World J Microbiol Biotechnol (2008) 24:1215–1222
DOI 10.1007/s11274-007-9598-x

ORIGINAL PAPER

Immobilization of laccase from *Streptomyces psammoticus* and its application in phenol removal using packed bed reactor

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Received: 5 September 2007 / Accepted: 30 October 2007 / Published online: 15 November 2007

Abstract Laccase was produced from *Streptomyces psammoticus* under solid-state fermentation. The enzyme was partially purified by ammonium sulphate precipitation and was immobilized in alginate beads by entrapment method. Calcium alginate beads retained 42.5% laccase activity, while copper alginate beads proved a better support for laccase immobilization by retaining 61% of the activity. Phenol and colour removal from a phenol model solution was carried out using immobilized laccase. Batch experiments were performed using packed bed bioreactor, containing immobilized beads. Reusability of the immobilized matrix was studied for up to 8 successive runs, each run with duration of 6 h. The system removed 72% of the colour and 69.9% of total phenolics from the phenol model solution after the initial run. The immobilized system maintained 50% of its efficiency after eight successive runs. The degradation of phenolic compounds by immobilized laccase was evaluated and confirmed by Thin layer chromatography and nuclear magnetic resonance spectroscopy.

Keywords ^{1}H NMR · Immobilization · Laccases · Phenol degradation · *Streptomyces psammoticus* · Thin layer chromatography

Introduction

Environmental pollution has become the subject of much research in the recent years. The expanding scenario of industrialization inevitably poses some kind of harmful effect on nature, especially to the water bodies. Aromatic compounds, including phenols are one of the major pollutants of the aquatic ecosystem and these compounds are found in the effluents of a wide variety of industries including coal conversion, petroleum refining, resins and plastics, wood preservation, metal coating, dyes and other chemicals, textiles, and pulp and paper (Nicell et al. 1993). Removal of phenols from industrial water effluents is an important practical problem, since many of these compounds are toxic and their presence in drinking and irrigation water is a health hazard. Tremendous efforts have been taken world wide to minimize the pollution of water bodies by phenolic effluents.

The removal of phenolics from waste water is achieved either by physico-chemical processes or by biological (enzymatic) processes of which the enzymatic treatment is gaining more prevalence. There is a growing recognition that enzymes can be used to target specific pollutants for treatment (Karam and Nicell 1997). The current trend in enzyme research is to develop methods to increase the efficiency of the enzymes used for different applications, including waste water treatment. Enzyme immobilization is one such technique that has attracted the attention of enzymologists globally. Immobilized enzymes are generally preferred over free enzymes for many of the application purposes due to its potential advantages like increased stability and reusability. Enzymes are immobilized on solid matrices by different methods. The chemical immobilization includes attachment of enzyme to matrix by either covalent bonds or cross-linking, while the physical method involves the entrapment of enzyme molecules within different types of matrices (Duran et al. 2002).

The enzymatic removal of phenols from the effluents is effected with the use of oxidative enzymes like peroxidases,

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laccases and tyrosinases. These enzymes catalyze the oxidative coupling of phenolic compounds resulting in the formation of water insoluble oligomeric and polymeric products, which are then removed by sedimentation or filtration (Shuttleworth and Bollag 1986; Dec and Bollag 1990). Laccases (E.C.1.10.3.2.) are the most reliable enzyme for phenol removal and large numbers of studies have been carried out on fungal laccases on this aspect (Duran et al. 2002; Hublik and Schinner 2000; D'Annibale et al. 2000). Laccases are multicopper phenol oxidases that catalyze the one-electron oxidation of four reducing-substrate molecules concomitant with the four-electron reduction of molecular oxygen to water (Piontek et al. 2002). These enzymes were known to catalyze the oxidation of a wide range of phenolic compounds and aromatic amines (Thurston 1994). Because of their relative non-specificity, laccases induce the cross-coupling of pollutant phenols with naturally occurring phenols and they can also oxidize phenolic compounds to their corresponding anionic free radicals which are highly reactive (Bollag et al. 1988).

Removal of phenolics and other aromatic compounds using fungal laccases immobilized on different matrices has been very well established. (Davis and Burns 1990; Hublik and Schinner 2000; Crecchio et al. 1995). However, the fungal laccases, which are mostly acidic in nature, could not be used universally for the treatment of effluents from different industries, largely due to the alkaline nature of the effluents. Under such situations the enzymes from actinomycetes with wide pH range play a better role than the fungal counterparts. In this paper we report, for the first time, the immobilization of an alkaline-stable laccase from *Streptomyces psammoticus* by alginate entrapment method. The efficiency of the immobilized system was evaluated by monitoring the phenol and colour removal from a phenol model solution. The reusability of the immobilized system was also evaluated by performing batch runs in packed bed reactor. The degradation of phenols by laccase was verified with the help of ^{1}H NMR and thin layer chromatography techniques.

Materials and methods

Microorganism and inoculum preparation

Streptomyces psammoticus MTCC 7334 used for the present study is an aerobic filamentous bacterium isolated from a mangrove swamp (Niladevi and Prema 2005). The culture was grown and maintained on starch casein agar slants and subcultured regularly. One-week old fully grown slants were used for inoculum preparation. The culture was aseptically transferred to the inoculum medium (Niladevi et al 2007) and was allowed to grow in the medium for 48 h and used as the inoculum.

Laccase production by Solid-state fermentation (SSF)

Five grams of rice straw were added to a 250 ml Erlenmeyer flask and was moistened with a salt solution containing (gl^{-1}): yeast extract—3.5, $(NH_4)_2SO_4$—0.2, Mg SO4—0.2, $CaCO_3$—0.04, $CuSO_4$—0.002. The initial moisture level in the substrate was adjusted to 65% by adding an adequate quantity of distilled water. After sterilization by autoclaving at 121°C for 45 min, the medium was cooled to room temperature and inoculated with 1.75×10^7 CFU of inoculum and incubated at 32°C for 48 h. The fermented material was extracted with distilled water to get a final extraction volume of 100 ml. The contents were mixed thoroughly by keeping the flasks on a rotary shaker at 200 rpm for 1 h. The mixture was centrifuged at 10,000 rpm for 20 min at 4°C.

Partial purification of laccase

The supernatant obtained after centrifugation was concentrated by fractionated precipitation between 30 and 60% $(NH_4)_2SO_4$ saturation. After centrifugation the precipitate was resuspended in minimal quantity of 50 mM phosphate buffer, pH 7.0 and extensively dialyzed against the same buffer and used for immobilization studies. Laccase activity of the dialyzed sample was measured by monitoring the oxidation of 500 µM ABTS (2, 2′-azino-bis-[3-ethyl benzothiazoline-6-sulphonic acid]) (SIGMA) buffered with 0.2 M sodium phosphate buffer (pH 7.5) at 420 nm for 1 min (Bourbonnais and Paice 1990). The reaction mixture (3 ml) contained 1 ml of culture filtrate. One unit of enzyme activity was defined as 1 µM of ABTS oxidized per min. To calculate enzyme activity an absorption coefficient of 3.6×10^4 M^{-1} cm^{-1} was used.

Immobilization of laccase in alginate beads

Laccase (100 U) was added to sodium alginate solution (2.5%) and mixed thoroughly by mild shaking on a rotary shaker. The viscous alginate-enzyme mixture was taken in a syringe fitted with a luer-lock needle and the solution was extruded drop by drop from the syringe into a 0.05 M solution of $CaCl_2$ or $CuSO_4$ under magnetic stirring to produce calcium and copper alginate beads respectively. The beads were kept for hardening at 4 °C for two h. After two h, the beads were filtered and washed thoroughly until there was no detectable protein in the wash out solution. The hardening (curing) solution

and washings were collected to calculate unbound enzyme activity.

Optimization of immobilization conditions

Attempts were made to optimize the conditions for laccase immobilization in alginate beads. The crucial parameters like concentration of sodium alginate, $CaCl_2$ and $CuSO_4$ were varied and subjected to study.

Activity measurements

The laccase binding efficiency of the immobilized system was calculated using the following equation:

$$\text{Laccase binding} = \frac{A - B}{A} \times 100$$

where A = Added enzyme (U) and B = Unbound enzyme (U)

Reactor studies using immobilized enzyme

Removal of phenols from the sample was carried out by performing batch studies in packed bed column bioreactor. The immobilized beads were loaded in a glass column (50 cm × 2 cm), leaving a headspace of 5 cm. A phenol model solution was prepared by mixing different phenolic compounds such as gallic acid, tannic acid, ferulic acid, resorcinol, guaiacol, catechol, vanillic acid and pyrogallol. The compounds were selected owing to the fact that they represent the common phenolic compounds in the industrial effluents, especially from paper industry and coir retting industry. The phenol model solution was passed through the column using a peristaltic pump (Gilson Minipuls 3, France) at a flow rate of 1 ml/min for 6 h and the treated sample collected at the outlet was subjected to total phenol estimation at regular intervals and was compared with the phenol content of the sample solution. The column temperature was maintained at 30 ± 2°C. Colour removal from the treated samples was monitored at 280 nm in a UV-visible spectrophotometer (Shimadzu UV-2401 PC) and compared with the untreated sample solution.

Total phenol estimation

Total phenol estimation of the untreated and treated samples was done by the method of Singleton and Rossi (1965). The total phenolic content of the samples was expressed as milligram of gallic acid equivalents (GAE) per milliliter of the sample. Phenol concentration of the untreated sample was taken as 100% and % phenol removal was calculated from the phenol remaining in the treated sample after each treatment.

Thin layer chromatography (TLC)

TLC was performed on silica gel sheets of size10 × 20 cm (Merck, Germany). The phenolic compounds present in the untreated and treated samples were analyzed by comparison with the standards run simultaneously. The untreated sample was phenol model solution that contained tannic acid, gallic acid, ferulic acid, resorcinol and pyrogallol (1 mg ml^{-1} concentration). Other phenolic compounds like catechol, vanillic acid and guaiacol were omitted from the sample for TLC owing to the difficulties in developing all the compounds by the solvent system used. Chloroform: ethyl acetate: acetic acid (50:50:1) was used as the solvent system. Detection of the compounds was done by keeping the air dried TLC sheet in the chamber pre-equilibrated with iodine or by spraying with 1% solution of ferric chloride.

Nuclear Magnetic Resonance spectroscopy (NMR)

The samples for NMR spectroscopy were evaporated to dryness in a rotavapor (Buchi R-200, Switzerland) and then dissolved in appropriate NMR solvents. Deuterated methanol and $CDCl_3$ were used as the solvents for untreated and treated samples respectively. ^1H NMR spectra of the untreated and treated samples were recorded using 300 MHz Bruker Avance DPX Spectrometer. The number of scans for the untreated and treated samples was 16 and 48, respectively. Variation in the number of scans was unavoidable since the concentration of the treated sample was very low to elicit peaks to required intensity. In NMR, chemical shift δ is expressed in parts per million (ppm) by frequency. In the present study, scanning was done in the range of 0–10 ppm.

Results and discussion

Enzyme immobilization

Partially purified laccase from *S. psammoticus* was immobilized by physical entrapment in alginate beads. Alginate is a natural polymer and can easily be converted into hydrogels via cross-linking with divalent cations. In the present study two different cations such as calcium and copper were used for the preparation of calcium alginate

and copper alginate beads respectively. Calcium alginate is the most commonly used support for enzyme immobilization while copper alginate gels are used specifically for laccases (Palmieri et al. 1994). The formation and the mechanical and structural properties of alginate beads depend upon different parameters such as the alginate concentration, nature of the cations and concentration of the cations (Ouwerx et al. 1998).

Optimization of immobilization conditions

Figure 1 shows the effect of alginate concentration on the laccase binding efficiency of the immobilised beads. The laccase binding efficiency was maximum when the alginate concentration was 2.5% for both calcium and copper alginate beads, although the laccase binding efficiencies observed with copper alginate beads were higher than the calcium alginate beads. Low concentration of alginate resulted in beads with reduced strength and increased enzyme leaching. The beads were more transparent and the bead shape was also affected at lower concentration of alginate. Increasing the alginate concentration beyond 2.5% failed to enhance the laccase binding efficiency which indicated that 2.5% sodium alginate concentration was the optimum for good bead formation. The nature of the cation is one of the other crucial factors that determines the functional properties of the beads. Extensive studies have been carried out on the type of cations and their interaction with alginate during bead formation(Ouwerx et al. 1998; Rodrigues and Ricardo 2006). For the present study, concentrations of both the cations (Ca and Cu) were varied from 0.01–0.2 M and the effect was studied. In the case of calcium alginate beads, the maximum laccase binding efficiency was observed at 0.05 M $CaCl_2$ concentration while the copper alginate beads exhibited maximum laccase binding efficiency at 0.1 M $CuSO_4$ concentration (Fig. 2).

When the overall efficiency of the two immobilized systems were compared, copper alginate beads proved to be the better support for the immobilization of laccase, exhibiting higher binding efficiency (Fig. 3). This was probably because copper having a high affinity for alginates, was strongly complexed in the polymer network (Ouwerx et al. 1998). It has been reported that Ca alginate beads have higher porosity and lower chemical stability than Cu alginate which, resulted in higher enzyme leakage than Cu alginate beads (Brandi et al. 2006). The operational efficiency (efficiency at which the system performs) of any immobilized system depends upon the amount of enzyme retained by the system (laccase binding efficiency). Hence, we chose copper alginate beads for further studies.

Reactor studies using immobilized enzyme

The schematic diagram of the packed bed bioreactor system used in the study is shown in Fig. 4. Batch experiments were performed in the bioreactor that contained laccase-immobilized Cu alginate beads. Figure 5 shows the removal of phenols and colour from the phenol model solution in a batch experiment run for 6 h. It was observed that 95% of the total phenols and 99% of the colour was removed from the system at the initial stage of 30 min. The extent of phenol removal and colour reduction was maintained almost in the same level up to 180 min. The flow of phenol sample through the immobilized beads resulted in the formation of water insoluble aggregates in the packed

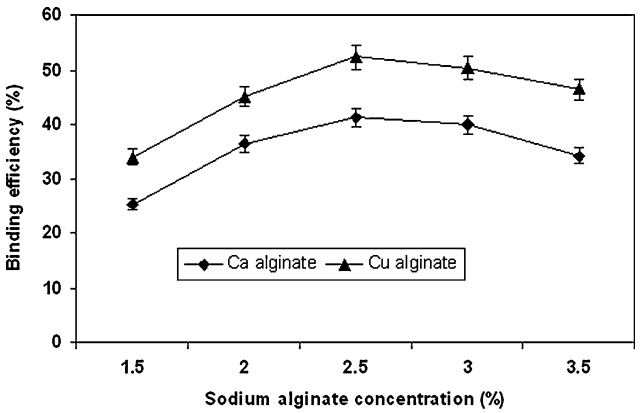

Fig. 1 Effect of sodium alginate concentration on laccase binding efficiency of calcium (♦) and copper alginate (▲) beads. Concentration of $CaCl_2$ and $CuSO_4$ was 0.05 M. Concentration of laccase was 100 U. Values presented are the mean from three independent experiments

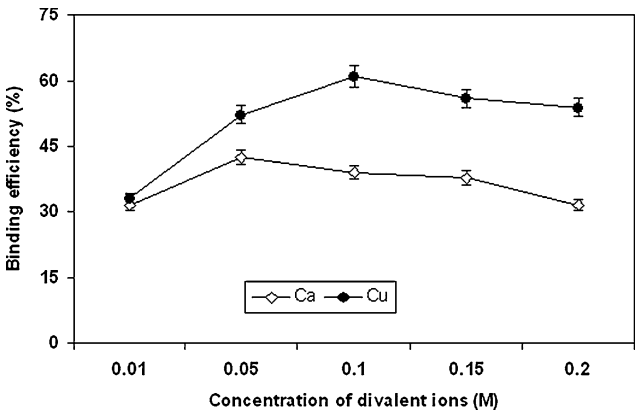

Fig. 2 Effect of calcium chloride (◇) and copper sulphate (●) concentrations on laccase binding efficiency of alginate beads. Concentration of sodium alginate and laccase were 2.5% and 100 U, respectively. Values presented are the mean from three independent experiments

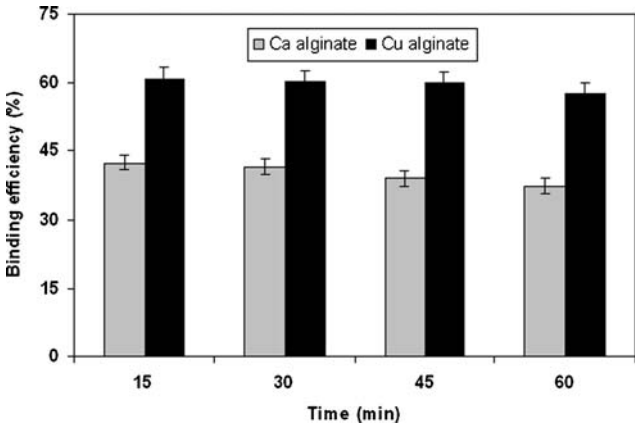

Fig. 3 Comparison of calcium alginate (□) and copper alginate (■) beads with respect to the laccase binding efficiency. Concentrations of sodium alginate and laccase were 2.5% and 100 U, respectively. Concentration of $CaCl_2$ and $CuSO_4$ were 0.05 M and 0.1 M, respectively. Values presented are the mean from three independent experiments

bed reactor. Precipitation of phenolic compounds as aggregates on reaction with phenol oxidases is a well-established phenomenon. (Dec and Bollag 1990). Formation of these polymerization products was an indication of typical laccase activity. These precipitates were easily separable from the beads by filtration after each catalytic cycle. At the end of the first run (6 h), phenol and colour removal was reduced to 70% and 72%, respectively.

The operational stability of the immobilized laccase was estimated by reusing the same matrix for successive runs. A total of eight runs, each with duration of 6 h were performed. The phenol model solution of same composition was used for the successive catalytic cycles and the result of the same is given in Fig. 6. Enzyme leaching has been reported as the major problem with alginate beads. However, in the present study it was observed that the system maintained 50% of operational efficiency even after eight successive runs. The result indicated that the enzyme leaching was prominent only during the initial run that reduced the phenol removal from 95% (at 30 min) to 70% (at 360 min). After the initial run, the enzyme leaching was relatively low, resulting in 50% operational efficiency after eighth run (48 h). This was probably due to the fact that the enzyme coated on the surface was leached out easily during the initial run while the enzyme entrapped deep within the alginate beads is retained relatively for long duration.

Degradation of individual phenolic compounds by laccases has been reported by many authors (Lante et al. 2000). However, the industrial effluents usually contain mixture of phenols in different proportions. Phenolic compounds are present in the wastewater generated from petroleum and petrochemical, coal conversion, pharmaceutical, plastic, rubber proofing, disinfectant, steel and phenol production industries (Nayak and Singh 2007). The use of co-immobilized laccases and tyrosinases has been suggested as a method to achieve the complete degradation of phenolic mixtures (Krastanov 2000). The results of our studies on phenol removal from phenol model solution (Figs. 5, 6) have proved that the laccase from *S. psammoticus* was active against a wide range of phenolic compounds. The effective degradation of phenolic compounds by laccase from *S. psammoticus* could be attributed mainly to its alkaline nature. The partially purified laccase was found to be stable at the alkaline pH range of 6.5–9.5 with pH optimum of 8.5, which was in good agreement with the pH of the phenolic solution used in the study (pH 9.2). In this respect the enzyme was different from the fungal laccases, most of which

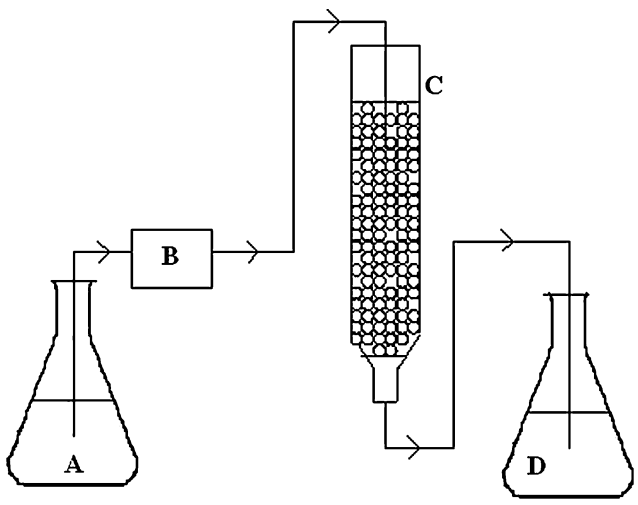

Fig. 4 Schematic representation of the packed bed bioreactor system for phenol removal using laccase immobilized on alginate beads. (**a**) Untreated sample (phenol model solution); (**b**) Peristaltic pump; (**c**) Column bioreactor packed with immobilized beads; (**d**) treated sample

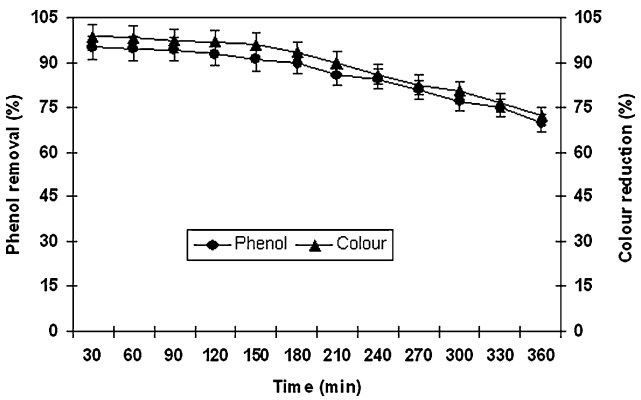

Fig. 5 Phenol (●) and colour (▲) removal from the phenol model solution in a batch experiment of 6 h duration. Values presented are the mean from three independent experiments

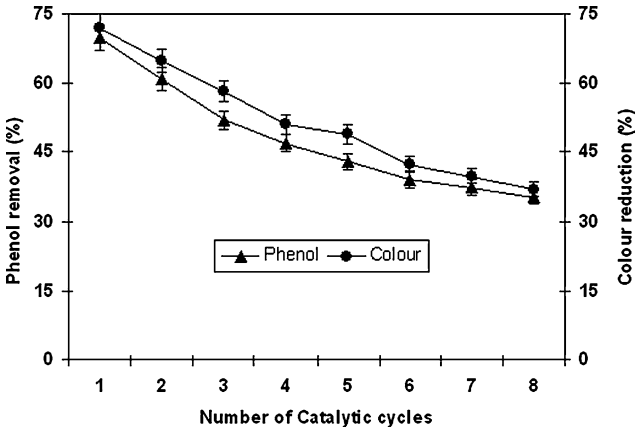

Fig. 6 Phenol (▲) and colour (●) removal from the phenol model solution over different catalytic cycles. The duration of each catalytic cycle was 6 h. Values presented are the mean from three independent experiments

are acidic in nature. The alkaline pH makes the enzyme suitable for use in the treatment of effluents from paper industries, which usually has alkaline pH (Calvo et al. 1998) and also for other industrial applications such as bio bleaching.

Thin layer chromatography (TLC)

The authenticity of the results was confirmed by thin layer chromatography technique (TLC). TLC was performed with a phenol model solution that contained five selected phenolic compounds such as pyrogallol, tannic acid, gallic acid, resorcinol and ferulic acid. Figure 7 shows the result of TLC. Lane 1 shows the untreated phenolic sample solution. Lane 2–6 corresponds to different phenol standards such as pyrogallol, tannic acid, gallic acid, resorcinol and ferulic acid respectively. The spots of resorcinol and ferulic acid appeared at the same region as it was evident from the figure (Lane 5 and 6) and hence it emerged as a single spot in the sample solution (Lane 1). No mobility was observed for tannic acid with the solvent system used in this study (Lane 3) and similar results using the same mobile phase has already been established (Singh et al. 2001). None of the phenolic compounds in the sample solution were observed in the treated sample (Lane 7) although; an unknown compound was detected in the treated sample. The disappearance of spots corresponding to phenolic compounds from the treated sample was a clear indication of phenolic compounds degradation. The unknown compound detected in the treated sample might be some breakdown product of phenolic degradation.

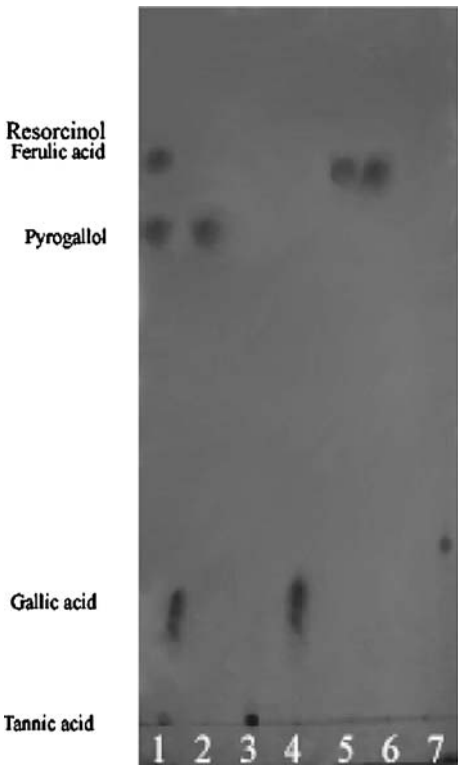

Fig. 7 TLC of untreated and treated phenolic solutions. Lane 1 indicates untreated phenolic sample (control), Lane 2–6 corresponds to standard phenolic compounds; pyrogallol, tannic acid, gallic acid, resorcinol and ferulic acid respectively. Lane 7 indicates the treated phenolic sample (test)

Nuclear magnetic resonance (NMR)

The results were further confirmed with the help of NMR spectroscopy. The ^{1}H NMR spectrum of the untreated sample solution (Fig. 8a) showed the presence of different peaks in the aromatic region (6–7.5 ppm), which corresponded to the different substituted phenols. It was obvious from the ^{1}H NMR spectrum of the treated sample (Fig. 8b) that the phenols in the sample solution have undergone significant degradation, which resulted in considerable chemical shifts in the aromatic region. The different peaks were visible in the aromatic region of the untreated sample, especially between 6.1 and 6.7 ppm (Fig. 8c) and these peaks disappeared in the treated sample (Fig. 8d); a strong indication of aromatic compounds degradation. The massive chemical shifts which occurred in the aromatic region indicated that the enzyme was active against a wide range of phenolic compounds.

Another significant difference in the spectra of treated and untreated samples were in the aliphatic region. There were no intensive peaks in the aliphatic region of the untreated sample except the solvent peaks (Fig. 8a), whereas the treated sample (Fig. 8b) showed large number

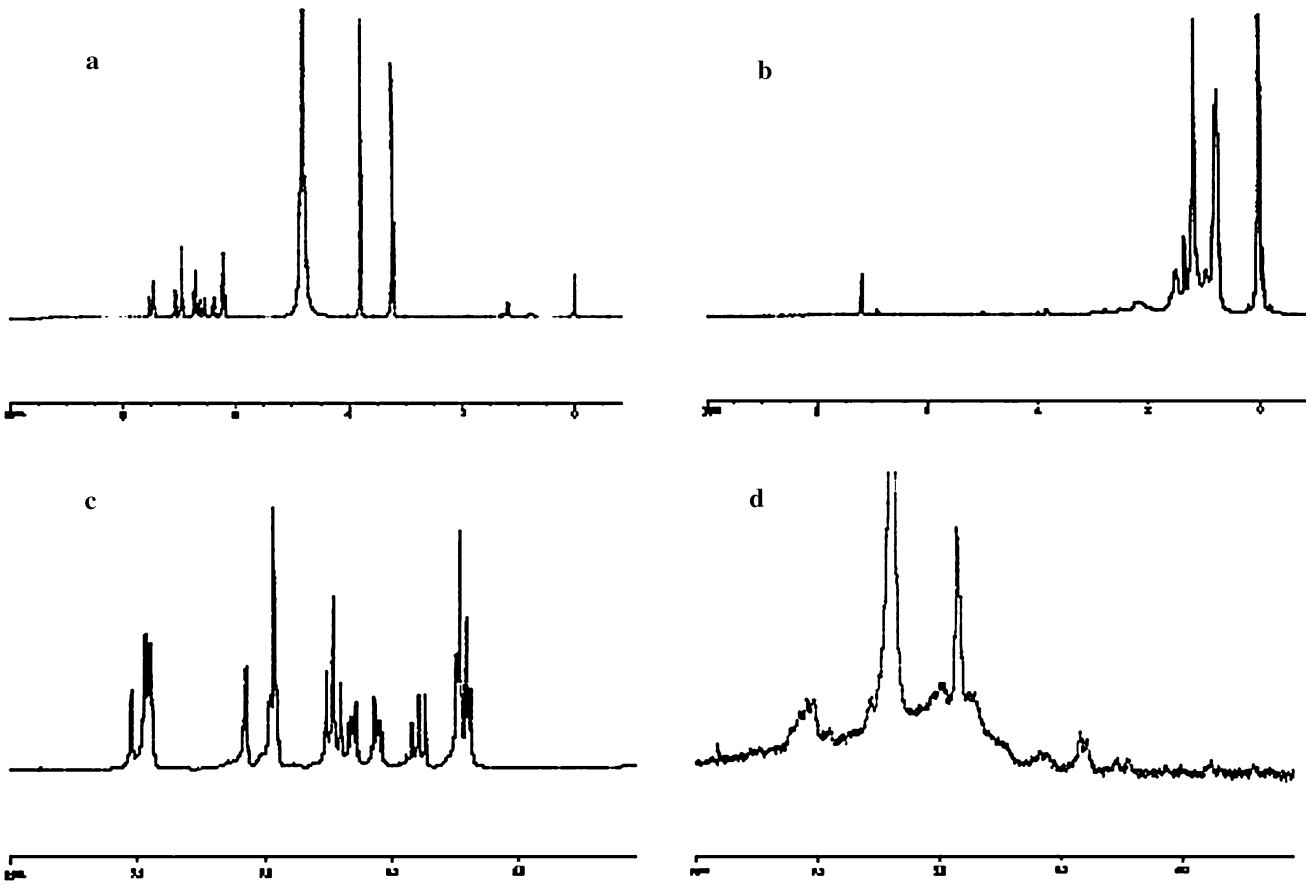

Fig. 8 ^{1}H NMR spectra of untreated and treated phenol samples. (**a**) Full ^{1}H NMR spectrum of untreated phenolic sample. (**b**) Full ^{1}H NMR spectrum of treated phenolic sample. (**c**) ^{1}H NMR spectrum showing the aromatic region of untreated phenolic sample. (**d**) ^{1}H NMR spectrum showing the aromatic region of treated phenolic sample

of signals between 0 and 5 ppm in addition to the expected solvent peaks. These peaks were characteristics of the aliphatic compounds. In a typical laccase catalyzed reaction, the phenols are converted presumably to quinones, which are reactive and can undergo subsequent conversions to form other intermediates (Lante et al. 2000). The possibility for further degradation of the break down products into aliphatic chains cannot be ruled out. Therefore, the presence of large number of peaks within the aliphatic regions might be due to various degradation products of laccase-catalyzed reactions. In order to identify the exact degradation products of these phenolic compounds, more studies are required.

The present study has successfully evaluated the advantages of using immobilized laccase system to degrade selected phenolic compounds. The pattern of phenolic sample degradation showed that *S. psammoticus* laccases are capable of degrading different phenolic compounds. The structure of the phenolic compounds present is similar in many industrial effluents and hence, it can be concluded that immobilized *S. psammoticus* laccases may be used for effective bioremediation of phenolic effluents from different sources.

Ackowledgements The authors are grateful to the Council of Scientific and Industrial Research, Government of India, for the Research Fellowship given to K.N. Niladevi.

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