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EFFICIENCY OF PURE LACCASE AND LIGNIN PEROXIDASE FROM PHANEROCHAETE CHRYSOSPORIUM BW808 (MTCC 787) IMMOBILIZED ON SOLID MATRIX IN DEGRADATION OF PHENOLICS FROM SEWAGE WASTE WATER

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ABSTRACT

Active laccase and LiP fractions purified from Phanerochaete chrysosporium broth cultures designed for degradation of phenols in sewage samples were immobilized by entrapping in Sol-Gel matrix of trimethoxysilane (TMOS) and propyltetramethoxysilane (PTMS). A maximum of 90.7% and 92.3% of immobilization efficiencies were achieved with a 2 mg/mL pure laccase and pure LiP each. Immobilized laccase and LiP retained 80% and 84% of their activities at pH 4.5 and 5.5 respectively compared to free enzymes. The capacity of the enzyme laccase to withstand the effect of inhibitors (cystein, EDTA, and Ag+) was also enhanced by up to 80% by immobilization. Immobilized Laccase was active in the reaction mixture for three hours, but degraded only 45% of the phenol in one hour whereas immobilized LiP which remained active for one and half hour degraded 95% of phenol in the stipulated one hour time. When immobilized laccase was treated with divalent metal ions Ca^{++} and Cu^{++} it worked faster equal to immobilized LiP and degraded 96% phenol in one hour and remained active for 3 hours.

KEYWORDS: Phanerochaete chrysosporium,Laccase,Lignin Peroxidase,Manganese Peroxidase, Purification and Enzyme Immobilization.

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INTRODUCTION

Wood rot fungi are very well known for their extraordinary capability to entirely degrade/mineralize lignin and phenolic organic substrates. This ability is because their extracellular enzymes function on a wide variety of substratesand are not selective towards the stereochemistry as well. They function together with H2O2 and secondary metabolites. The physiological aspect of enzyme production of white rot basidiomycetes, like *Trametes versicolor*, *Phanerochaete chrysosporium*, *Ganoderma leucidum,* and some others, have been investigated to degrade lignin components, especially lignocellulosic agro-industrial waste materials.[1,2] These fungi produce two major categories of enzymes, generally termed ligninolytic enzymes, *i.e.*, extracellular peroxidases (MnP, manganese peroxidase; LiP, lignin peroxidase; and VP, versatile peroxidases), and phenol oxidases (laccases). Recalcitrant phenolic/non-phenolic compounds and many environmental pollutants that create deleterious effects on the living environment, especially animals and humans are acted upon by laccases and manganese peroxidases[1] The phenolics are oxidized to non toxic residues which are further degraded to minimize the harmful effects. Industrial effluents which pollute the planet as a whole contain several types of chemicals, including pigments, azo-dyes and non degradable pollutants that are highly toxic in nature. Some are carcinogenic, and extremely harmful for living organisms. Such industrial effluents were being discharged into rivers, oceans and other water bodies by authorities of these industries without or after only partial treatments, causing water pollution and dangerously affecting the aquatic life. Among the various major environmental pollutants, the entire spectrum of untreated wastewater effluents is one of them. If cost effective resources are utilized in the production of these wonderful enzymes the biotreatment of the recalcitrant pollutants can be effectively done. Various agro-industrial waste materials and their byproducts, such as straw from food crops, husk, banana peel, citrus peel, leftover corncobs and fruit pulp and sugar cane bagasse. [3, 4, 5] were being used by many researchers over the past several years in the production of ligninolytic- and cellulose-degrading enzymes. In the present day industry, the wastes are not fully rendered harmless in developing countries before they are discarded and this greatly contributes to ecological pollution. Multitude of processes, such as bioremediation of industrial effluents, lignin hydrolysis for ethanol production, de-lignification, oxidation of pollutants, biosensors development, bio-fuels, bio-finishing, beverage processing, bio-bleaching, and detergent manufacturing[6] employ MnP and laccases. Many attempts were made to convert lignocellulosic materials, especially the waste biomass to valuable products with the help of ligninolytic enzymes (LiP, MnP, and laccase) from wood rot fungi, many of which have been successful. Analysis of the secretome revealed enzymatic disposition for lignocellulosic biomass degradation in some of the Trichoderma reesei strains by quantitative profiling methods. According to Adav SS et al., [7] diverse groups of all these enzymes, cellulases, glycoside hydrolases, hemicellulases, lignin degrading enzymes, peroxidases, esterases, lipases, chitinases, peptidases, and hypothetical proteins were quantified from Phanerochaete chrysosporium secretions, of which several were novel enzymes that hydrolyze lignocellulosic residues. Since the wood rot fungi are naturally adapted to degrade the complex polymers consisting of lignin and cellulose, the nonspecific nature of their extracellular ligninolytic enzyme system (lignin peroxidases, manganese peroxidases, and laccases) they are able to degrade wide range of contaminants. Decolorization of textile dyes was successfully carried out by laccase from a novel strain of Trametes modesta with the help of appropriate methods by Nyanhongo et al., [8].Therefore, biological treatment processes which are more cost effective and eco-friendly can be applied to a wide range of industrial wastewater effluents as they provide an alternative to existing physico-chemical water purifying technologies. A lot of techniques, such as gel entrapment, chemical engineering modification, mutation and surface binding have been followed by different researchers to increase the efficacy and stability of enzymes leading to better functionality with a broad spectrum of substrates [9,10] . Among these techniques, gel entrapment is a chosen model to produce and operate enzymes in defined thin films that are thermo-stable and have the ability to catalyze reactions under mild environmental conditions.[9] Since physical and nutritional factors during culture and growth play an important role in enzyme function many novel strains of white rot fungi have been tried and in association with optimization of for the production of lignin degrading enzymes.[11] The immobilization of enzymes has revolutionized the entire world of industrial biotechnology because it provides techniques that can efficiently accomplish bioremediation of industrial effluents which are otherwise very recalcitrant. Management of agro-wastes, degradation and decolorization textile effluents can be done in an ecofriendly way without resorting to health hazards of chemical treatments with the help of immobilized enzymes. Immobilization in xerogels was used for Hyperactivation and thermostablization of *Phanerochaete chrysosporium* lignin peroxidase, [12] and the efficacy and suitability of the glutaraldehyde derivatives consisting of Calix(n)arene as cross linking reagents was checked on immobilized lipase [13]. A magnetic nano-composite was amine terminated and employed for the immobilization of laccase which was explored by glutaraldehyde cross linking methods.[14] Wood rot fungi, Trametes versicolor and Kuehneromyces mutabilis were also responsible for the degradation of toxic residues, phenanthrene and pyrene possibly in a period of 63 days.[15] *Trametes trogii*[16] and *Ganoderma lucidum*[17] and Trametes versicolor[6] are very well known for their potential among the various lignin-degrading microorganisms. In addition *Lentinula edodes* and *Flavodon flavus* can produce laccase[18&19] luxuriantly and the lignocellulosic enzymes[20] they produce are very well characterized and many researchers concluded that they are suitable for a wide range of bioremediation applications. Some bioreactor oriented solutions that solve problems associated with bioremediation of azo dye reactive red

are also available[21] so that biological degradation of azo dyes can be effectively tackled. Therefore, in this article Phanerochaete chrysosporium laccase was immobilized by entrapping it in a sol-gel matrix with the objective of enhancing its tolerance to high temperatures and inactivating agents for industrial application. Lastly, another important objective was to investigate the practicability of sol-gel matrix-entrapped laccase for the decolorization of phenol-containing waste water. To explore the phenol degrading capacity, the immobilized laccase was tested against two different sewage samples and pure phenol samples.

MATERIALS AND METHODS

A.Organism

P. chrysosporium BW808 (MTCC 787) was obtained from IMTECH, Chandigarh India. It was maintained at 37°C on 1% malt extract agar slants and sub cultured every week.

B. Chemicals and Samples

All the chemicals were procured from Sigma-Aldrich, India. The potato dextrose agar and yeast, malt extract and dextrose YMD agar media and other fungal growth media were taken from Hi media. The waste water samples were collected from Vijayawada city, Andhra pradesh.

C. Inoculation and media preparation

The inoculum was taken from stationary cultures containing fresh conidial suspension with a conidium concentration of 5×10^5 spores per ml. The inoculation ratio of the experimental cultures was 2% (v/v), and the incubation temperature was maintained at 37°C in all cases. The growth and maintenance medium used was YMD broth medium [1% (w/v) malt extract, 1% (w/v) yeast extract, 0.1% (w/v) KH2PO4, 2% (w/v) dextrose and YMD agar contain 1.5% agar] at pH 6.5. Basal medium used for growth curves and enzyme production contained 3% (w/v) starch (carbon source), 0.3% (w/v) NaNO3 (nitrogen source), 0.05% (w/v) KCl, 0.1% (w/v) KH2PO4 and 0.01% (w/v) FeSO4.7H20 and maintained at 37°C in an orbital shaker (120 rpm) for 24 to 96 h. At the end of incubation, the whole fermentation medium was filtered, centrifuged at 5000 rpm for 15 min and the clear supernatant was used as crude enzyme preparation.

Laccase and LiP Activities and Protein Contents Estimation

A. Laccase activity

Laccase activity was monitored according to Ander and Messner methodology (18) using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), (ABTS) as substrate at 40ºC.

B. LiP activity

LiP was measured with veratryl alcohol (vacuum distilled prior to use; Aldrich) at 25^0C. The reaction mixture contained 0.1 M sodium tartrate buffer (pH 3.0), 0.4 mM veratryl alcohol, and 1.65 ml of culture filtrate in a total volume of 3 ml. The reaction was started by adding H_2O_2 to a final concentration of 0.2 mM, and *A*310 was monitored. LiP activity was expressed as units per milliliter.This procedure is for the determination of Lignin Peroxidase enzymatic activity using veratryl alcohol VA as the substrate. One unit of lignin peroxidase will form 1.0 milligram of veratryl aldehyde from veratryl alcohol in 20 seconds at pH 3.0 at 20 °C. This VA (20 second) unit is equivalent to ~18 µM units per minute at 25 °C.Bovine serum albumin (BSA) was used as a standard to determine the protein contents of crude and purified laccase extracts by adopting the UV/Visible spectrophotometric method.

Purification of Laccase

Crude laccase extract was centrifuged (3,000×g) for 15 min at 4 °C to attain maximum clarity, followed by ammonium sulfate fractionation purification methodology as described by Asgher *et al.,* (2012)[7]. The ammonium sulfate-treated crude laccase fractions were dialyzed against buffer to remove extra salt, lyophilized, and subjected to further purification by gel filtration chromatography using a freshly packed 120×2 cm Sephadex G-100 column. To determine the molecular weight of the laccase, SDS-PAGE was performed on a 5% stacking and a 12% resolving gel according to the method as described previously[7]

Sol-Gel Immobilization of Laccase

To prepare gel for laccase entrapment in a sol-gel matrix, TMOS and PTMS were used in molar TMOS:PTMS (T:P) ratios of 1:1. Five different purified laccase fractions, ranging from 2 to 10 mg/mL were suspended in deionized water and centrifuged (4,000 ×g) for 15 min at 4 °C. The separated supernatant fluid (400 µL) from each fraction was added to an equal ratio mixture of aqueous sodium fluoride, polyvinyl alcohol, and water. The solution was shaken and PTMS was added, followed by TMOS. The reaction mixture was gently mixed for 20 sec in a vortex mixer and placed in an ice bath until gelation occurred (Iqbal *et al.,* 2012). At the end, the activity of sol-gel-entrapped laccase was determined as described in the previous section. The entrapped enzyme fraction having the highest specific enzyme activity (U/mg) was selected for further decolorization study.

Characterization of Free and Sol-Gel Matrix-Entrapped Laccase and lignin peroxidase

pH and thermal stability of laccase and lignin peroxidase

An active pH profile of free and entrapped laccase was determined by studying the effect of different pH buffers ranging from 2 to 10. For a stability assay, laccase was incubated at 25 °C for up to 4 h without substrate. To find out the thermal stability profile, both the purified free and sol-gel-entrapped laccase were incubated at varying temperatures ranging from 20 to 80 ºC for up to 4 h in the absence of substrate. Residual activity of both enzyme forms (free and immobilized) was checked for pH and temperature profiles after every hour using the standard activity assay described above.

Effect of stimulators/inhibitors

The effect of various organic compounds and metal ions, mainly including Cu^{2+},Fe^{2+} , Ag^{+}, EDTA, and cystein, as possible activators or inhibitors on purified free and sol-gel-entrapped laccase and lignin

peroxidase was also studied. A standard activity assay protocol as described earlier was followed to determine the residual activities in each case.

Degradation of phenols in waste water by Immobilized Laccase and Lignin peroxidase

To investigate the degradation capacity of of sol-gel-entrapped laccase and LiP, two different sewage wastewater effluents were collected from Vijayawada, Andhra Pradesh. The working conditions of a single continuous operation were: triplicate flasks containing 5 g of sol-gel-entrapped laccase as a biocatalyst, 100 mL of each waste sample with 1 mL of 1 mM ABTS as laccase mediator, and incubated in a temperature-controlled shaker (100 rpm) for up to 24 h reaction time period. After every 8h reaction time, samples were collected from each flask to determine the percentage enzymatic removal of phenol by considering the initial and final reduction in the optical density. All the collected samples were centrifuged at 5,000 × g for 15 min at room temperature (30 °C) and clear supernatants were analyzed spectrophotometrically at λ_{max} (510 nm) in order to determine the percent degradation. Percent degradation of phenols was calculated by,

$$\% \text{ Degradation} = (Aini - Afin) \times 100 / Aini$$

where *Aini* is the initial absorbance of phenol before incubation and *Afin* is the final absorbance of phenol after incubation.

Statistical Analysis

All the experimental data were statistically evaluated using the student's T-test and performed in triplicate. The means and standard errors of means (mean ± S.E.) were calculated for each treatment and each analysis was carried out in triplicate.

RESULTS AND DISCUSSION

Figure 1
Growth of Phanerochaete chrysosporium (A) and production of LiP and laccase (B) Production of LiP and Laccase

The results showed that laccase and lignin peroxidase enzymes were produced in YMD medium with glucose repression in the presence of lignin and the activities recorded were 550±4 U/ml and 530±2 mU/ml respectively. The nature and amount of the available nutrients/microelements in the fermentation growth substrate had a strong effect on extracellular ligninolytic enzyme production. The combined effect of the addition of lignin and repression of glucose greatly enhances the production of enzymes Laccase and Peroxidases by Phanerochaete chrysosporium in cultures that were designed for the degradation of phenolics in waste water samples. More importantly the purpose of achieving enhanced degradation of phenol in the waste water samples is served by addition of lignin and the repression of glucose.[22]

Purification of Laccase and LiP

This was achieved by ammonium sulphate fractionantion and ion exchange chromatography**.** The crude cell free supernatant enzyme solution was subjected to partial purification by with unit activity and specific activity of 123.2 U/mL and 0.11 U/mg, respectively for Laccase ; 94U/ml and 0.087U/mg respectively for LiP. After gel filtration through a Mono Q sepharose and DEAE

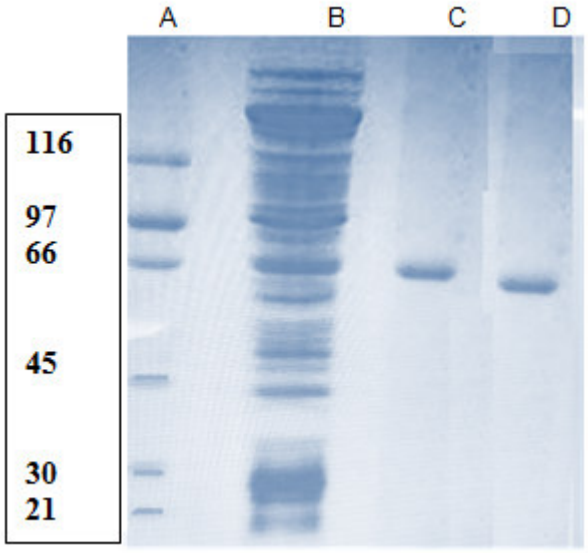


Figure 2
SDS PAGE of pure laccase and LiP
Lane A: Standard protein molecular weight markers
Lane B: crude extract on the 5th day of incubation
Lane C: Laccase purified fraction
Lane D: LiP purified fraction

sepharose columns, the laccase and LiP were maximally purified to a level up to 88.7% and 87.2% purity respectively. The specific activities of the pure laccase and LiP were observed to be 0.172 U/mg and 0.14 U/mg respectively. (Tables 1&2).

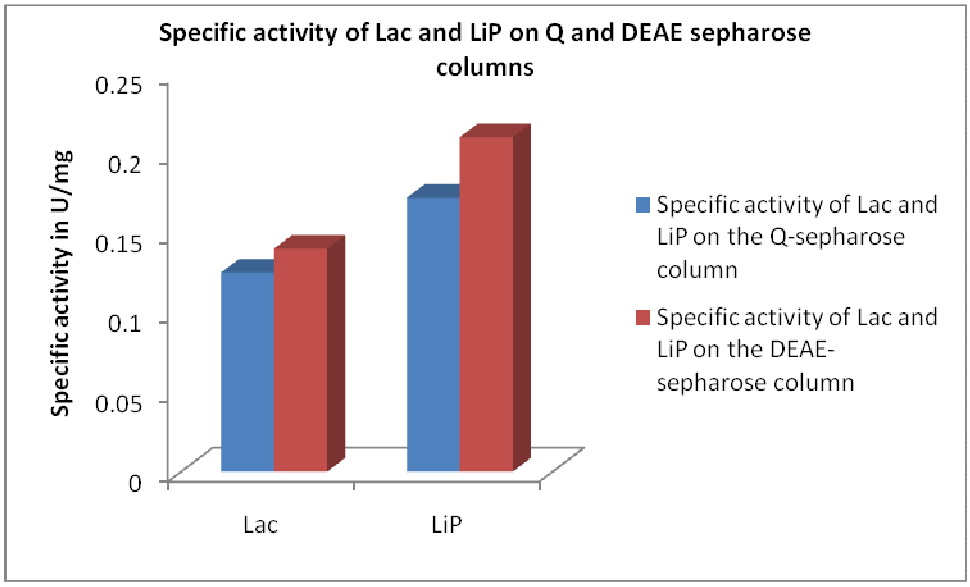


Figure 3
Graphical representation of Specific activity of Lac and LiP on Q and DEAE sepharose columns

Table 1
Laccase purification

Purification Step	Total protein in culture(mg)	Total activity (Units)	specific activity (Units/mg)	Yield(%)	Relative purification
Crude extract	1080	123.2	0.11	100	1
Mono Q- Sepharose	575	72	0.125	88.7	1.136
DEAE Sepharose	360	62	0.172	76.3	1.56

Table 2
LiP purification

Purification Step	Total protein in culture(mg)	Total activity (Units)	specific activity (Units/mg)	Yield(%)	Relative purification
Crude extract	1080	94	0.087	100	1
Mono Q- Sepharose	575	82	0.14	87.23	1.6
DEAE Sepharose	360	78	0.21	82.9	2.41

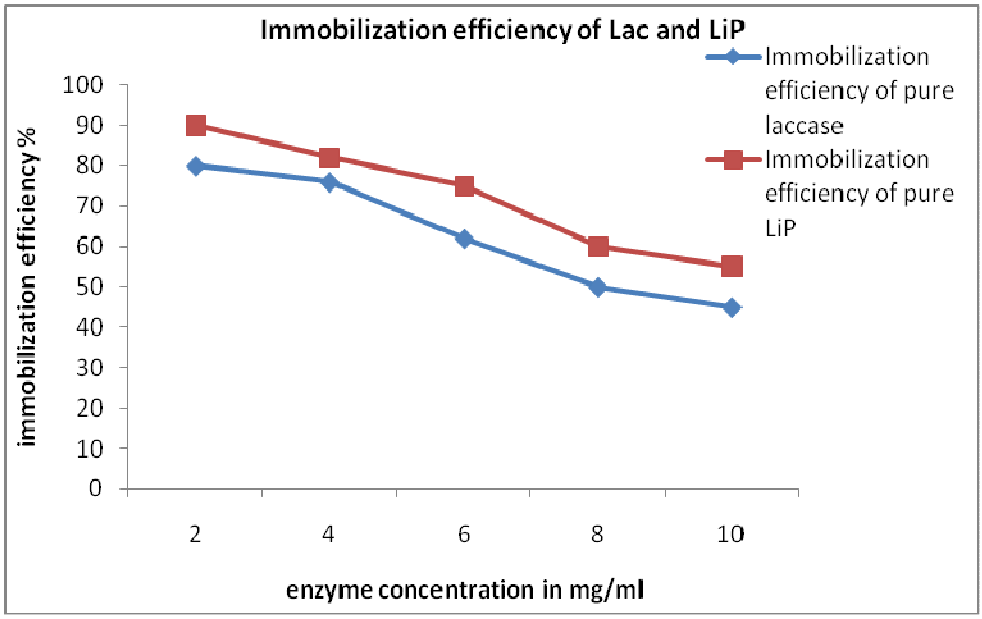


Figure 4
Graph showing Immobilization efficiency of Lac and LiP

To confirm the purity of laccase at homogeneity level, the purified active fraction obtained from the gel filtration column was resolved on a 5% stacking and 12% running gel. Laccase was found to be a homogenous monomeric protein, as evidenced by the single band corresponding to 64 kDa on SDS-PAGE (Fig. 1), which is within the range of the laccase family (Asgher *et al.,* 2012). The LiP had a molecular weight of 60Kda and was observed as a single band too.

Immobilization efficiency of free Laccase and LiP and immobilized Laccase and LiP

Immobilization efficiency was studied relative to enzyme concentration over the range of 2 to 10 mg/mL with purified laccase and Lignin peroxidase. Figure 2 reveals that the trial fractions with enzyme concentrations of 2 mg/ml showed maximum immobilization efficiency (80%) with Laccase and (90%) LiP respectively. Any further increase in the enzyme concentration caused a decrease in the immobilization efficiency. An activity profile showed that the specific activity of sol-gel-immobilized enzyme fractions was higher than the purified free laccase and LiP. Previously, Asgher *et al.* (2007) reported hyper-activation of LiP from *P. chrysosporium* in xerogels up to optimum gel hydrophobicity limits. The immobilization results from the present study by the sol-gel entrapment method are in the range of those attained by other methods. Many of the earlier reported methods (covalent binding on siliceous cellular foams, sepa beads,and amine-terminated magnetic nano-composite) require glutaraldehyde as a coupling agent (Xiao *et al.,* 2006; Nwagu *et al.,* 2012). In contrast, the present immobilization approach did not require any coupling agent, making the process more economical, chemical-free, and eco-friendly.

Characterization of Free and immobilized Laccase and LiP

pH and residual activity of laccase and LiP

A residual pH activity profile showed that immobilized Laccase showed 80% residual activity whereas free laccase showed 60% at the optimum pH 4.5.The results also showed that immobilized LiP showed 80% residual activity whereas free LiP showed 75% at the optimum pH 5.5.This showed that immobilization has remarkably increased the activity of Laccase upto 20% whereas immobilization has slightly increased the activity of free LiP upto 5%.

	Percent Residual Activity			
pH	**Imm. Laccase**	**Free Laccase**	**Imm. LiP**	**Free LiP**
3.5	22	22	21	22
4.5	80	60	60	62
5.5	60	45	80	75
6.5	42	35	68	60
7.5	18	15	50	54
8.5	10	10	40	42

Table 3
Percent residual activity of free and immobilized enzymes

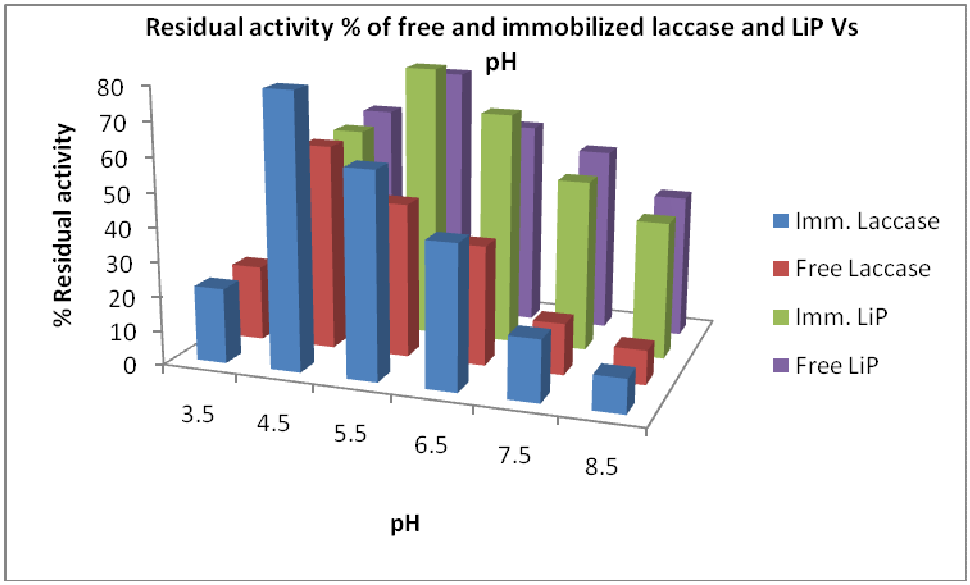


Figure 5
pH activity profile of free and immobilized laccase and LiP

Effect of activators/inhibitors

It would appear that the effects of different organic compounds and metal ions as possible activators/inhibitors on the stability of immobilized Phanerochaete laccase and LiP simultaneously are being investigated for the first time in our study. Results showed that free laccase was almost completely inhibited by EDTA and to a certain extent with cystein. Among the metal ions used, Ag+ caused 40% inhibition in the case of LiP and 30% was inhibited in the case of laccase.80% activity was inhibited by EDTA in the case of LiP whereas laccase it was inhibited by 90% with EDTA. Both the enzymes were fully stimulated by Cu2+ and Ca2+ as shown in the table below. The immobilized laccase and LiP showed 94% and 95% of the residual activity respectively in the presence of cu2+ or ca2+ whereas the free LiP and free Laccase showed 90% and 72% activity in the presence of either of the divalent metal ions. The LiP was 90% active even in the free state and the activity slightly increased in the immobilized state. More importantly laccase activity has increased significantly from 72% to 94% in the presence of cu++ and ca++The efficiency of immobilized laccase in the presence of activators, which are cu++ and ca++ has reached up to the level of LiP which was more active than laccase in the free state itself. The sol-gel matrix-entrapped laccase and LiP demonstrated a higher tolerance than free enzymes against inactivation by cystein and Ag+. What is remarkable is that in the presence of Ag+ the laccase retained 40% of its activity whereas the LiP retained only 30% both in free and immobilized states. The LiP and laccase both in the free and immobilized states showed 30% and 40% of activity respectively in the presence of cystein. The metal chelating activity of EDTA seems to be inhibiting the activity of the enzymes in both free and immobilized states upto 90% in the case of laccase and 80% in the case of LiP.

Activator/inhibitor	Imm. laccase	Free Laccase	Imm. LiP	Free LiP
Ca^{++}	94	72	95	90
Cu^{++}	94	74	95	90
Fe^{++}	82	70	82	80
Ag^{+}	60	60	70	70
EDTA	10	10	20	20
Cystein	40	40	30	30

Table 4
Residual activity percent of Immobilized and free Laccase and LiP in the presence of Ca^{++}, Cu^{++}, Fe^{++}, Ag^{+}, EDTA, and Cystein

The activity of enzymes with respect to time

The objective of improving the activity of pure enzymes with immobilization and using divalent metal ions in this study is to achieve the degradation of phenolics in the waste water samples. So we have tested the phenol degrading capacity of immobilized enzymes with cu++ with respect to different time intervals at optimum pH and at 37^0C at which maximum activity of both the enzymes observed keeping other conditions unchanged. The results showed that a sharp rise in the activity of both the immobilized Laccase and LiP was seen after 60 minutes at which higher amount of phenol was degraded which was confirmed by both

absorbance measurements and HPLC of phenols on a c18 reverse phase column.

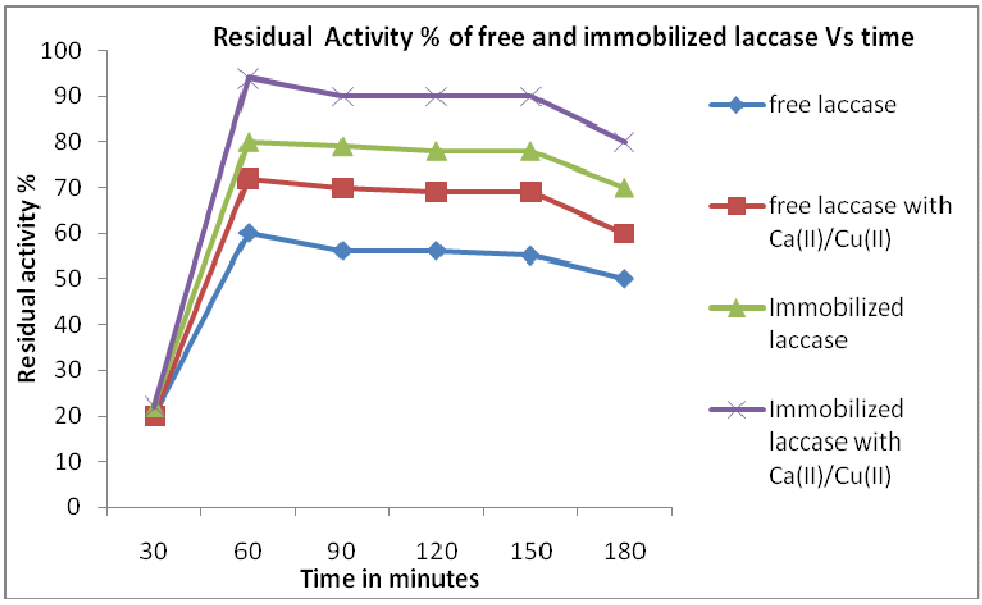


Figure 6
Graph showing Residual Activity % of free and immobilized Laccase Vs time

After 1 hour time interval the 1ug/ml free laccase showed 60% of residual activity and the same amount showed 72% in the presence of cu/ca whereas immobilized laccase showed 80% of residual activity and the same amount showed 94% activity in the presence of cu/ca. In addition after one hour time interval the 1ug/ml free LiP showed 75% of residual activity and the same amount showed 80% in the presence of cu/ca whereas immobilized LiP showed 90% of residual

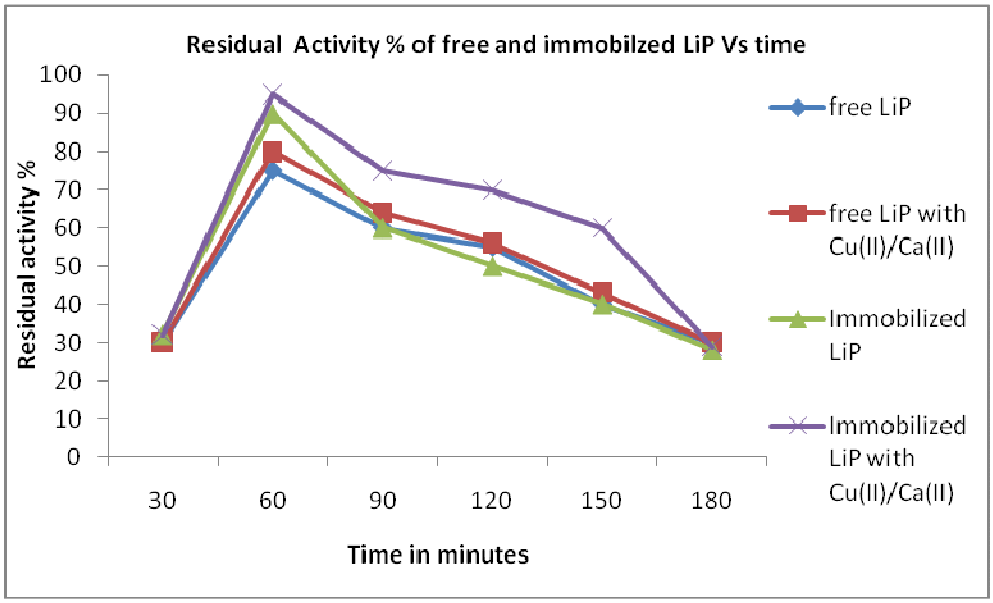


Figure 7
Graph showing Residual Activity % of free and immobilized LiP Vs time

activity and the same amount showed 95% activity in the presence of cu/ca. This result clearly demonstrates that the divalent metal ions are playing a key role in enhancing the activity of both free and immobilized enzymes. Immobilized laccase continues to show 80% activity at 180 min and shows a plateau of nearly constant activities whereas Immobilized LiP reaches a peak at 60 min and drops down to 30% as the time passes to 180 min.

Degradation of phenolics from sewage samples by immobilized laccase and lignin peroxidase

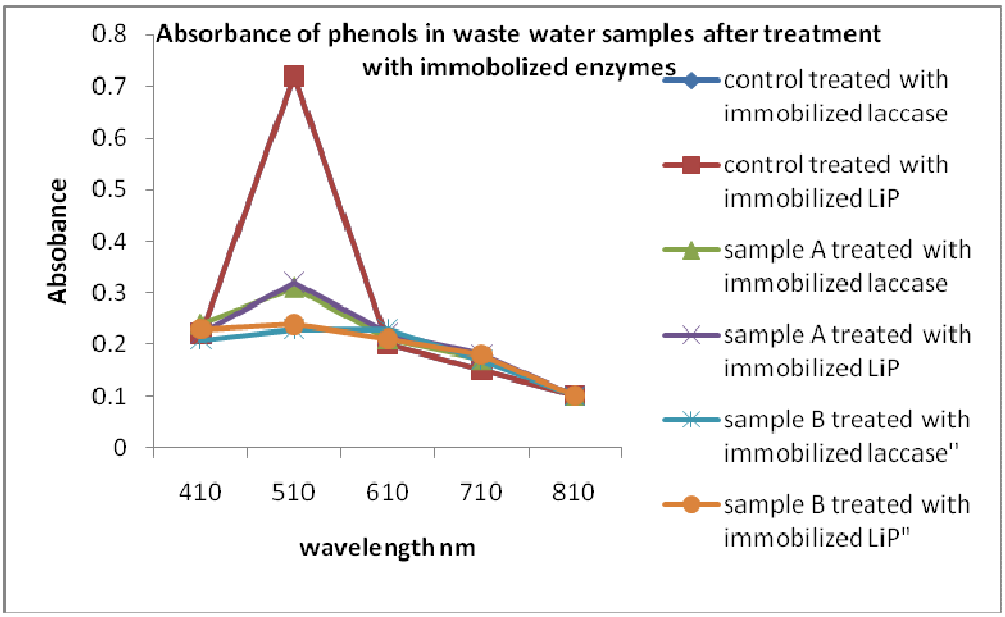


Figure 8

Graph showing Absorbance of phenols in waste water samples after treatment with immobilized enzymes

The degradation of phenolics was carried out with free and immobilized Laccase and LiP purified from broth cultures of Phanerochaete chrysosporium with and without the divalent cations ca/cu. 10ml each of the samples A and B containing 100 ug phenol were added to 1 ml of immobilized enzyme preparations at optimum conditions and the degradation of phenols was monitored for 3 hours and the absorbance was recorded at 410 to 810 nm and controls without the enzyme preparations were simultaneously run and the results were as shown in the table and graph.

OD VALUES OF IMM ENZ ACTIVITY CONTROL AND TEST SAMPLES A&B					
Wavelength nm	**410**	**510**	**610**	**710**	**810**
control laccase	0.22	0.72	0.2	0.15	0.1
control LiP	0.22	0.72	0.2	0.15	0.1
sample A laccase	0.24	0.31	0.21	0.17	0.1
sample A LiP	0.22	0.32	0.22	0.18	0.1
Sample B laccase	0.21	0.23	0.23	0.17	0.1
sample B LiP	0.23	0.24	0.21	0.18	0.1

Table 5

showing absorbance values of immobilized enzyme activity in the optimum conditions for the control and test samples A&B

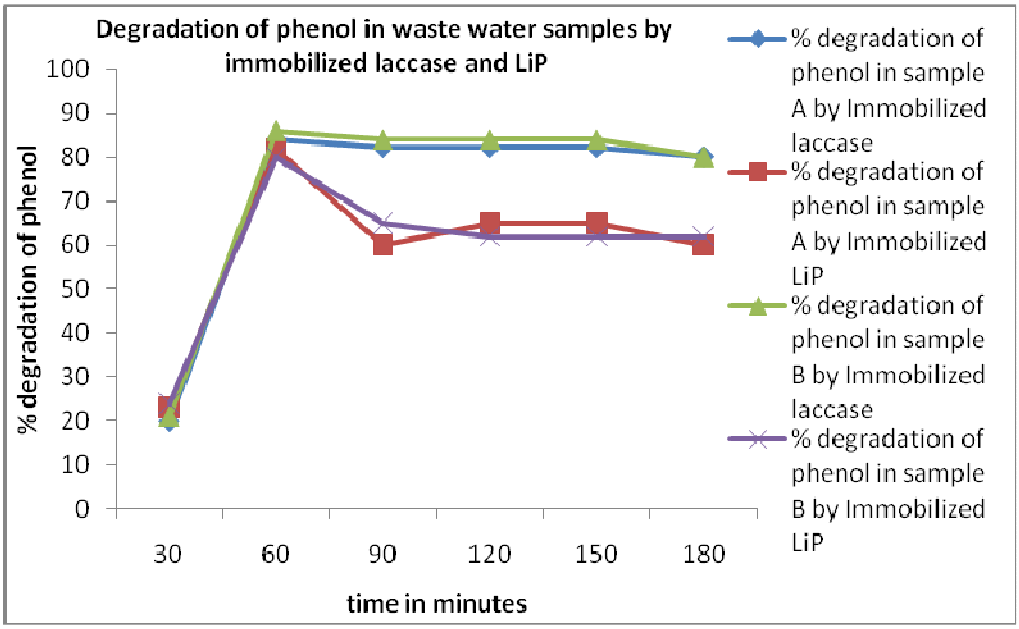
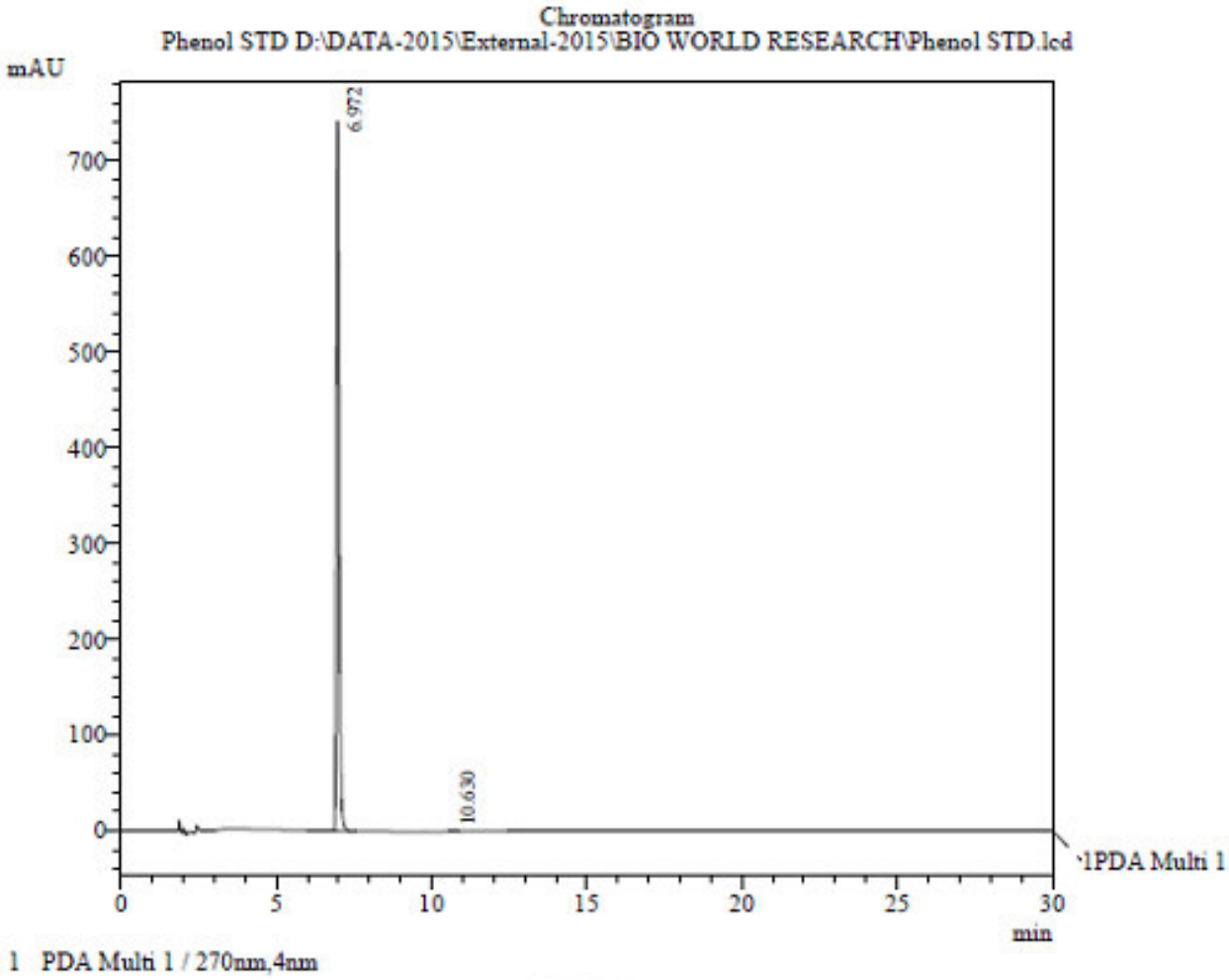


Figure 9
Graph showing Degradation of phenol in waste water samples by immobilized laccase and LiP

HPLC analysis of phenol degradation in the waste water samples by immobilized enzymes

Laccase and LiP which were immobilized as described in materials and methods were used to degrade the phenols present in waste water samples A and B collected from different locations in Vijayawada. The residual mixture after one hour long treatment was centrifuged at 5000 rpm and the clear supernatant was collected and used for HPLC on a C18 reverse phase column as described in materials and methods. The HPLC profiles of control and immobilized enzyme treated samples A and B with respect to phenol degradation are shown below.



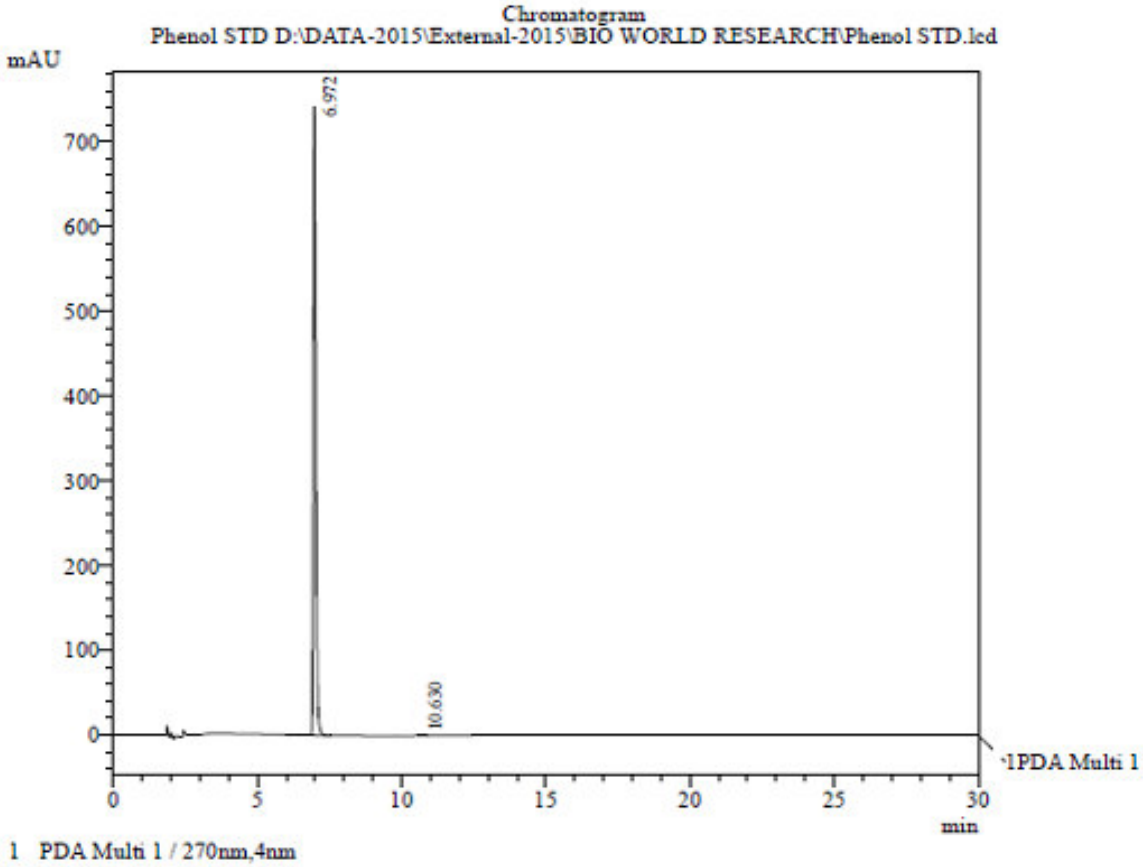
1 PDA Multi 1 / 270nm,4nm

PeakTable

PDA Ch1 270nm

Peak#	Ret. Time	Area	Theoretical Plate#	Relative Retention Time	Area %
1	6.972	4454500	25807.608	1.000	99.935
2	10.630	2881	37841.062	1.525	0.065
Total		4457380			100.000

Figure 10
Control incubated without laccase

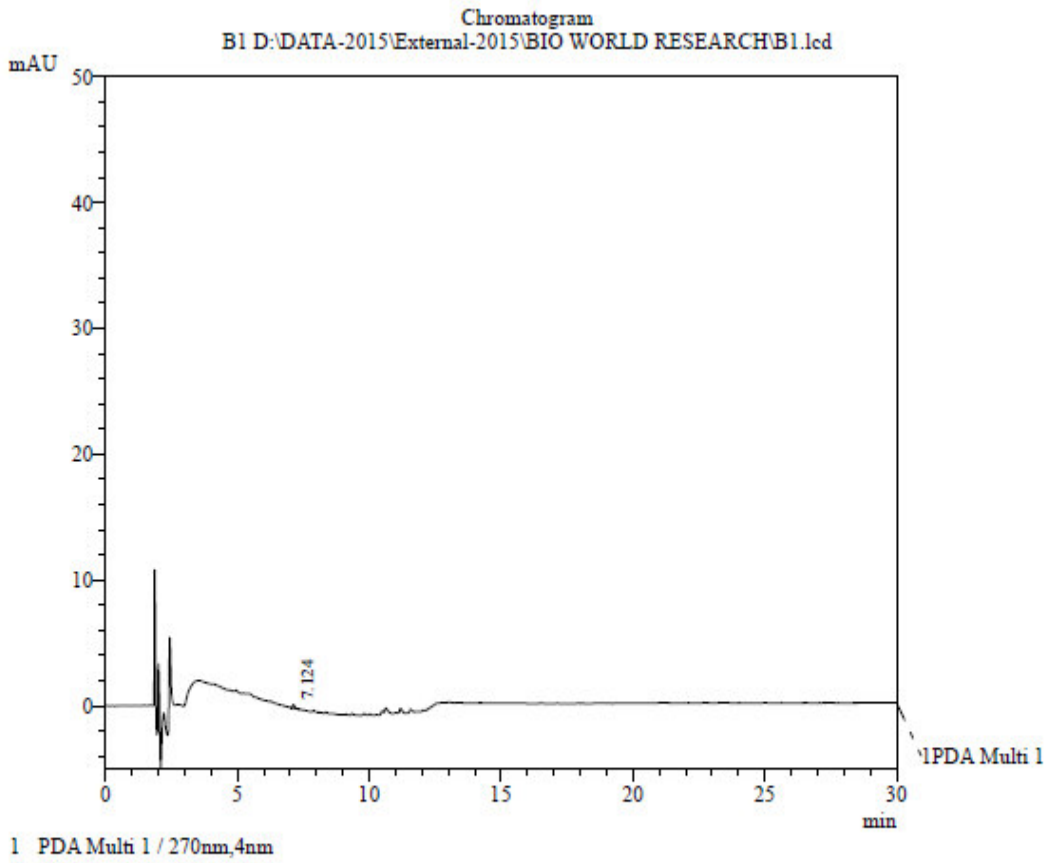


PeakTable

PDA Ch1 270nm

Peak#	Ret. Time	Area	Theoretical Plate#	Relative Retention Time	Area %
1	6.972	4454500	25807.608	1.000	99.935
2	10.630	2881	37841.062	1.525	0.065
Total		4457380			100.000

Figure 11
Control incubated without Lip

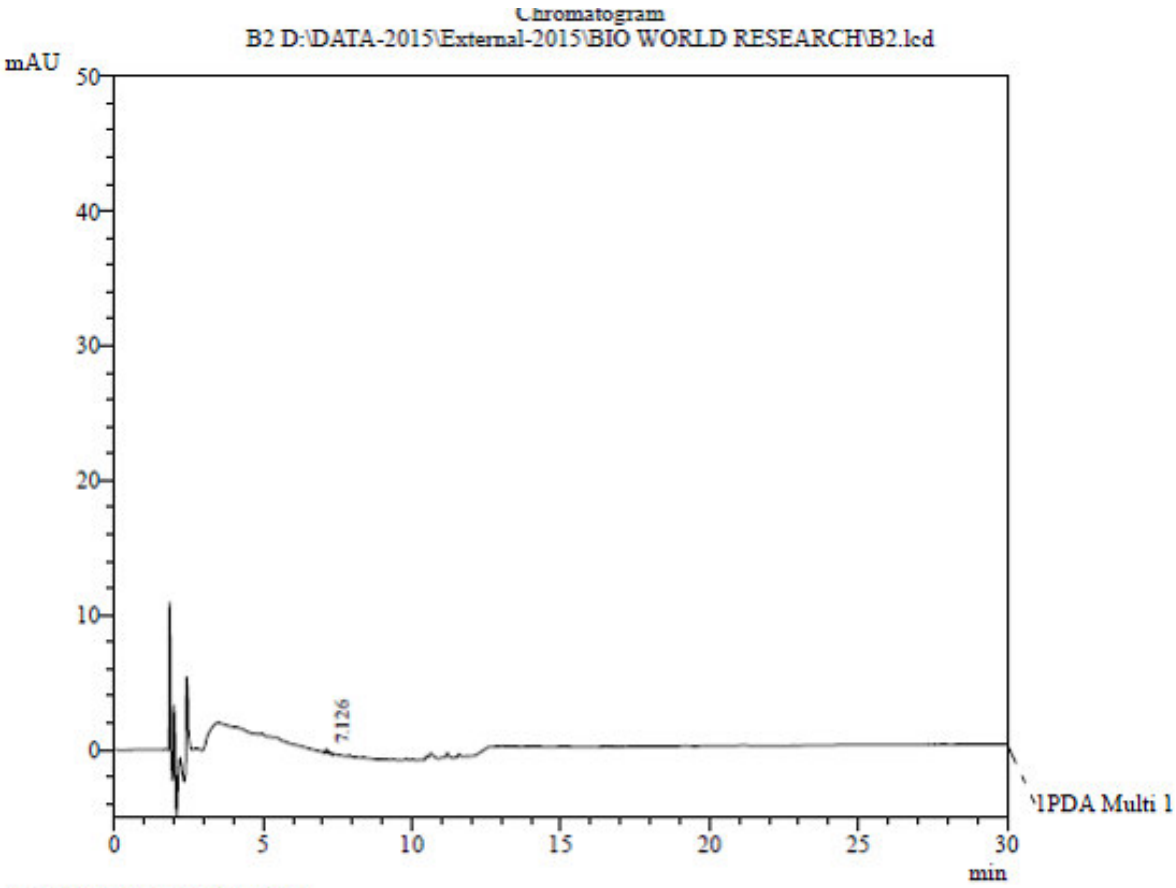


PeakTable

PDA Ch1 270nm

Peak#	Ret. Time	Area	Theoretical Plate#	Relative Retention Time	Area %
1	7.124	1579	29094.885	1.000	100.000
Total		1579			100.000

Figure 12
Sample A treated with immobilized laccase



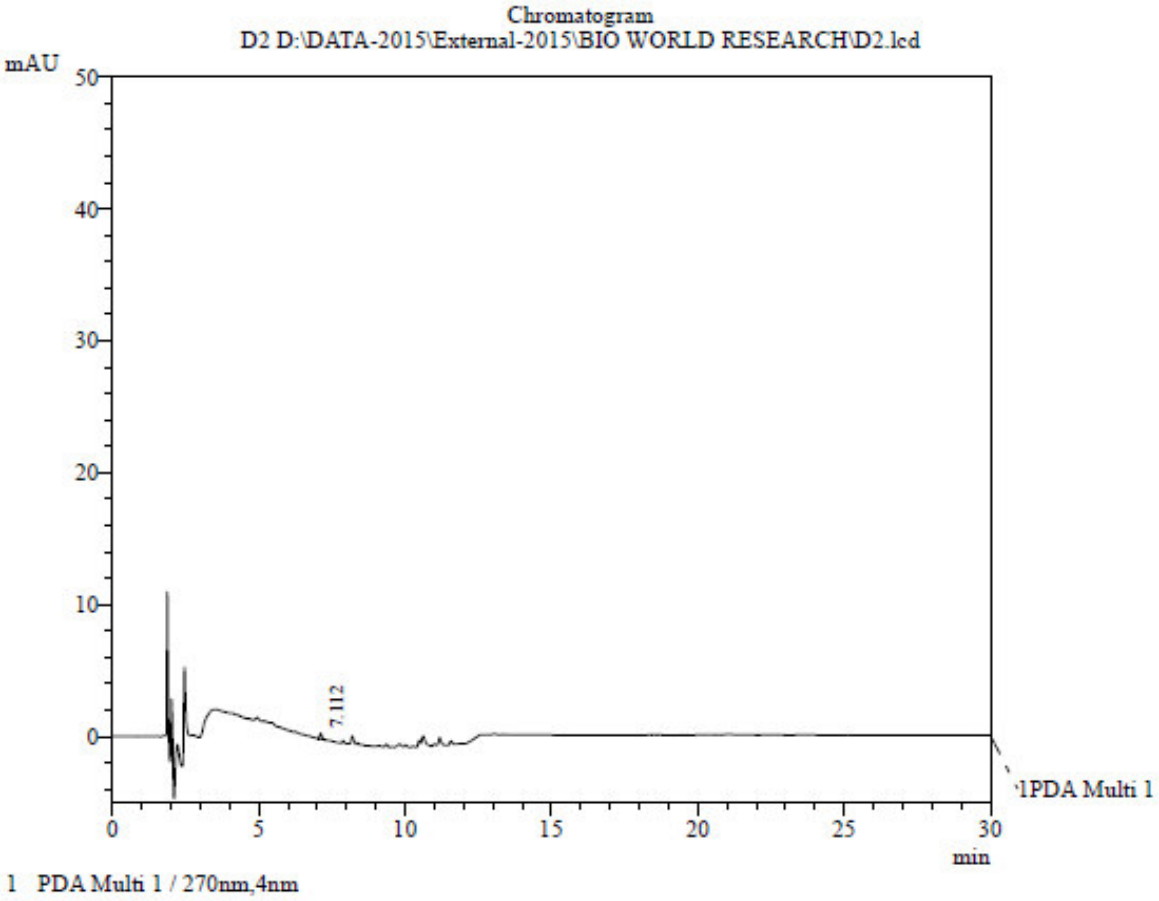
1 PDA Multi 1 / 270nm,4nm

PeakTable

PDA Ch1 270nm

Peak#	Ret. Time	Area	Theoretical Plate#	Relative Retention Time	Area %
1	7.126	1430	32400.455	1.000	100.000
Total		1430			100.000

Figure 13
Sample A treated with immobilized lip

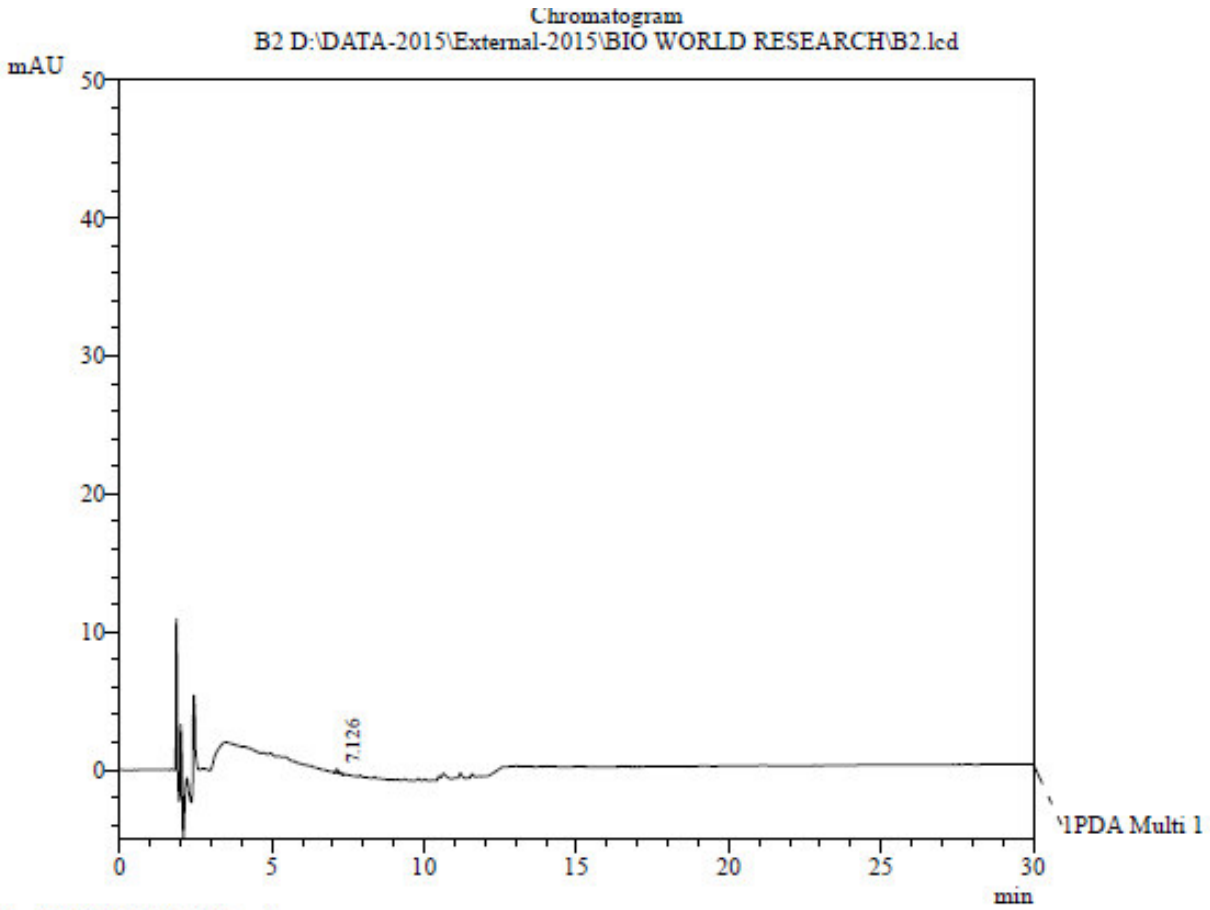


PeakTable

PDA Ch1 270nm

Peak#	Ret. Time	Area	Theoretical Plate#	Relative Retention Time	Area %
1	7.112	2323	30780.645	1.000	100.000
Total		2323			100.000

Figure 14
Sample B treated with immobilized laccase



PeakTable

PDA Ch1 270nm

Peak#	Ret. Time	Area	Theoretical Plate#	Relative Retention Time	Area %
1	7.126	1430	32400.455	1.000	100.000
Total		1430			100.000

Figure 15
Sample B treated with immobilized Lip

The results showed that control samples containing added amount of phenol not treated with the enzymes showed the same standard peak for phenol whereas the sewage samples incubated with the immobilized enzymes did not show phenol peak but other peaks which seem to indicate the degradation products of phenols. The appearance of similar peaks in the enzyme treated samples shows that both the enzymes have degraded the added phenol up to 99%.The results revealed that the controls showed the phenol standard peak and the phenol content obtained by area under the peaks of the samples were 0.04% and 0.03% in sample A by immobilized laccase and LiP respectively. Similar result was obtained in sample B which showed phenol content as 0.05% and 0.03% degraded by immobilized laccase and LiP respectively.

CONCLUSION

Immobilized Phanerochaete chrysosporium laccase and LiP retained 80% and 84% of their activities at pH 4.5 and 5.5 respectively compared to free enzymes. Immobilization has greatly increased the activities of pure enzymes and tolerance capacity of laccase against (cystein, EDTA, and Ag+) was also enhanced by up to 80% by immobilization. Although immobilized Laccase was active in the reaction mixture for three hours it degraded only 45% of the phenol in one hour where as immobilized LiP which remained active for one and half hour degraded 95% of phenol in the stipulated one hour time. When immobilized laccase was treated with divalent metal ions Ca^{++} and Cu^{++} the activity dramatically improved and it worked faster equal to immobilized LiP and degraded 96% phenol in one hour and remained active for 3 hours. Immobilization and addition of divalent cations have enhanced the activity of Laccase and made to as efficient as Lignin peroxidase.

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CONFLICT OF INTEREST: None

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