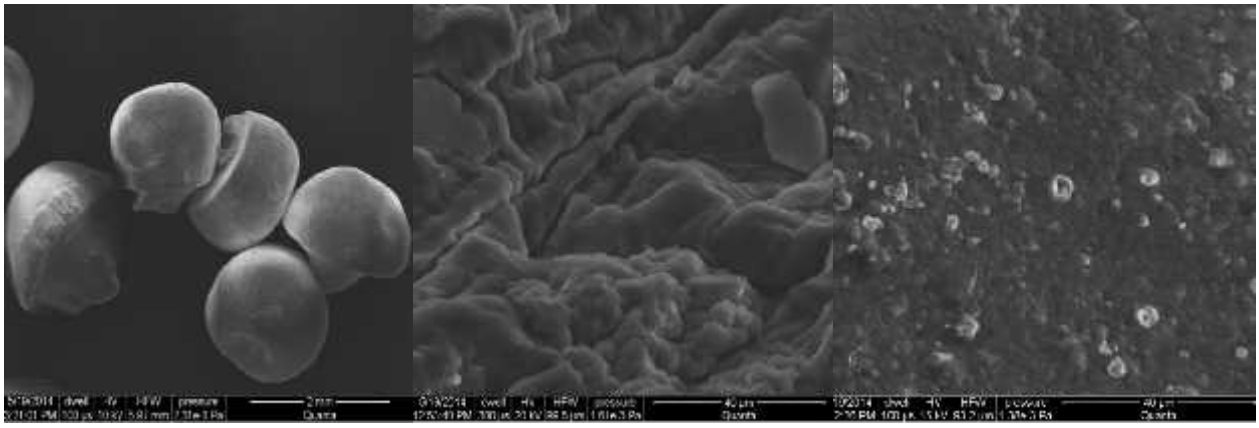


Fig.1 : SDS-PAGE for LiP produced by *schizophyllum commune* IBL-06[Lane L, Molecular weights of standard marker (14.4-116kDa); Lane C, crude enzyme; Lane-1, partial purified LiP after dialysis; Lane-2 LiP after ion exchange chromatography; Lane-3 purified LiP after gel filtration]

(a) **(b)**

Fig.2 Free chitosan beads (b) Chitosan beads with immobilized LiP



(A) (B) (C)

Fig. 3 (A). SEM imaging of chitosan beads,(B); the chitosan beads without immobilized enzyme, (C); the chitosan beads with immobilized LiP

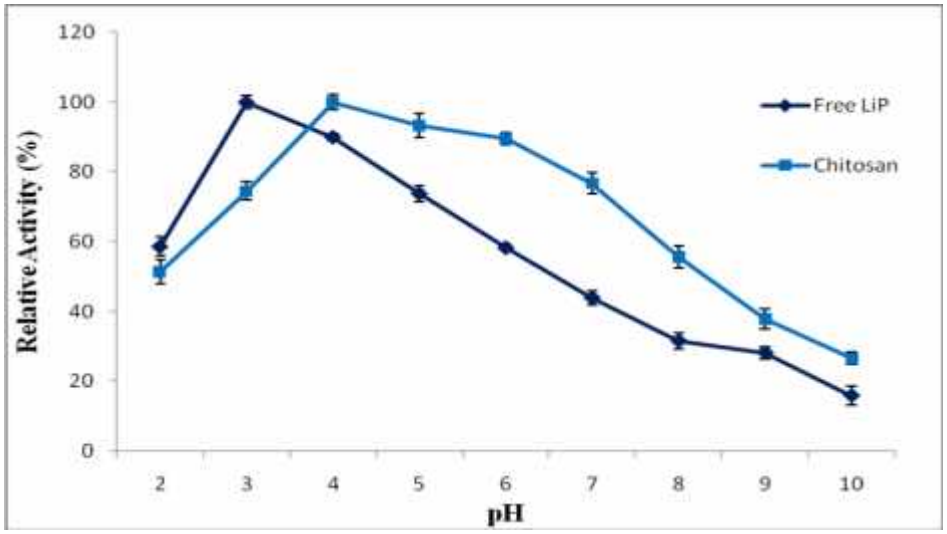


Fig.4 Effect of varying pH on free and chitosan beads immobilized LiP from *S. commune* IBL-06

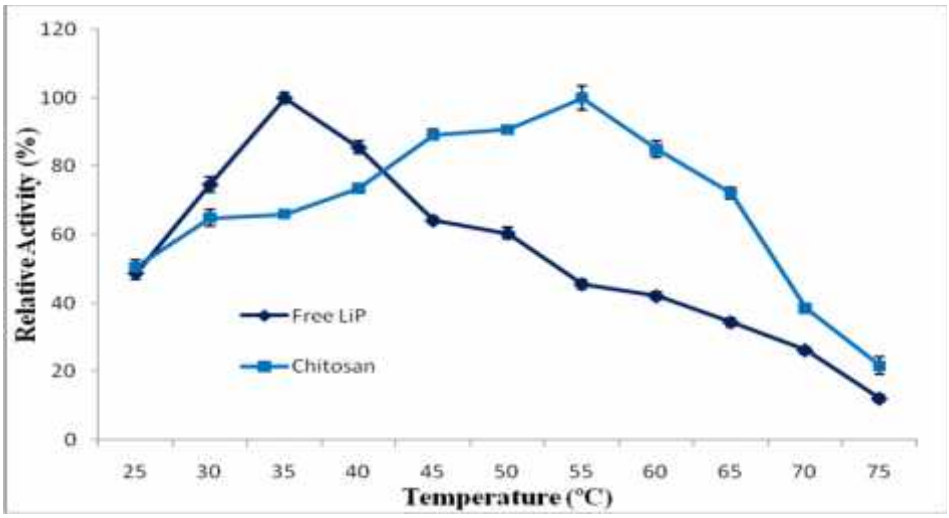


Fig.5 Effect of temperature on activities of free and chitosan beads immobilized LiP from *S.commune* IBL-06

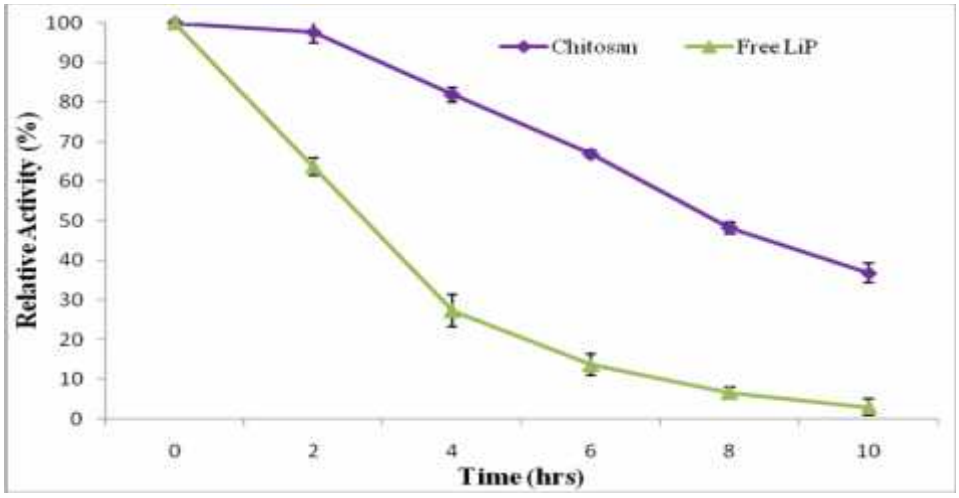


Fig.6 Thermal inactivation of free and chitosan-immobilized LiP

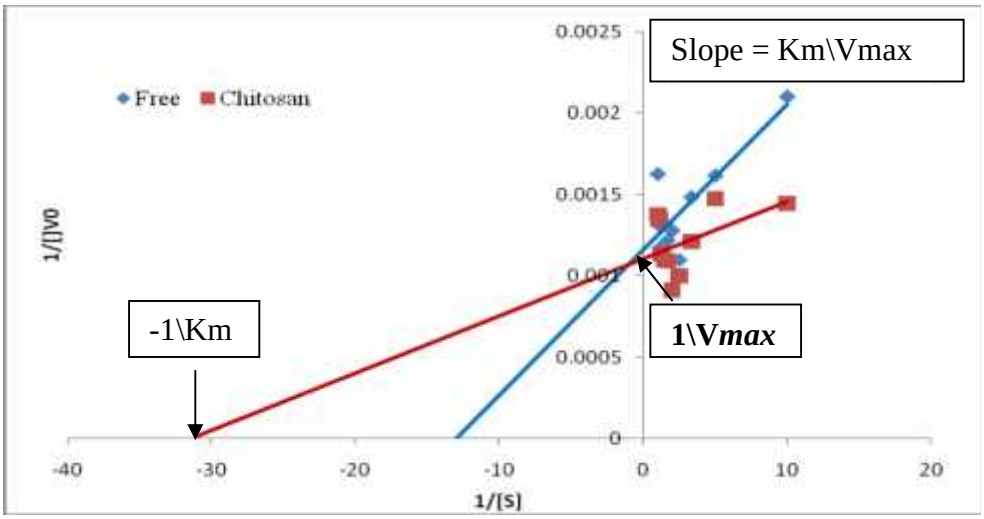


Fig.7 Determination of K_m & V_{max} for free and immobilized LiP through Lineweaver-Burk double reciprocal plot

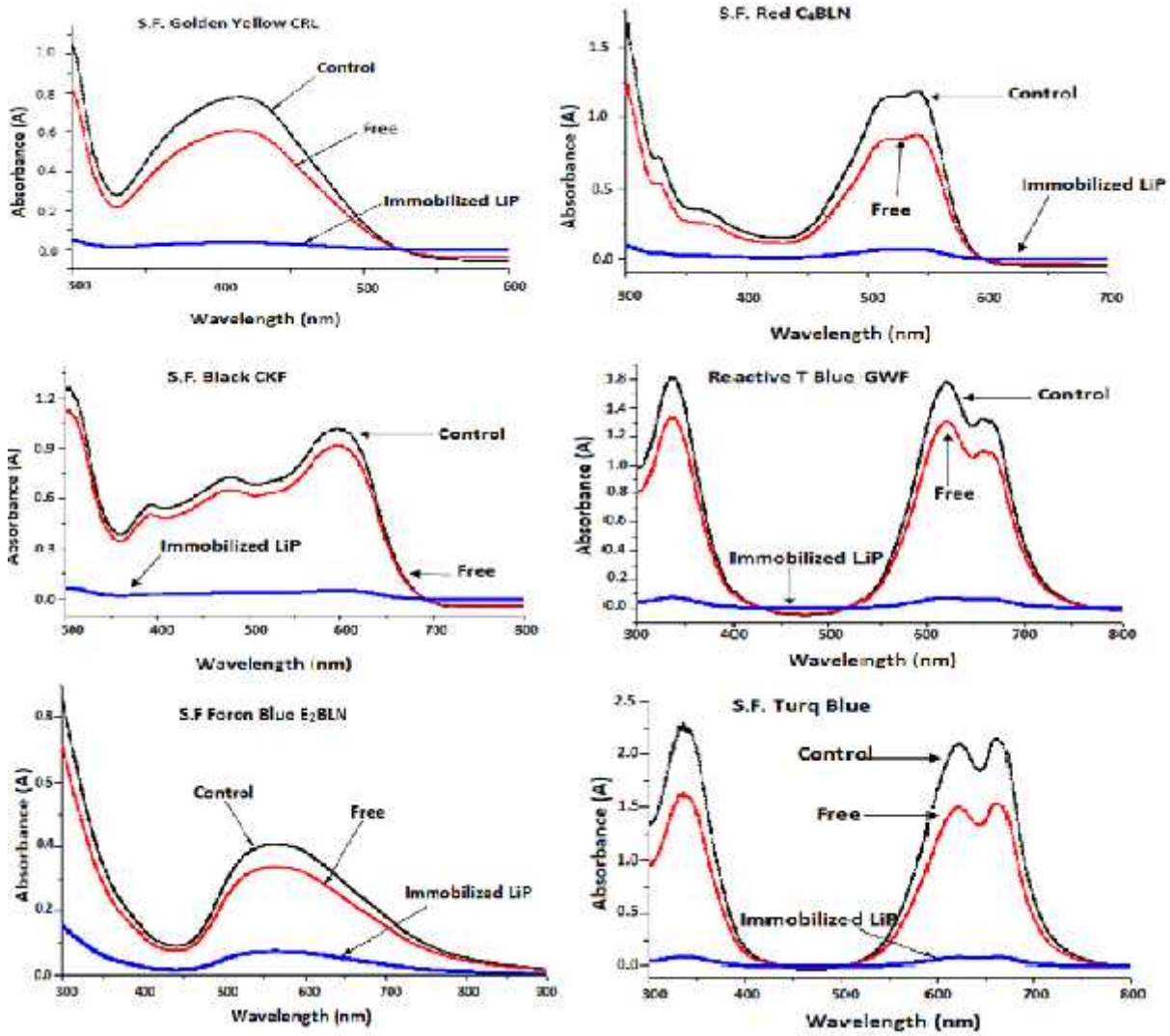


Fig.8 Comparative UV-Vis absorption spectra of textile dyes after 6h treatment with free (red curve); immobilized lignin peroxidase (blue curve) and control (black curve)

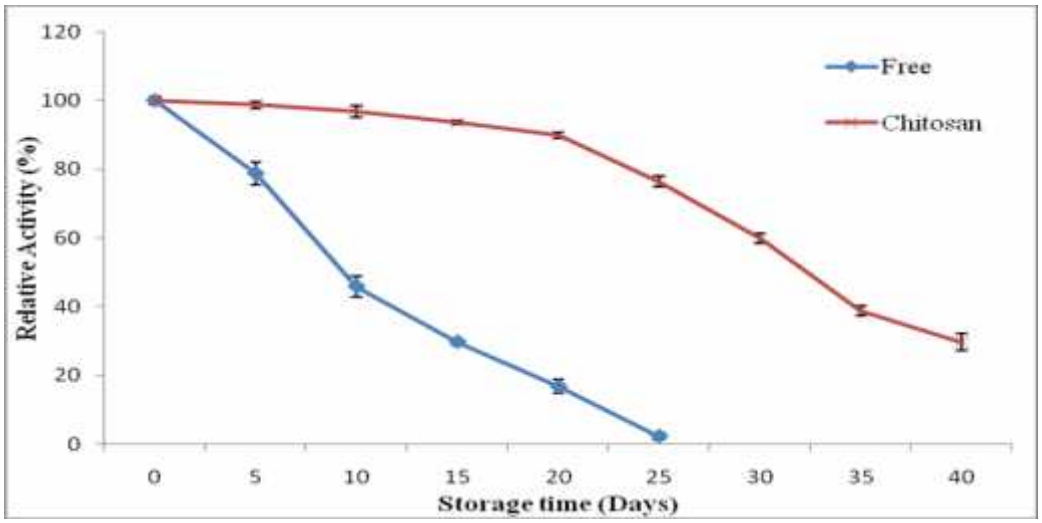


Fig.9Storage stability of free and immobilized Lignin peroxidase

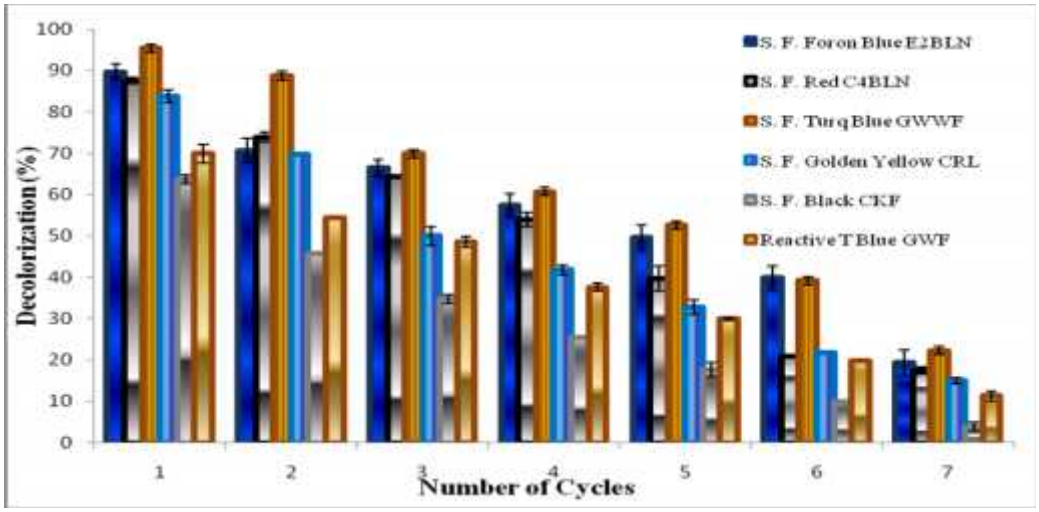


Fig.10. Reusability of chitosan immobilized LiP for decolorization of textile dyes

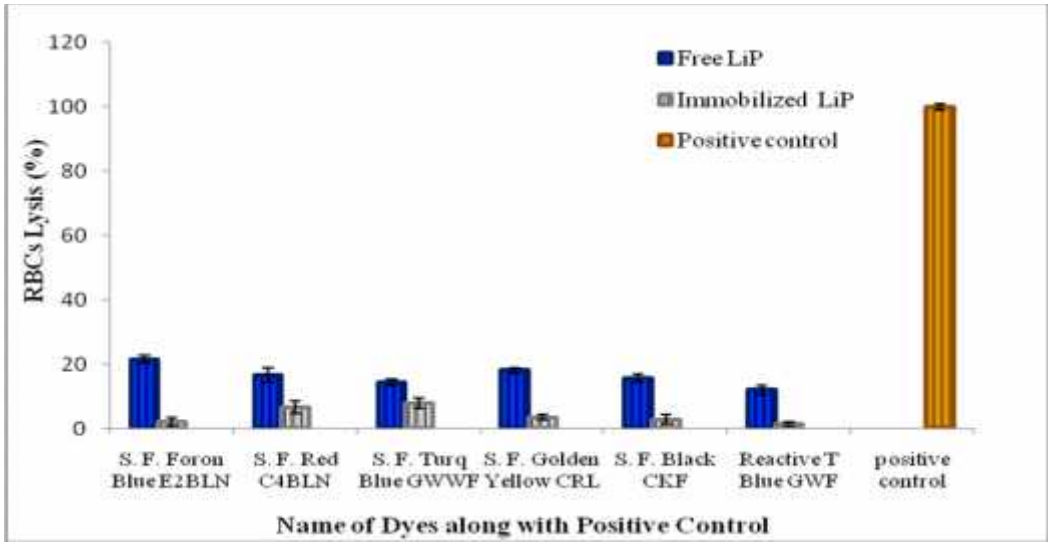


Fig.11. Haemolytic toxicity assay of industrial dyes treated with soluble and immobilized LiP.

Conclusion: From the findings of the study it was concluded that extracellular *Schizophyllum commune* IBL-06 lignin peroxidase can be successfully immobilized using chitosan beads as immobilization support and glutaraldehyde as cross-linking agent. Immobilization of liP enhanced catalytic efficiency, thermostability, storage stability, dye decolorization ability and reusability in repeated dye decolorization batches suggesting its effective and economic storage and reuse in industrial batch operations.

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REFERENCES

Alam, M. Z., F. Mariatul, K. C. Mansor,and A. Jalal (2009). Optimization of lignin peroxidase production and stability by *Phanerochaete chrysosporium* using sewage-treatment plant sludge as substrate in a stirred-tank bioreactor. J. Industrial Microbiology and Biotechnology. 36:757–764.

Asgher, M., B. Aslam and H.M. N. Iqbal (2013a). Novel catalytic and effluent decolorization functionalities of sol gel Immobilized *Pleurotus ostreatus* IBL 02 manganese peroxidase Produced from bioprocessing of wheat straw. Chinese J. Catalysis. 34:1756-1761.

Asgher, M., H.N. Bhatti, Ashraf M, and R.L. Legge (2008b) Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system. Biodegradation 19:771–783.

Asgher, M., H. M. N. Iqbal, and M. J. Asad (2012c). Kinetic characterization of purified laccase produced from *Trametes versicolor* IBL-04 in solid state bio-processing of corncobs. Bioresources 7:1171–1188.

Asgher, M., H. M. N. Iqbal, and M. Irshad (2012d) Characterization of purified and Xerogel immobilized Novel Lignin Peroxidase produced from *Trametes versicolor* IBL-04 using solid state medium of Corncobs. BMC Biotechnology 12:46.

Asgher, M., S. Kamal, and H. M. N. Iqbal (2012e) Improvement of Catalytic Efficiency, Thermo-stability and Dye Decolorization Capability of Pleurotus ostreatus IBL-02 laccase by Hydrophobic Sol Gel Entrapment. Chemistry Central J. 6:110.

Asgher, M., M.Shahid, S. Kamal, and H. M. N Iqbal (2014). Recent trends and valorization of immobilization strategies and ligninolytic enzymes by industrial biotechnology. J. Molecular Catalysis B: Enzymatic, 101, 56-66.

Asgher, M., Q. Yasmeen, and H.M.N.Iqbal (2013) Enhanced decolorization of Solar brilliant red 80 textile dye by an indigenous white rot fungus *Schizophyllum commune* IBL-06. Saudi J. Biological Sci., 20(4): 347–352.

Bailey, A.J, and S.P.Robins(1972) Age-related changes in collagen: The identification of reducible lysine-carbohydrate condensation products. Biochemical and Biophysical Research Communications 46(1): 76–84

Batool, S, M. Asgher, M.A.Sheikh, and S. U.Rahman (2013) Optimization of physical and nutritional factors for enhanced production of lignin peroxidase by *Ganoderma lucidum* IBL-05 in solid state culture of wheat straw. The J. Anim. and Plant Sciences 23(4):1166-1176.

Bhushan, B., A. Pal,and V. Jain (2015). Improved Enzyme Catalytic Characteristics uponGlutaraldehyde Cross-Linking of Alginate Entrapped Xylanase Isolated from *Aspergillus flavus* MTCC 9390. Hindawi Publishing Corporation Enzyme Research2015:9.

Bilal, M., and M. Asgher (2015) Sandal reactive dyes decolorization and cytotoxicity reduction using manganese peroxidase immobilized onto polyvinyl alcohol-alginatebeads. Chemistry Central J. 9:47.

Bilal, M., M. Asgher, and M. Ramzan (2015) Purification and biochemical characterization of extracellular manganese peroxidase from *Ganoderma lucidum* IBL-05 and its application. Scientific research and Essays. 10:456-464.

Bilal M, M. Iqbal, H. Hu, and X. Zhang (2016) Mutagenicity and cytotoxicity assessment of biodegraded textile effluent by Ca-alginate encapsulated manganese peroxidase. Biochemical Engineering J. 109:153–161.

Bradford, M. M. (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.Analytical Biochemistry.72: 248–254.

Cabana, H., J.J. Peter, and N.A. Spiros (2007). Preparation and characterization of cross-linked laccase aggregates and their application to the elimination of endocrine disrupting chemicals. J. Biotechnology 132:23–31.

Chang, M.Y., and R. S. Juang (2007) Use of chitosan–clay composite as immobilization support for improved activity and stability of –glucosidase. Biochemical Engineering J. 35: 93–98.

Chen, Q., M.N.Marshall, S. M.Geib, M. Tien, and T. L.Richard (2012) Effects of laccase on lignin depolymerization and enzymatic hydrolysis of ensiled corn stover. Bioresource Technology.117:186–192.

Cheng, J., R. Shiqian, and L. Tang (2007) Purification of a new manganese peroxidase of the white-rot fungus *Schizophyllum sp*. F17, and decolorization of azo dyes by the enzyme. Enzyme and Microbial Technology 41: 258-264.

Datta, S., R L. Christena, Y. Rani, and S. Rajaram (2013) Enzyme immobilization: an overview on techniques and support materials. Biotech 3:1–9.

Dwevedi, A., and A.M Kayastha (2009) Optimal immobilization of β-galactosidase from Pea (PsBGAL) onto Sephadex and chitosan beads using response surface methodology and its applications. Bioresource Technology 100: 2667–2675.

Kahraman, M.V., G. Bayramoglu, N. Kayaman- Apohan, and A. Gungor, (2007) Alpha-amylase immobilization on functionalized glass beads by covalent attachment. Food Chemistry 104: 1385-1392

Krajewska, B., M. Leszko, and W.J.Zaborska (1990) Urease immobilized on chitosan membrane: preparation and properties. J. Chemical Technology and Biotechnology. 48:337–350.

Kumar, V.P., C. Naik, and M. Sridhar (2014) Production, Purification and Characterization of Novel Laccase Produced by *Schizophyllum commune* NI07 with Potential for Delignification of Crop Residues. Applied Biochemistry and Microbiology 51(4):432–441.

Laemmli, 1970. Cleavage of structural proteins during assembly of head of bacteriophage T4. Nature (227):680–685

Maciel, M. J. M., C. S. Ademir, C. Helena, and R. Telles (2010). Industrial and biotechnological applications of ligninolytic enzymes of the basidiomycota. A review. Electronic J. Biotechnology, 0717-3458.

Missau, J., A.J.cheid, E.L. Foletto, S.L. Jahn, M.A. Mazutti and R.C. Kuhn (2014) Immobilization of commercial inulinase onalginate–chitosan beads. Chemical Processes 2:13.

Munir, N., M. Asgher, I. M. Tahir, M. Riaz, M. Bilal and S. M. A. Shah (2015). Utilization of agro-wastes for production of ligninolytic enzymes in liquid state fermentation by *Phanerochaete chrysosporium*-IBL-03. Intl J. Chemical and Biological Sci., 7:9-14.

Nasir, A. H. A, F. S. M. Asri, A. M. Zaina, M. S. Suhaimi, and A. Idris (2013). Textile Effluent Discoloration by Immobilized *Phanerochaete Chrysosporium* into PVA-Alginate-Sulfate Beads. J. Technology (Sciences & Engineering) 62(2):1–5.

Parshetti, G.K., S. Parshetti, D.C.Kalyani, R.A.Doong, and S.P.Govindwar (2012) Industrial dye decolorizing lignin peroxidase from Kocuria rosea MTCC 1532. Annals Microbiology. 62(1):217–223.

Placido J., X. Chanaga, S. O. Monsalve, M. Yepes and A. Mora (2016) Degradation and detoxification of synthetic dyes and textile industry effluents by newly isolated Leptosphaerulina sp. from Colombia. Bioresour. Bioprocess. 3:6.

Qiu, H.Y., G. Li, G. Ji, X. Zhou, Y. Huang, andP. Gao (2009) Immobilization of lignin peroxidase on nanoporous gold: Enzymatic properties and in situ release of H_2O_2 by co-immobilized glucose oxidase. Bioresource Techno., 100:3837-3842.

Shi, Q. H., Y. Tian, X.Y.Dong, S. Bai, and Y. Sun (2003). Chitosan-coated silica beads as immobilized metal affinity support for protein adsorption. Bioch. Engineer. J. 16:317–322.

Srivastava, P.K., and A. Anand (2014) Immobilization of acid phosphatase from Vigna aconitifolia seeds on chitosan beads and its characterization. International J. Biological Macromolecules. 64:150-4.

Varalakshmi, T.V., T.N. Suseela, T.G.G. Sundaram, T.S. Ezhilarasi and T.B. Indrani, (2004) Statistics, 1:218-237.

Thakur, V., P. Kumar, A. Verma, and D. Chand (2015) Decolorization of dye by alginate immobilized laccase from *Cercospora* SPF-6: Using compact 5 stage plug flow reactor. Intl. J. Current Microbiology and Applied Sci., 4(1): 183-200

Tien, M., and T. K. Kirk (1988) Lignin degrading from *Phanerochaete chrysosporium* characterization and catalytic properties of unique H_2O_2 requiring oxygenase. . Proceedings of the National Academy of Sciences. USA 81:2280-2284.

Wang, X., K. Zhu, and H.M.Zhou (2011)Immobilization of Glucose Oxidase in Alginate-Chitosan Microcapsules. Intl. J. Molecular Sci., *12*: 3042-3054

Wentworth, D.S, D. Skonberg, D.W.Donahue, and A. Ghanem (2004). Application of chitosan-entrapped b-galactosidase in a packed-bed reactor system. J. Applied polymer Sci., 91: 1294–299.

Yadav, M, P. Yadav, and K.D.S.Yadav (2009a) Purification and characterization of lignin peroxidase from *Loweporus lividus* MTCC-1178. Engineer. in Life Sci., 9 (2): 124–129.

Yadav, M., S. K. Singh, and K.D.S. Yadav (2009b) Purification and characterisation of lignin

peroxidase from *Pleurotus sajor caju* MTCC-141. J. Wood Chem. Technol. 29:1-15

Yang, J., H. Yuan, H. Wang, and W. Chen (2005) Purification and characterization of lignin bperoxidases from *Penicillium decumbens* P6. World J. Microbiol. Biotechnology. 21:435–440.

Yasmeen, Q., M. Asgher, M.A. Sheikh, and H. Nawaz (2013) Optimization of ligninolytic enzymes production through response surface methodology. Bioresources 8(1): 944-968.

Zhan, J. F., S.T.Jiang, and L.J.Pan (2013) Immobilization of phospholipase using a polyvinyl alcohol-alginate matrix and evaluation of the effects of immobilization. Brazilian J. Chemi. Engineering 30(04):721 – 728.