DECOLORIZATION APPLICABILITY OF SOL-GEL MATRIX-IMMOBILIZED LACCASE PRODUCED FROM *Ganoderma leucidum* USING AGRO-INDUSTRIAL WASTE

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With the extensive industrial applications of laccase in mind, this study was performed to investigate the decolorization applicability of sol-gel matrix-immobilized laccase. Indigenous *G. leucidum* laccase (592±6 U/mL) was purified 6.5-fold with a specific activity of 875 U/mg. The purified laccase had a molecular weight of 66 kDa, as evidenced by a single band on SDS-PAGE. Active laccase fractions were immobilized by entrapping in Sol-Gel matrix of trimethoxysilane (TMOS) and propyltetramethoxysilane (PTMS). A maximum of 90.7% immobilization efficiency was achieved with a purified fraction containing 2 mg/mL laccase. An activity profile revealed that immobilized laccase retained 78 to 80% of its original activity at a pH of 4 and a temperature of 80 °C, compared to free laccase. The tolerance capacity of laccase against inactivating agents (cystein, EDTA, and Ag^{+}) was also enhanced by up to 80% by sol-gel immobilization. To explore the decolorization applicability, the immobilized laccase was tested against four different textile industry effluents. After the stipulated reaction time (24 h), varying decolorization percentages of wastewater effluents (with a maximum of 97.3% decolorization) were achieved.

Keywords: G. leucidum; laccase; Sol-gel immobilization; Stabilization; Textile effluent; Decolorization

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INTRODUCTION

White rot fungi (WRF) have the capability to entirely degrade/mineralize lignin and phenolic organic substrates. This capability is due to the nonspecific and non-stereo-selective nature of their extracellular enzyme system that functions together with H_2O_2 and secondary metabolites. The aptitude of white rot basidiomycetes, including *Trametes versicolor*, *Phanerochaete chrysosporium*, *Ganoderma leucidum*, and some others, has been investigated from a physiological point of view to degrade lignin components, especially lignocellulosic agro-industrial waste materials (Wesenberg *et al.* 2003; Sadhasivam *et al.* 2008). These microorganisms produce two major families of enzymes, generally termed ligninolytic enzymes, *i.e.*, extracellular peroxidases (MnP, manganese peroxidase; MIP, manganese independent peroxidase; LiP, lignin peroxidase; and VP, versatile peroxidases), and phenol oxidases (laccases). Laccases and manganese peroxidases have the ability to oxidize phenolic/non-phenolic compounds and some toxic

environmental pollutants that create deleterious effects on the living environment, especially animals and humans (Wesenberg *et al.* 2003).

Over the past several years, there has been great interest among researchers in the production of ligninolytic- and cellulose-degrading enzymes from various agro-industrial waste materials and their byproducts, such as wheat straw, rice husk, banana waste, citrus peel, rice straw, corncobs, corn stover, apple pomace, and sugar cane bagasse (Iqbal *et al.* 2011b; Asgher and Iqbal 2011; Irshad *et al.* 2011). These wastes are not fully discarded in developing countries and have become a major source of ecological pollution. Recently, MnP and laccases have been applied to various 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 (Stoilova *et al.* 2010; Asgher *et al.* 2012). Significant efforts have been made to convert lignocellulosic residues to valuable products with the help of ligninolytic enzymes (LiP, MnP, and laccase) from WRF, many of which have been successful.

Industrial textile wastewater effluents contain several types of chemicals, including dyes that are toxic, carcinogenic, and dangerous for the living ecosystem. Such wastewater effluents are being discharged into water streams by textile industries without or after only partial treatments, causing water pollution and negatively affecting the aquatic life. Among the various major environmental concerns, the treatment of textile wastewater effluents is one of them. WRF has the ability to degrade contaminants by virtue of the nonspecific nature of its extracellular ligninolytic enzyme system (lignin peroxidases, manganese peroxidases, and laccases) (Nyanhongo *et al.* 2002). Therefore, biological treatment processes provide an alternative to existing physico-chemical water purifying technologies because they are more cost effective, eco-friendly, and can be applied to a wide range of dye-containing industrial wastewater effluents.

The immobilization technique has modernized the field of enzyme biotechnology because it offers a noteworthy solution that can meet the industrial and environmental challenges mainly including agro-waste management, textile effluents decolorization, alternatives of health hazardous chemical based procedures, and industrial applicability of enzymes. According to published reports, various techniques, such as chemical engineering modification, mutation, gel entrapment, and surface binding, have been adopted for enhancing the operational stability of enzymes (Iqbal *et al.* 2012; Nwagu *et al.* 2012). Among these techniques, gel entrapment is preferred because of its ability to produce enzymes in defined thin films that are thermo-stable and have the ability to catalyze reactions under mild environmental conditions (Iqbal *et al.* 2012).

Among the various potent lignin-degrading microorganisms, *Ganoderma leucidum* is one that can produce abundant extracellular peroxidase and phenol oxidase that are suitable for a wide range of environmental applications. Therefore, in this article, *G. leucidum* 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 textile industrial dye-containing waste effluents.

EXPERIMENTAL

Fungal Culture, Chemicals, and Agro-Industrial Substrate

A pure culture of locally isolated white rot fungal strain *G. leucidum* was available in the Molecular Biotechnology Laboratory, University of Gujrat, Pakistan, and was used for the present study. 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), polyvinyl alcohol, trimethoxysilane (TMOS), and propyltetramethoxysilane (PTMS) and all other chemicals were of analytical laboratory grade and used as such. The lignocellulosic agricultural waste peanut shells were collected from the local market in Gujrat, Pakistan. To avoid free moisture, the substrate was oven dried (60 °C), ground to fine particle size, and stored in airtight plastic jars. For decolorization studies, four different textile industry effluents were collected onsite from local textile industries in Faisalabad, Pakistan.

Fungal Spore Suspension Development

To develop homogeneous fungal spore suspension (10^7-10^8 spores/mL), *G. leucidum* was cultivated at 30±1 °C for 5 days in an Erlenmeyer flask (250 mL) containing a basal salt medium. The main constituents of the media were: $(NH_4)_2SO_4$, 10 gL^{-1}; KH_2PO_4, 4 gL^{-1}; $MgSO_4 \cdot 7H_2O$, 0.5 gL^{-1}, and $CaCl_2$, 0.5 gL^{-1}. Before fungal inoculation, the medium was sterilized at 121 °C and 15 lb/in^2 pressure in a laboratory scale autoclave for 15 min (Iqbal *et al.* 2011a).

Laccase Production, Extraction Protocol

The production of laccase by *G. leucidum* was carried out in 500 mL Erlenmeyer flasks using peanut shell waste under optimized growth conditions. Erlenmeyer flasks containing growth medium were autoclaved (120 °C) and inoculated with 5 mL of freshly prepared homogeneous fungal spore suspension in triplicate. The inoculated flasks were kept at 30 °C in a temperature-controlled still culture incubator for the optimum fermentation time period (4 days). Extracellular laccase was extracted by adding 100 mL of distilled water to the 4 day fermented solid-state cultures, followed by shaking at 120 rpm for 30 min (Iqbal *et al.* 2011a).

The contents were filtered using Whatman No.1 filter paper and the filtrates were centrifuged at 3,000×g for 10 min to get clear and biomass cell debris free supernatant. The resulting supernatants were collected and used as crude enzyme extract for laccase activity estimation, purification, and sol-gel immobilization.

Laccase Activity and Protein Contents Estimation

The crude enzyme extract obtained was used for extracellular laccase activity estimation by adopting the UV/Visible spectrophotometric method as described earlier (Asgher *et al.* 2012). The recorded activities were expressed as U/mL, while one unit of laccse activity was defined as the amount of enzyme forming 1 μmol of $ABTS^{\bullet+}$ per min. 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). 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 (Asgher *et al.* 2012).

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

pH and thermal stability of laccase

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

Decolorization of Textile Industry Effluents by Immobilized Laccase

To investigate the decolorization applicability of sol-gel-entrapped laccase, four different textile industry wastewater effluents were collected from Magna, Crescent, Arzoo and Chenab textiles in Faisalabad. 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 textile effluent with 1 mL of 1 mM ABTS as laccase

mediator, and incubated in a temperature-controlled shaker (120 rpm) for up to 24 h reaction time period. After every 6h reaction time, samples were collected from each flask to determine the percentage enzymatic removal of textile effluent 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 λmax (590 nm) in order to determine the percent decolorization. Percent decolorization of effluents was calculated by,

$$\% \text{ Decolorization} = 100 \times \frac{(A_{ini} - A_{fin})}{A_{ini}} \quad (1)$$

where A_{ini} is the initial absorbance of dye before incubation and A_{fin} is the final absorbance of dye after incubation.

Statistical Analysis

All the experimental data were statistically evaluated using the statistical software Minitab, version 15, and performed in triplicate. The means and standard errors of means (mean ± S.E.) were calculated for each treatment and used to draw figures.

RESULTS AND DISCUSSION

Production of Laccase

A large amount of indigenous laccase was obtained from locally isolated white rot fungal strain *G. leucidum* from solid-state bioprocessing of peanut shells under optimized fermentation growth conditions. Maximum laccase activity (592±6 U/mL) was attained when peanut shell was supplemented with 2% fructose, inoculated with 5 mL of freshly prepared spore suspension of *G. leucidum*, and incubated at 30 °C. The nature and amount of the available nutrients/microelements in the fermentation growth substrate had a strong effect on extracellular ligninolytic enzyme production. *G. leucidum* produced higher level of laccase activity (592±6 U/mL) in 4 days as compared to other reported WRF strains producing significantly lower laccase activities after 10 and 17 days, respectively (Sack et al. 1997; Levin et al. 2003). Stajić *et al.* (2010) noted maximum laccase activity (181.4 U/L) in the crude extract of *G. lucidum* HAI 447.

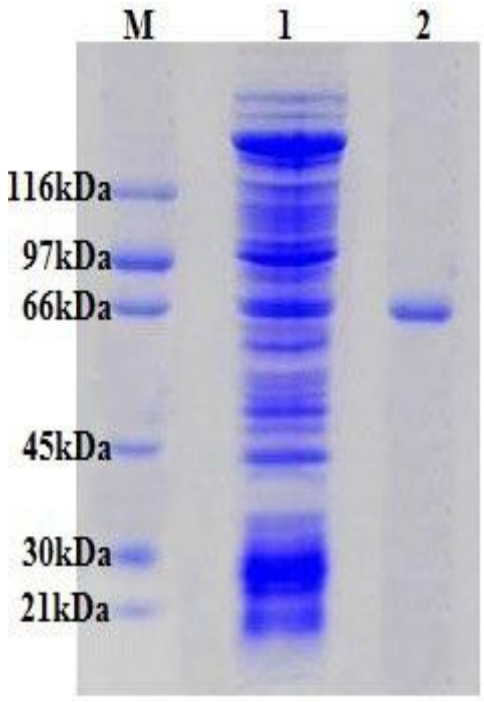
Purification of Laccase

The crude cell free supernatant enzyme solution was subjected to partial purification by ammonium sulfate fractionation with unit activity and specific activity of 118400 U/200 mL and 135 U/mg, respectively. After gel filtration through a Sephadex G-100 column, the laccase was maximally purified to a homogeneity level up to 6.5-fold with a specific activity of 875 U/mg (Table 1). In an earlier study, Mtui and Nakamura (2008) achieved 50 to 80% $(NH_4)_2SO_4$ saturation followed by chromatographic purification for the recovery of pure ligninolytic enzymes. Iqbal *et al.* (2011b) also successfully used the gel filtration chromatographic technique for the purification of

fungal cellulase. 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 66 kDa on SDS-PAGE (Fig. 1), which is within the range of the laccase family (Asgher *et al.* 2012).

Table 1. Purification Summary of *G. leucidum* Laccase

No.	Purification Steps	Volume (mL)	Enzyme Activity (U)	Protein Content (mg)	Specific Activity (U/mg)	Purification Fold	% Yield
1	Crude Enzyme	200	118400	875	135	1	100
2	$(NH_4)_2SO_4$ Precipitation	25	15500	105	148	1.1	13.1
3	Dialysis	20	13500	62	218	1.6	11.4
4	Sephadex-G-100	13	9620	11	875	6.5	8.1



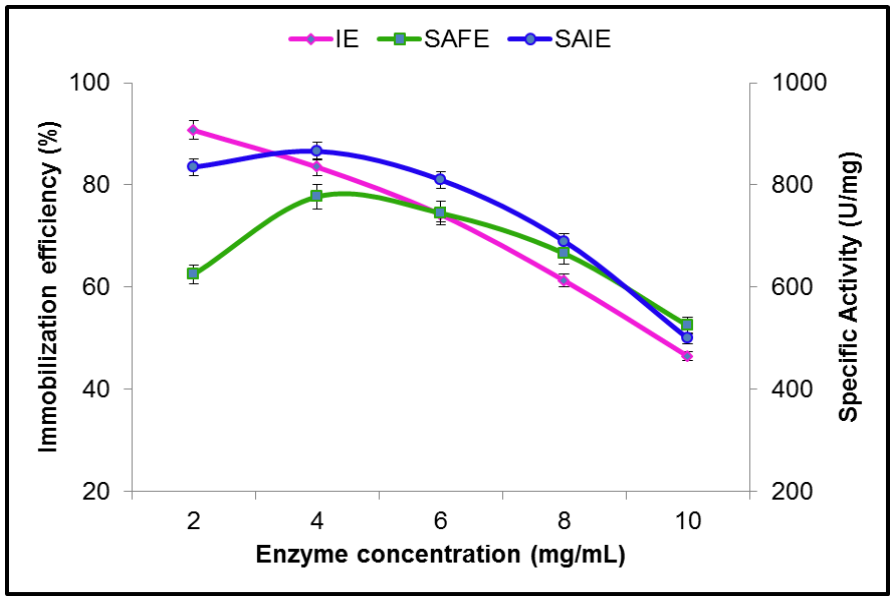
[(Lane M, Molecular weights in kDa of standard marker; lane 1, Crude extract; lane 2, Purified laccase (66kDa)]

Fig. 1. Molecular mass determination of purified laccase by SDS-PAGE

Immobilization of Laccase

Immobilization efficiency was studied relative to enzyme concentration over the range of 2 to 10 mg/mL purified laccase. Figure 2 reveals that the trial fraction with an enzyme concentration of 2 mg/mL showed maximum immobilization efficiency (90.7%). 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 fraction was higher than the purified free laccase. 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.



[IE, Immobilization efficiency; SAFE, specific activity of free enzyme; SAIE, specific activity of immobilized enzyme], Specific activities were calculated in U/mg of triplicate means while, Immobilization efficiency was calculated as the ratio of the enzyme entrapped (difference between the enzyme loaded and the enzyme in the supernatant after washing) × 100

Fig. 2. Immobilization efficiency and specific activities of free and immobilized laccase

Characterization of Free and Sol-Gel Matrix-Entrapped Laccase

pH and thermal stability of laccase

A residual pH activity profile showed that immobilization slightly shifted the pH optima toward more acidic, *i.e.* 4, compared to the purified laccase that was initially pH 6. Results of the stability assay showed that free enzyme was only stable for 1 h in a pH range of 5.0 to 7.0, but started steadily losing much of its activity at pH values higher or lower than this range. Gel entrapment enhanced the laccase pH stability for up to 4 h (Fig. 3 A, B). The slight shift in pH optima after immobilization might be due to the buffering effect of the carrier surface, as was also reported earlier in the literature by Erdemir *et al.* (2009), who observed a slight pH shift from 7 to 6 for lipase.

A thermal stability profile revealed that purified free laccase was optimally active at 55 °C. At higher temperatures (up to 80 °C), free laccase was only 18% active, while sol-gel-entrapped laccase showed better thermo-stability and retained almost 73% of its initial activity at 80 °C after 4 h incubation (Fig. 4 A, B). High thermo-stability of an enzyme is an attractive and required characteristic for a variety of industrial applications

(Iqbal *et al.* 2011b). Immobilization in a solid support secures the enzyme structure and creates increased stability against high temperatures in comparison to the free enzyme structure that may be altered by high temperatures.

Most of the previously reported research on fungal laccase found that it lost activity rapidly at temperatures around 60 °C or higher. According to Nagai *et al.* (2002), most of the WRF laccases are stable and almost fully active in a temperature range of 30 to 50 °C. To overcome this crucial drawback for industrial application, the present sol-gel matrix-immobilized laccase showed noteworthy thermo-stability, as it retained almost 73% of its original activity at 80 °C for up to 4 h, suggesting its potential for biotechnological applications.

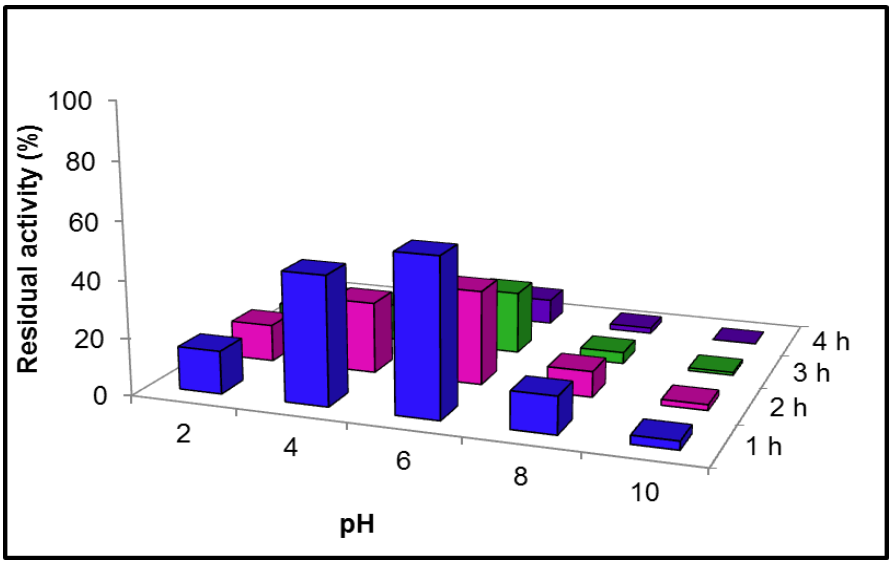


Fig. 3A. Effect of different pH on stability of free laccase

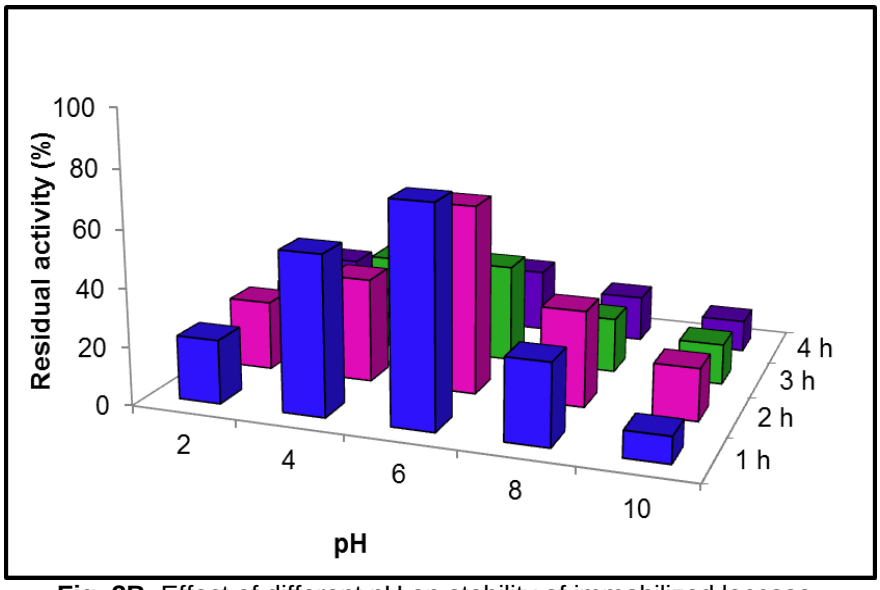


Fig. 3B. Effect of different pH on stability of immobilized laccase

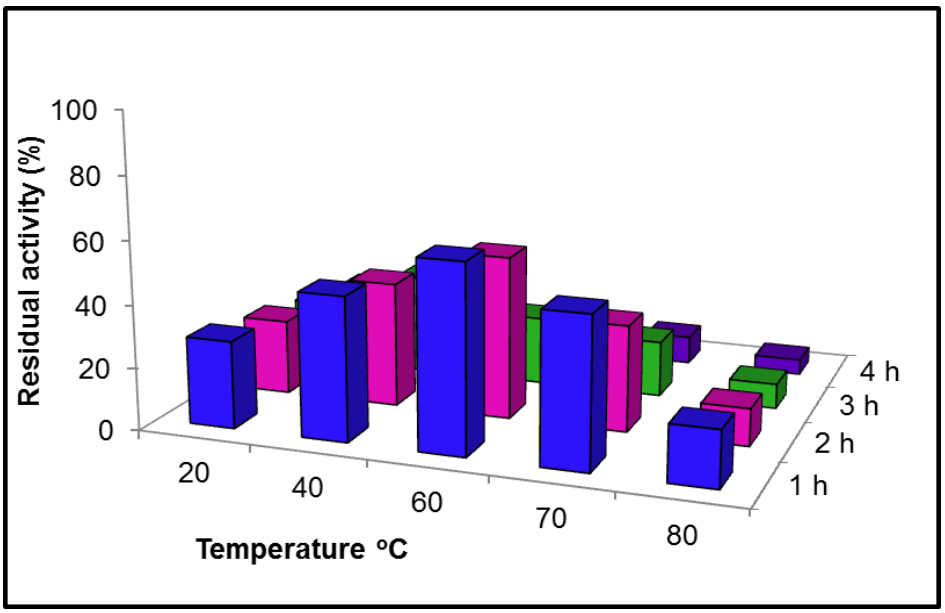


Fig. 4A. Effect of different temperatures on stability of purified free laccase

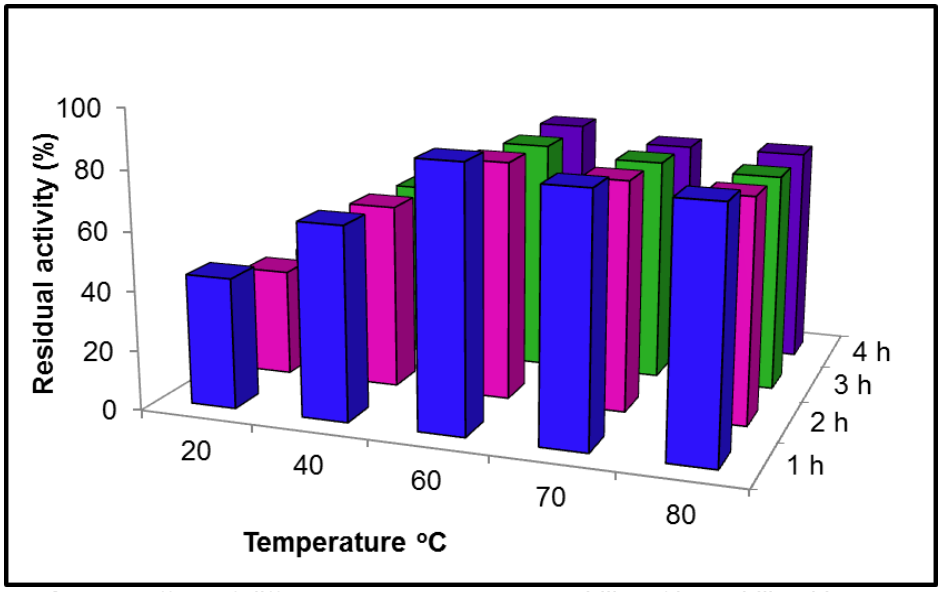


Fig. 4B. Effect of different temperatures on stability of immobilized laccase

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 *G. leucidum* laccase are being investigated for the first time in this article. Results showed that free laccase was almost completely inhibited by cystein and to a certain extent with EDTA. Among the metal ions used, Ag+ caused complete inhibition, whereas *G. leucidum* laccase was fully stimulated by Cu^{2+} and Fe^{2+} and retained 100% of its original residual activity (Fig. 5). The sol-gel matrix-entrapped laccase was observed to have a higher tolerance than free enzyme against inactivation by cystein, EDTA, and Ag+ (residual activity of immobilized laccase increased from 7 to 36% for cystein, from 28 to 75% for EDTA, and

from 12 to 48% for Ag^{+}). EDTA has the ability to form a complex with inorganic active groups of enzymes and therefore an inhibitory effect was revealed because of its metal chelating ability (Asgher *et al.* 2012). This phenomenon has also been reported by Sadhasivam *et al.* (2008) and Asgher and Iqbal (2011). In another study, Jung *et al.* (2002) reported L-cysteine and dithiotheitol as effective inhibitors of *Trichophyton rubrum* LKY-7 laccase. In this article, and for the first time, characterization of the purified and sol-gel-immobilized laccase from *G. leucidum* has been investigated in the presence of various activators/inhibitors.

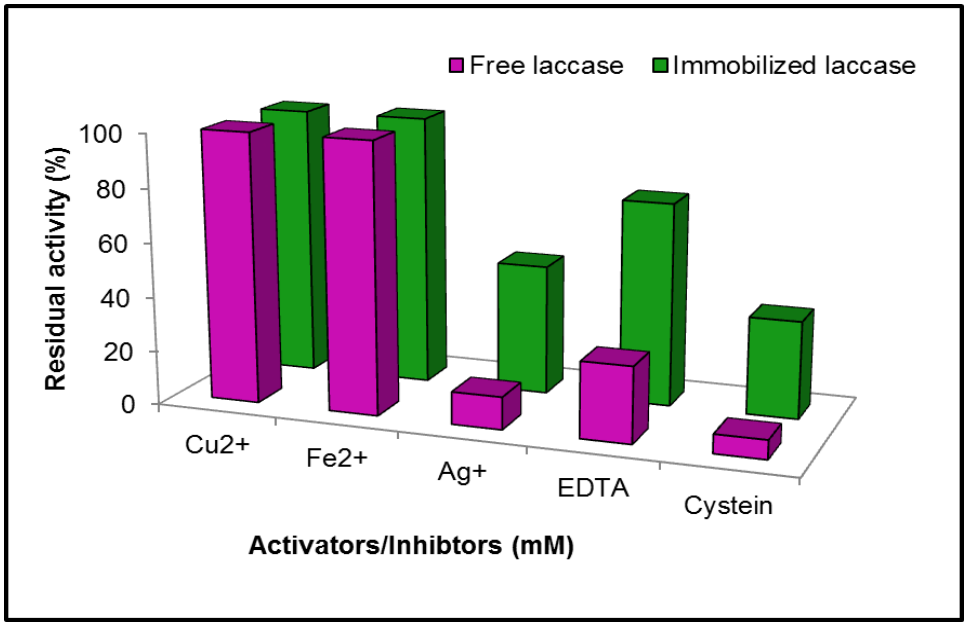


Fig. 5. Effect of different activators/inhibitors on free and immobilized laccase

Decolorization of Textile Industry Effluents by Immobilized Laccase

After 24 h reaction time under continuous shaking culture conditions, the sol-gel-immobilized enzyme mixture was found to maximally decolorize the Magna textile effluent to 97.3%, followed by Crescent textile (91.5%), Arzoo textile (85%), and Chenab textile (77.8%) industry effluents, as shown in Fig. 6. The effluent decolorization increased with an increase in reaction time. At the end of 24h reaction time, maximally decolorized effluent samples were analyzed at the wavelength of maximum absorbance at λmax (590 nm) by UV-Vis spectrophotometric spectrum (Fig. 7). WRF grown in synthetic textile dye solutions and industrial effluents takes more time to decolorize dyes, compared to isolated enzymes, because of the necessary lag phase before they grow and secrete ligninolytic enzymes for dye degradation. In a previous study, *Coriolus versicolor* was found to decolorize Arzoo textile industry effluents to 84% in 3 days (Asgher *et al.* 2009). The variation in effluent composition is also responsible for the difficulty of its decolorization by enzyme extracts from different fungi (Maas and Chaudhari 2005). The present sol-gel matrix-entrapped laccase seems to have potential capabilities to meet the challenges of the modern industrial sector, especially for bioremediation in the textile industry.

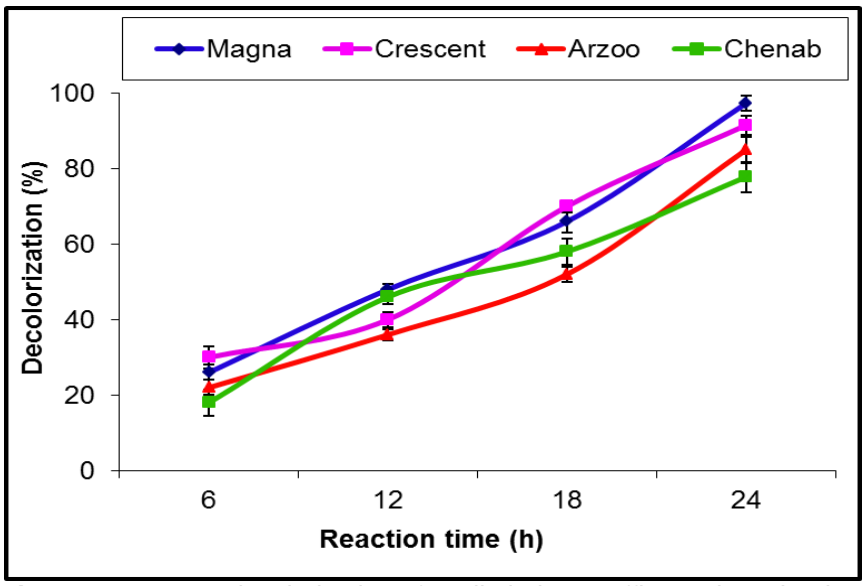


Fig. 6. Percentage decolorization of textile industry effluents by sol-gel-immobilized *G. leucidum* laccase

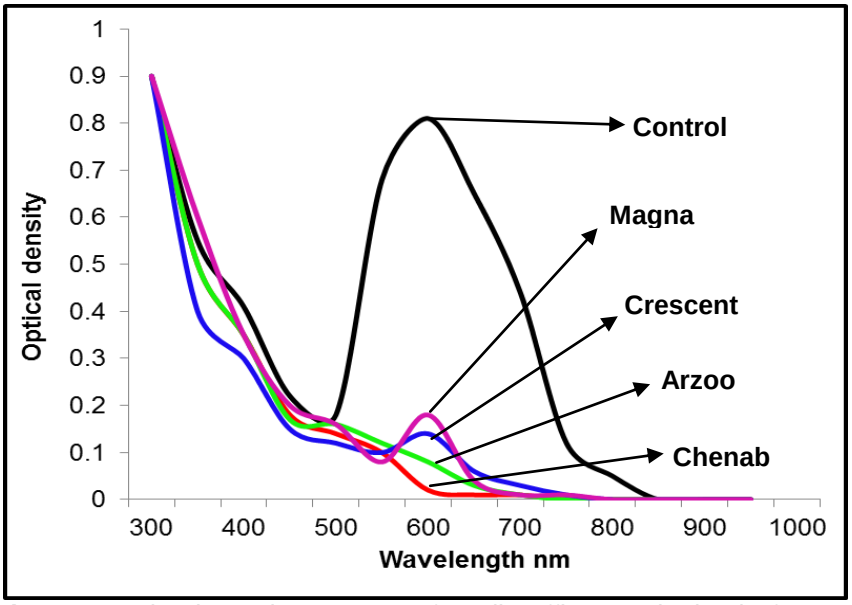


Fig. 7. UV-Vis absorption spectra of textile effluents obtained after 24h treatment with *G. leucidum* laccase

CONCLUSIONS

1. In this article and for the first time, *G. leucidum* laccase was immobilized by entrapping it in a sol-gel matrix.
2. Isolation of novel *G. leucidum* laccase was accomplished using inexpensive, lignin-based, solid waste peanut shells.

3. Immobilized *G. leucidum* laccase displayed enhanced tolerance against high temperatures and inactivating agents.
4. Sol-gel matrix seems to be a promising immobilization tool for enzyme engineering.
5. Maximum decolorization of textile wastewater effluents was achieved within a 24 h time period.

ACKNOWLEDGMENTS

The authors are grateful to the Department of Biochemistry and Molecular Biology, University of Gujrat, Pakistan for providing financial support and laboratory facilities.

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Article submitted: June 18, 2012; Peer review completed: July 11, 2012; Revised version received and accepted: July 18, 2012; Published: July 24, 2012.