JOURNAL OF Applied Polymer SCIENCE

Trametes versicolor laccase immobilized poly(glycidyl methacrylate) based cryogels for phenol degradation from aqueous media

Tuğba Doğan, Engin Bayram, Lokman Uzun, Serap Şenel, Adil Denizli
Department of Chemistry, Hacettepe University, 06381-Beytepe Ankara, Turkey
Correspondence to: S. Şenel (E-mail: senel@hacettepe.edu.tr)

ABSTRACT: This study aims removal of phenols in wastewater by enzymatic oxidation method. In this study, *Trametes versicolor* laccase was covalently immobilized onto a cryogel matrix by the nucleophilic attack of amino groups of laccase to epoxy groups of matrix. Glycidyl methacrylate was chosen as functional monomer to prepare poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) [p(HEMA-*co*-GMA)] cryogels. The enzyme immobilized matrix was characterized by FTIR, SEM, and swelling tests. The effect of pH, reaction time, temperature, substrate concentration, enzyme concentration, and storage period on immobilized enzyme activity was determined and compared with those of free enzyme. The model substrate was 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid (ABTS). Lineweaver-Burk plots were used to calculate K_m and V_m values. K_m values were 165.1 and 156.0 μ*M* while V_m values were 55.2 μ*M* min^{-1} and 1.57 μ*M* min^{-1} for free and immobilized laccase, respectively. Immobilized enzyme was determined to retain 82.5% and 72.0% of the original activity, respectively, after 6 consecutive use and storage period of 4 weeks. The free enzyme retained only 24.0% of its original activity following the same storage period. Lastly, decomposition products resulting from enzymatic oxidation of a model phenolic compound (3,5-dinitrosalicylic acid) in aqueous solution were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41981.

KEYWORDS: catalysts; membranes; properties and characterization

Received 15 September 2014; accepted 11 January 2015
DOI: 10.1002/app.41981

INTRODUCTION

Phenol is mainly used in pesticides, dyes, textiles production, explosives, and precursors production for plastics.[1] The use of phenol in several industries results in the emission of contaminated wastewater, if not treated.[2,3] Natural processes like the decomposition of organic matter or biosynthesis by fungi and plants are the other sources for phenol emission. Because of the toxicological and ecological effects, it was set as a priority pollutant into the list by the US Environmental Protection Agency (EPA), and EPA has set a water purification standard of less than 1 μg/L of phenol in drinking waters.[4]

Conventional methods for phenol removal include adsorption, solvent extraction, oxidation, ion exchange, stream stripping, and biological treatment.[5] Microorganisms (usually aerobes) or enzymes (such as peroxidases and polyphenol oxidases) are used in biological treatment. Peroxidases, horseradish peroxidase, lipase, laccase, and bilirubin oxidase, have the ability to oxidize the aromatic compounds to give aromatic radicals.[6–8] The resulting polymeric substances formed by combination of these radicals precipitate, and are easily removed by physical means. Horseradish peroxidase has been widely used to remove phenol and its derivatives. Since it functions in the presence of hydrogen peroxide, the risk of inhibition and deactivation, the formation of toxic soluble reaction by-products, and cost effects should be considered.[6] Tyrosinase is a cheaper alternative to peroxidase and it does not require the use of peroxide. But it has the disadvantages of smaller substrate spectrum and the risk of irreversible inactivation during the catalytic reaction.[9] In case of laccase (EC 1.10.3.2, multi copper blue oxidase), dissolved molecular oxygen is required for catalytic activity of enzyme, leading to the more advantageous use of it. Laccase can oxidize a broad range of substrates, including phenols, anilines, polyphenols, and even certain inorganic compounds with parallel reduction of oxygen to water.[10,11] The broad substrate specificity, very low redox potential, wide reaction capabilities, high efficiency for its action, no requirement for cofactors or peroxide, and low cost degradation of contaminants were the main concerns in several biotechnological applications.[12] Laccases are widely used in pulp and textile dye bleaching, polymer synthesis, bioremediation of water and soils, development of biosensors and biofuel cells.[13,14]

In the case of enzymes, the immobilized form is generally preferred since it offers several advantages such as catalyst reuse, easier product separation, high storage stability, and cost advantage, especially in case of large volume of wastewaters.[15,16]

Additional Supporting Information may be found in the online version of this article.

© 2015 Wiley Periodicals, Inc.

Natural or synthetic, inorganic, or organic several substances have been used as matrices for reversible or irreversible immobilization.[17–21] Epoxy activated carriers,[22] glass beads,[23] alginate hydrogels,[24] bimodal carbon-based mesoporous magnetic composites,[25] nanoparticles and kaolinite,[26] p(GMA-EGDMA) cryogel,[27] nanoporous gold,[28] and PVA cryogel[29] were used for laccase immobilization in several bioremediation applications. An attractive alternative form for the synthesis of polymeric matrix is cryogels due to the advantages such as macroporous structure, allowing separation at higher flowrate with lower backpressure, ease of synthesis, and physical and chemical stability.[30,31]

In this study, poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) [p(HEMA-*co*-GMA)] cryogels were used for covalent immobilization of *Trametes versicolor* laccase based on the substitution reaction between epoxy groups of the matrix and amino groups of the enzyme. The effect of several parameters (temperature, pH, substrate concentration, etc.) on enzyme activity was examined for both free and immobilized enzymes using ABTS as substrate. The potential use of immobilized enzyme was tested qualitatively by LC-MS/MS for a model phenol compound, 3,5-dinitrosalicylic acid (DNS).

EXPERIMENTAL

The enzyme, *Trametes versicolor* laccase, the substrate, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid) (ABTS), 3,5-dinitro salicylic acid (DNS), 2-hydroxyethyl methacrylate (HEMA), ethylene glycol dimethacrylate (EGDMA), *N*,*N*,*N*′,*N*′-tetra-methyl-ethylene diamine (TEMED), sodium lauryl sulphate (SLS), and ammonium persulphate (APS) were purchased from Sigma-Aldrich (Sigma Chem); sodium azide (NaN_3) was from Merck AG (Darmstadt, Germany) and glycidyl methacrylate (GMA) was from Fluka AG (Buchs, Switzerland).

All other chemicals were of reagent grade and were supplied from Merck AG (Darmstadt, Germany). All water used in experiments was purified using a Barnstead (Dubuque, IA) Ropure LP® reverse osmosis unit, organic/colloid removal unit, and ion-exchange column.

Synthesis of p(HEMA-*co*-GMA) Cryogel

The bulk polymerization method was performed for the synthesis under partially frozen-conditions. Two aqueous phases were prepared. Phase I contained 555.0 μL of GMA and 2.2 mL of HEMA in 3.3 mL of water. Phase II involved 0.50 g of SLS, surface active agent, and 1.2 mL of EGDMA, crosslinker, in 13.8 mL of water. SLS was used to make easier the dissolution of hydrophobic component, EGDMA, lowering the surface tension of water. Then, two phases were mixed and magnetically stirred and cooled in an ice-bath for 20 min. Following the addition of 0.01 g of APS, the initiator, and 50.0 μL of TEMED, the activator, the mixture was poured between two glass plates, and polymerization was conducted at −12.0°C for 24 h. The resulting polymer was thawed at room temperature and extensively washed with deionized water to remove unreacted monomers and stored at 4.0°C in the swollen form.

Laccase Immobilization

Cryogel membranes were equilibrated in acetate buffer (pH: 5.0) for 2 h. Then, 40 circular cryogel membranes (diameter 6 mm) were placed in 20 mL of enzyme solution having a concentration of 2 mg/mL in acetate buffer (pH 5.0) and stirred magnetically with a stirring rate of 100 rpm at room temperature for 24 h. Laccase concentration was determined by UV-visible spectrometer (UV mini-1240, Shimadzu, Tokyo, Japan) by measuring the absorbances at 280 nm. The incubation period was chosen as 3, 6, 12, and 24 h to evaluate effect of enzyme amount, and resulting polymers were encoded as P3, P6, P12, and P24, respectively. A membrane sample containing no enzyme was also synthesized as well for comparison, encoded as P0.

Characterization of Cryogels

FTIR spectra of p(HEMA-*co*-GMA) cryogels and immobilized enzyme containing cryogels were obtained by using a FTIR spectrophotometer (Spectrum One, Perkin Elmer, USA). The dried and crushed cryogel sample (about 2.0 mg) was thoroughly mixed with KBr (98.0 mg, IR Grade, Merck, Germany) and pressed into a pellet. The spectrum was then recorded over 4600–400 cm^{-1} wavelengths.

The surface morphology of the cryogels was examined by scanning electron microscopy (Jeol, SEM5600, Tokyo, Japan). The cryogel sample dehydrated at −60.0°C in lyophilizer (Christ Alpha, 1–2 LD Plus; M Christ GmbH, Germany), was coated with gold (about 200 Å in thickness) and examined using SEM instrument at different magnifications.

The swelling degree was determined by weighing the dry and wet membranes (4 pieces) following the incubation in 50.0 mL of deionized water at room temperature until a constant weight for wet sample was obtained.

The swelling ratio was calculated using the following equation:

$$SR(\%) = [(Ws - Wo)/Wo] \times 100\%$$

where Ws and Wo denote the masses of wet and dry membranes (g), respectively.

The porosity was estimated by weighing the wet swollen cryogel membrane and squeezed membrane:

$$\text{Porosity} = [(m_{\text{swollen}} - m_{\text{squeezed}})/m_{\text{swollen}}] \times 100\%$$

The preceding equation where "m" denoted mass was used in calculation. All measurements were performed in triplicate.

Activity Studies for Laccase

ABTS was chosen as substrate since laccases in general have high affinity for ABTS that is oxidized to a stable green colored cationic radical, $ABTS^+$. ABTS (4.5 mL) solutions in acetate buffer (0.1*M*, pH 5.0) having concentrations of 0.1–1.0 m*M* were interacted with 500 μL (0.148 mg) of laccase. Following the enzymatic reaction conducted at 25.0°C for 30 min, the inhibitor, NaN_3 (20 m*M*, 500 μL) was included (enzyme/inhibitor ratio was 1 : 1, V : V) to stop the reaction. The absorbance values for the product were recorded by an UV-visible spectrophotometer (Shimadzu, UV mini-1240, Tokyo, Japan) at 420 nm, and a calibration curve (absorbance vs. $ABTS^+$ concentration) was plotted to calculate enzyme activity.

Activity Measurement for Immobilized Laccase

Two pieces of membrane encoded as P24 were added to 1.0 m*M* ABTS solution in 0.1*M* pH 5.0 acetate buffers. The

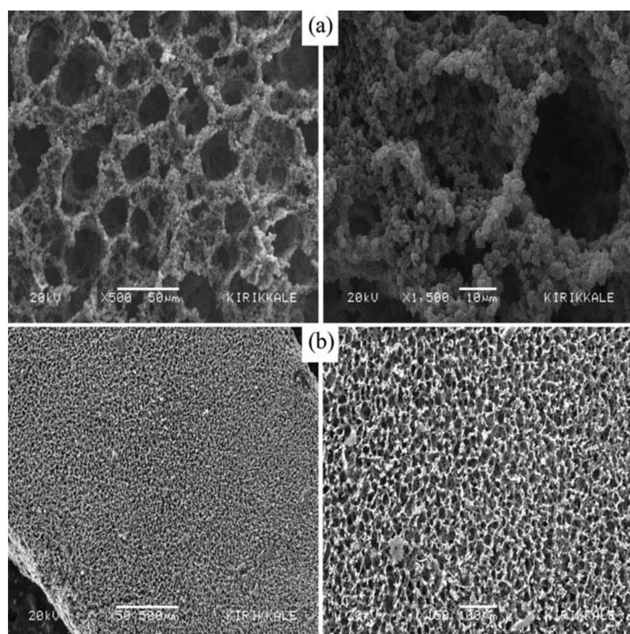


Figure 1. SEM images: (a) Surface morphology and (b) cross-section of of p(HEMA-*co*-GMA) cryogel.

reaction was conducted as 25.0°C for 30 min while the reaction vessel was placed in a shaker. The absorbance values at 420 nm were measured for the solutions before and after the reaction. Activity values were calculated using the following equation:

$$\text{Activity}: \Delta A_{420}/\Delta t \times \Delta C/\Delta A_{420}$$

where ΔC denotes the change in ABTS concentration (mM), t stands for reaction time (min), and ΔA_{420} stands for the change in absorbance values of $ABTS^{+}$.

Effective Parameters on Activity of Free and Immobilized Enzymes

The experiments were conducted in batch mode. pH effect was tested over 3.0–6.0 range for free and immobilized enzyme (P24; mass of laccase 0.148 mg) at 25.0°C where ABTS concentration and reaction time were as 1.0 m*M* and 30 min, respectively. The temperature was examined over 25.0–75.0°C range, the other parameters being the same as above. The substrate concentration effect was examined over 0.1–3.0 m*M* range. The temperature and pH were adjusted to 25.0°C and 5.0, respectively. Again P24 encoded sample was used for immobilized form of enzyme. P3, P6, P12, and P24 encoded membranes were also used to evaluate the effect of immobilized enzyme amount on activity. 0.017, 0.038, 0.073, and 0.148 mg of laccase containing 500 μL of enzyme solutions were used for the analysis of free enzyme activity for comparison. For the effect of reaction time, the range of 1–60 min was examined. The immobilized enzyme containing cryogel was used for 6 successive runs to test the reusability. The membrane was washed with sodium hydroxide (10 m*M*), acetate buffer (0.1*M*, pH 5.0) and water before each use. The storage period effect on activity was tested for four weeks for both free and immobilized enzymes.

DNS Degradation by Immobilized and Free Laccase

The degradation process was performed under optimum conditions leading to maximum activity for enzyme. The process was

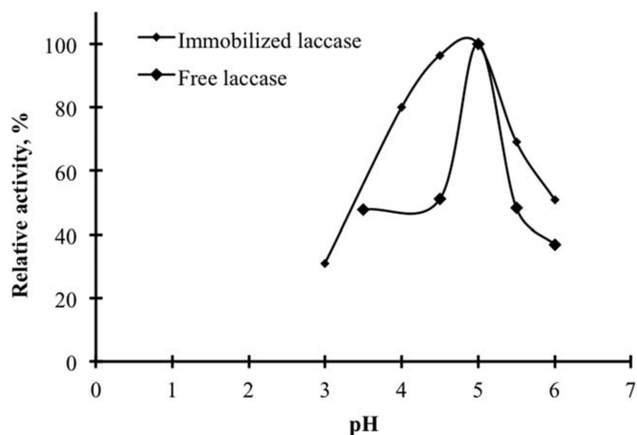


Figure 2. The effect of pH on laccase activity. Cryogel code: P-24; laccase amount: 0.148 mg; NaN_3 concentration: 20 m*M*; enzyme/inhibitor v : v, 1 : 1; ABTS concentration: 1.0 m*M*; time: 30 min; T: 25.0°C.

started by two pieces of P24 encoded membranes included in 2.5 mL of 1.0 m*M* DNS, and 2.5 mL of 0.5 m*M* ABTS (chemical mediator). The reaction was conducted at 55.0°C for 2 h. Following the reaction, 1.0 mL of solution was stored for LC-MS/MS analysis. The same procedure was applied to free enzyme including 500 μL (0.148 mg) of laccase dissolved in acetate buffer (pH 5.0) into the mixture containing ABTS and DNS.

LC-MS/MS Analysis

A triple quadrupole LC-MS/MS system (Shimadzu LC-MS-8030, Kyoto, Japan) in Q3 scan mode was used to carry out the analysis. The chromatographic separation was performed by inertsil ODS-4 HPLC column (150 × 2.1 mm, 3 μm). The mobile phases A and B were formic acid (pH 3.5) and acetonitrile, respectively. The gradient mixtures containing 10.0%A and 90.0%B and 80.0%A and 20.0%B were used for the first 13 min and the following 5 min of the analysis. The linear gradient for chromatographic separation was performed at a furnace temperature of 40.0°C for a flow-rate of 0.3 mL/min. The separation was realized at ESI-positive and negative ion modes over the mass

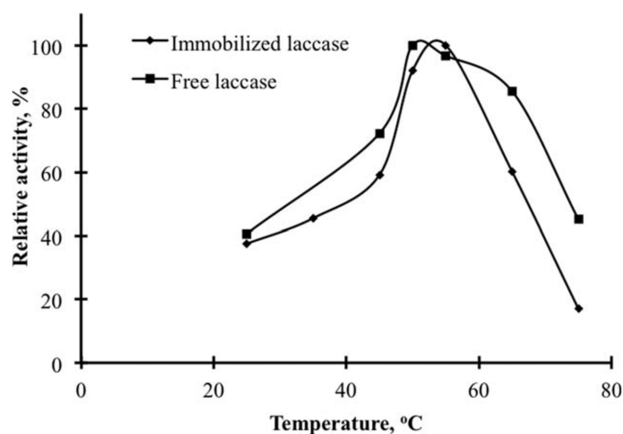


Figure 3. The effect of temperature on laccase activity. Cryogel code: P-24; laccase amount: 0.148 mg; NaN_3 concentration: 20 m*M*; enzyme/inhibitor v : v, 1 : 1; ABTS concentration: 1.0 m*M*; time: 30 min; pH: 5.0.

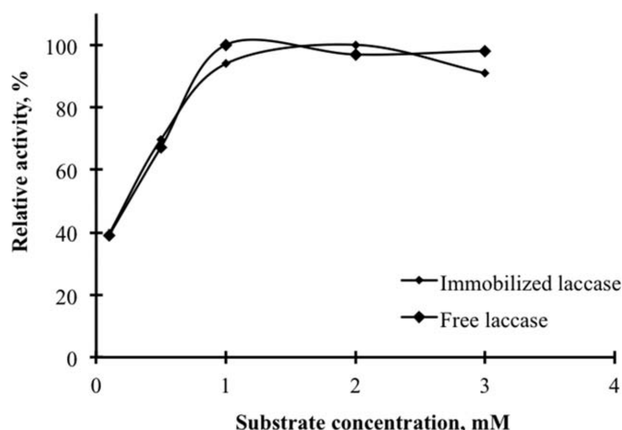


Figure 4. The effect of substrate concentration on laccase activity. Cryogel code: P-24; laccase amount: 0.148 mg; NaN_3 concentration: 20 m*M*; enzyme/inhibitor v : v, 1 : 1; time: 30 min; T: 25.0°C; pH: 5.0.

range of 75–250 m/z with a probe voltage of 3.5 kV and a scanning rate of 1250 µ/s.

RESULTS AND DISCUSSION

Amount of Immobilized Enzyme

The covalent immobilization of laccase was realized by nucleophilic attack of amino groups of enzyme to the epoxy groups of GMA, and the subsequent ring opening. Since the binding was strong, no leakage of enzyme from the support was noted. But, the immobilization method included the disadvantages such as the risk of chemical modification of enzyme and the small amounts of immobilized enzyme. Besides the affinity interaction, adsorption occurs to a lesser extent due to the secondary interactions between enzyme and matrix. The amount of immobilized enzyme was determined spectrophotometrically from absorbance measurements at 280 nm using the previously determined calibration curve, and considering the amounts of laccase before and after immobilization in solution and in washing water. Two circular pieces of membrane contained 0.017, 0.038, 0.073, and 0.148 mg of enzyme for P3, P6, P12, and P24 samples, respectively. The amount of immobilized enzyme increased with increasing immobilization period.

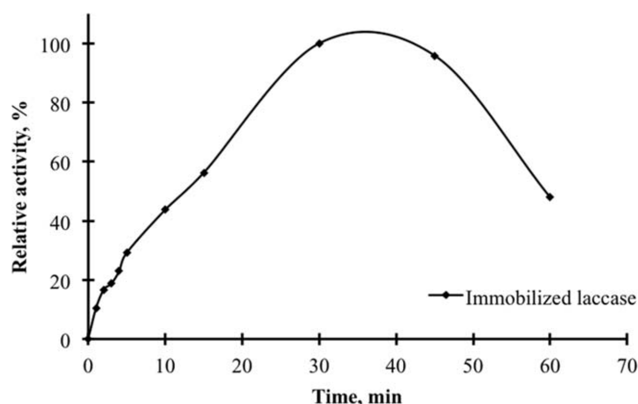
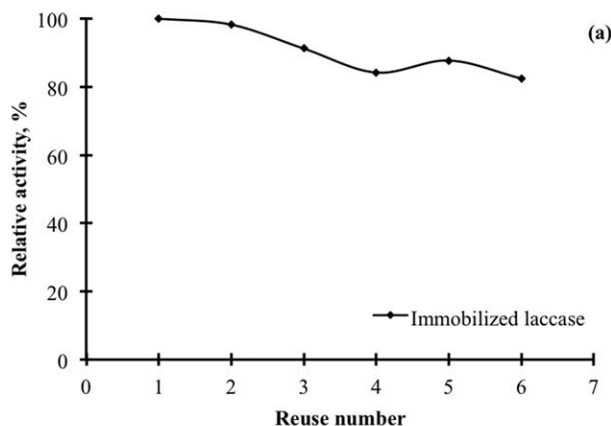


Figure 5. The effect of reaction time on laccase activity. Cryogel code: P-24; laccase amount: 0.148 mg; NaN_3 concentration: 20 m*M*; enzyme/inhibitor v : v, 1 : 1; ABTS concentration: 1.0 m*M*; T: 25.0°C; pH: 5.0.



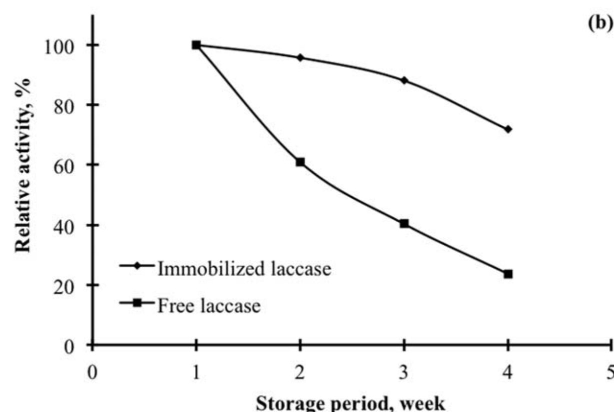


Figure 6. The repeated use (a) and storage stability (b) of the cryogels. Cryogel code: P-24; laccase amount: 0.148 mg; NaN_3 concentration: 20 m*M*; enzyme/inhibitor v : v, 1 : 1; ABTS concentration: 1.0 m*M*; time: 30 min; T: 25.0°C; pH: 5.0.

Characterization of Cryogels

GMA was chosen as the functional monomer due to the reactive epoxy groups to interact with amine groups of enzyme without requiring the activation of the matrix. FTIR spectra for p(HEMA-*co*-GMA) and immobilized enzyme containing p(HEMA-*co*-GMA) are used to characterize the chemical structure of cryogels (Figure SF-1, Supporting Information). A broad peak of —O—H stretching vibration around 3440 cm^{-1} has appeared for both of them. The characteristic methylene vibration around 2950 cm^{-1} belongs to GMA and HEMA. The peak around 1730 cm^{-1} corresponds to ester configurations (—COO—) of both monomers. The peak around 900 cm^{-1} belongs to epoxy groups for p(HEMA-*co*-GMA) cryogel whereas the existence of peaks around 1570 cm^{-1} for enzyme containing membranes proves the existence of C—N bonds. The increase in the intensity of —O—H stretching vibration was an indication of covalent attachment of laccase through epoxy ring opening, and increase in number of —OH functional groups due to bonding of oxygen atoms in epoxy rings with hydrogen atoms.

The swelling of cryogel membranes was rapid due to the capillary effect of water molecules into pores. The swelling ratios (%) were 523%, 531%, 559%, 570%, and 572% for P0, P3, P6, P12, and P24, respectively. Macroporosity (%) values were 54%, 55%, 58%, 59%, and 61%, in the same order. The hydrophilic

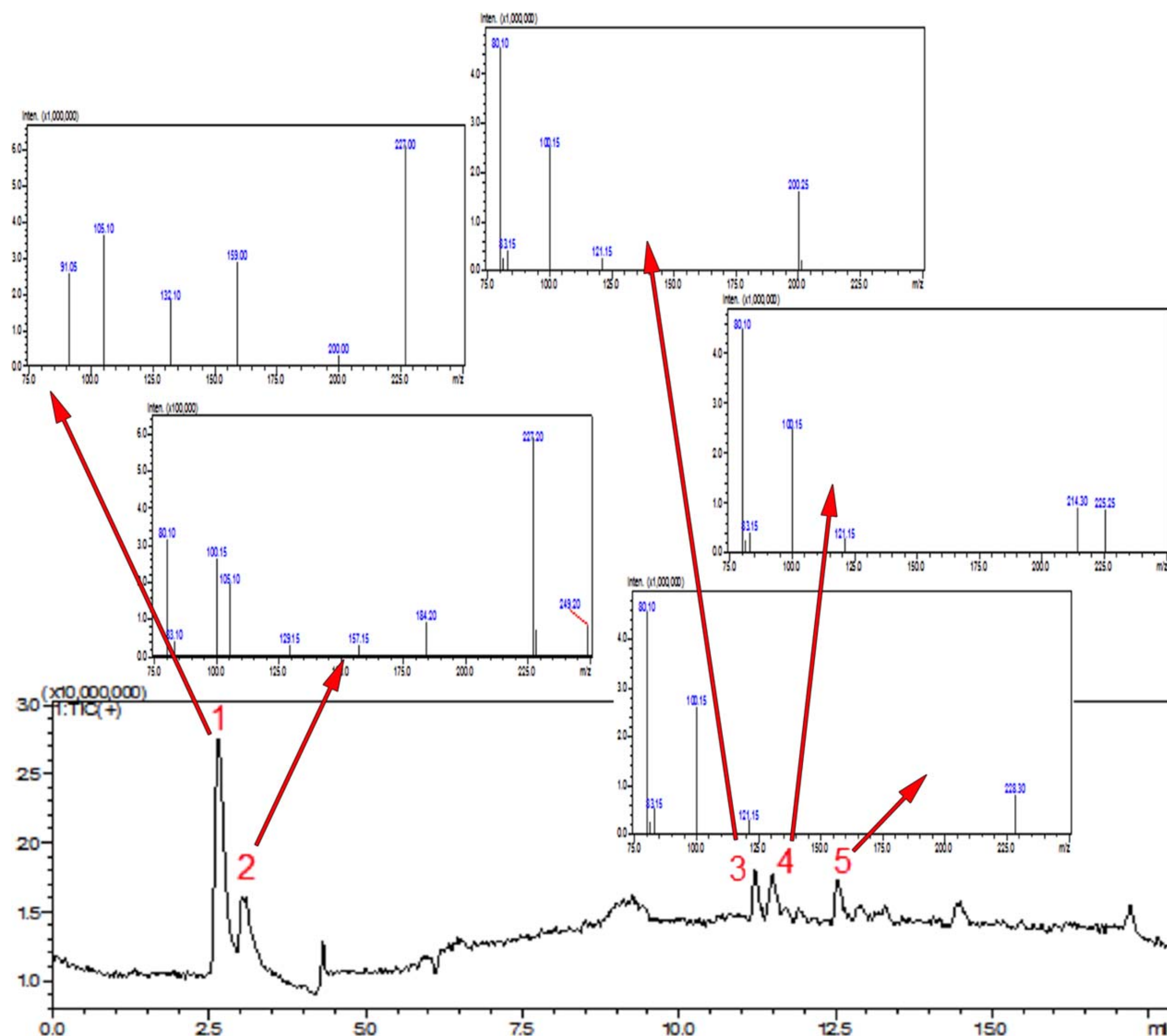


Figure 7. Positive ion chromatogram and MS spectra of DNS solution treated with laccase immobilized cryogel. Peaks belonged to 1: ABTS and 2–5: products. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

character of matrix and enzyme, the increase in surface area following immobilization, the polar groups for protein are probably responsible for the increase in swelling ratios and macroporosity.

The SEM images with different magnification ratios of p(HEMA-*co*-GMA) cryogel are given in Figure 1. The interconnected macropores are clearly seen. The size of pores was roughly 20–50 μm. Addition of surface-active agent (SLS) into polymerization recipe, probably caused the shapes of pores and surface of polymeric walls to become rougher than traditional cryogel images due to the change in distribution of water molecules throughout the network and freezing them into ice crystals.

Optimization of Enzyme Activity Conditions

Effect of pH on Activity. The pH value has significant effects on the ionic structure of active center, stability, and three-dimensional structure of enzyme, the maximum rate (V_m), Michealis-Menten constant (K_m), the functional groups involved in interactions and the affinity of enzyme for substrate. Since the electrostatic and hydrophobic interactions and hydrogen bonding are responsible for stability, tertiary, and quaternary structures of enzymes are strongly affected with a change in pH. For instance, the carboxyl groups are protonated when pH is less than 3.0 whereas amino groups are protonated when pH is less than 10.0. Electrostatic bond formation is prevented causing changes in enzyme activity.[32] Thus; enzymes are denatured in strongly acidic and alkaline media. When ABTS was used as substrate, laccases are active below pH 6.0. The pH optima are found in the range 3.0–5.0.[33] Also, Type III copper of laccases is inhibited by hydroxide ion for alkaline media, and laccase is converted into an inactive form due to the redox potential difference between Type I copper and substrate[34] That was the reason for choosing 4.0–6.0 range for pH analysis. The effect of pH on activity for both free and immobilized enzyme is given in Figure 2. The optimum pH was 5.0 for both, consistent with the values reported in literature.[35–37] But, the pH range for

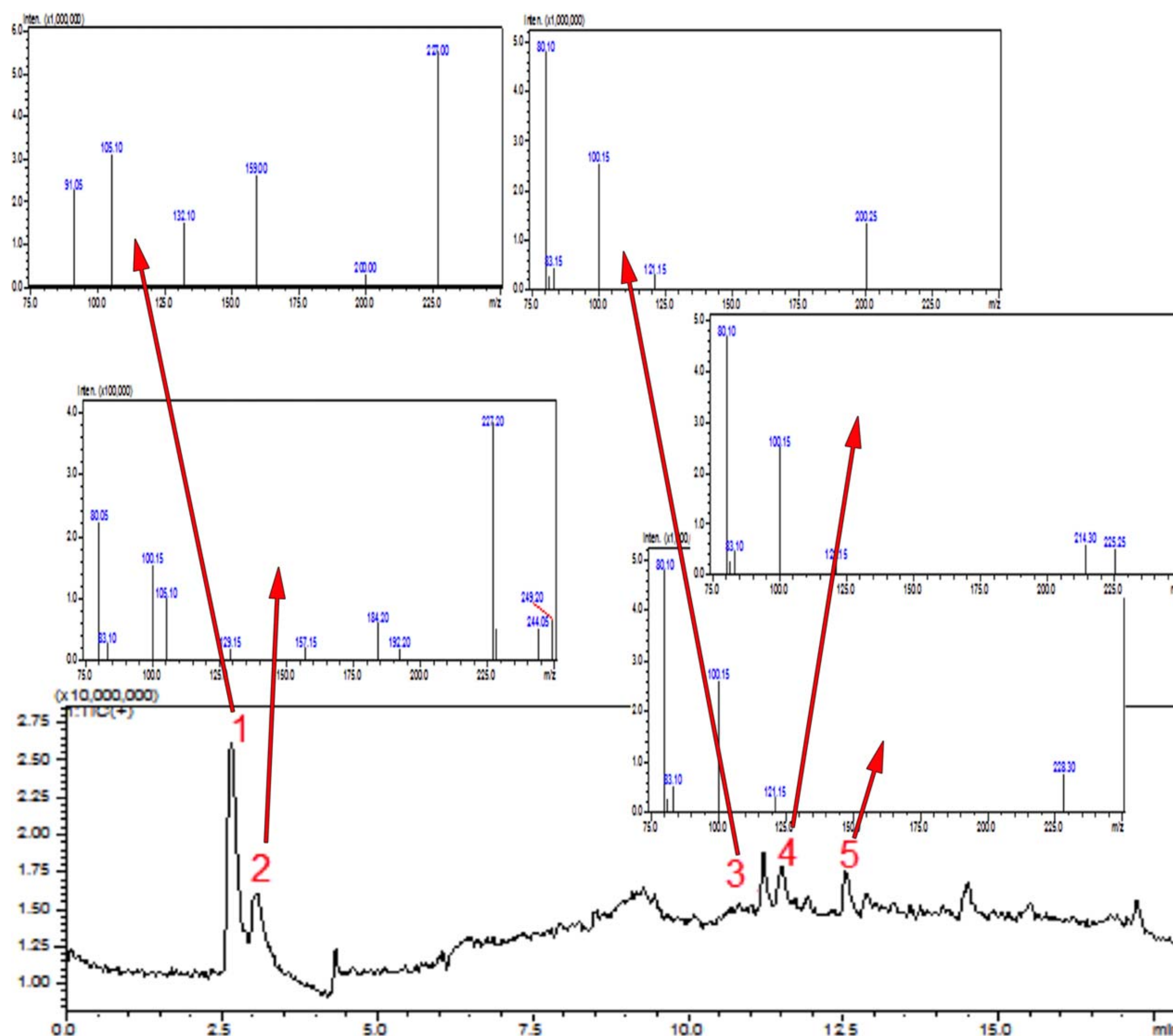


Figure 8. Positive ion chromatogram and MS spectrum of DNS solution treated with free laccase. The peaks belonged to 1: ABTS and 2–5: products. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

which the active enzyme was involved broadened in case of immobilized form which means immobilization decreased the sensitivity of enzyme with respect to pH variation.

Effect of Temperature on Activity. The temperatures for free and immobilized laccase were varied between 25.0 and 75.0°C (Figure 3). The optimum temperature range for fungal laccases was reported as 30.0–60.0°C in literature.[38,39] The decrease in activity at higher and lower temperatures was due to the possibility of denaturation and conformational changes in active center of enzyme. The enzymatic reaction rate is strongly affected with a change in temperature due to Arrhenius equation. For 298–323 K range, the activation energies were determined from Arrhenius plots as 27.0 and 28.8 kJ/mol for free and immobilized enzymes, respectively. The slight increase in activation energy for immobilized enzyme was due to the decrease in conformational flexibility of enzyme for binding to substrate. Optimum temperature for enzyme activity shifted from 50.0 to 55.0°C due to the stability caused by immobilization procedure.

Effect of Substrate Concentration on Activity. Lineweaver-Burk diagrams were plotted to get Michealis-Menten constant (K_m) and maximum reaction rate (V_m) for saturated substrate concentration from the slope and intercept, respectively, using following equation (Figure not given):

$$1/V = K_m/V_m \times (1/[S]) + 1/V_m$$

K_m values for free and immobilized laccase were 165.1 and 156.0 μ*M* while the V_m values were 55.2 and 1.57 μ*M*/min, respectively. The K_m values of laccases are in the range of 2–5000 μ*M*.[10] In this study, the change in K_m, a measure of enzyme's affinity for substrate was an indication of enzyme's affinity for ABTS not effected by immobilization procedure, instead increased, contrary to general trend followed by immobilized enzymes. V_m of free enzyme was 35-fold lowered in case of immobilized one. Compared with the free enzyme, the immobilized enzyme usually has its activity lowered. The change in V_m was probably caused by the change of microenvironment in which the enzyme works, different from the bulk solution,

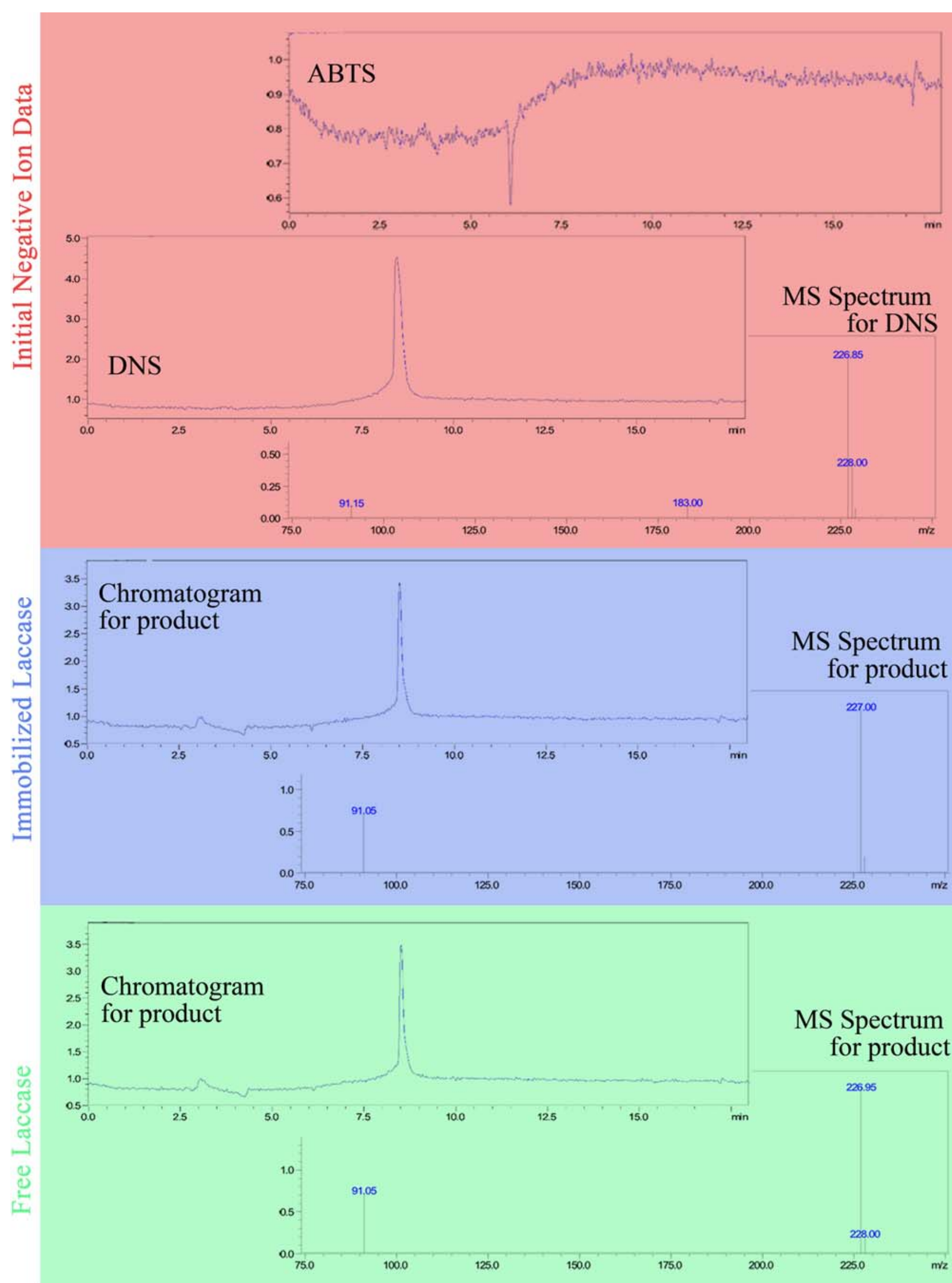


Figure 9. Negative ion chromatograms and MS spectra for ABTS and DNS solution before and after enzymatic treatments. Note: Figures have already been given as Supporting Information separately. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and structural changes introduced to the enzyme by the applied immobilization procedure.[40]

Effect of Amount of Enzyme on Activity. The relative activity of both free and immobilized enzyme increased with increasing amount of enzyme as expected (Figure 4). The lower rate of change of activity for immobilized one was probably due to the steric hindrance effect stemming from covalent immobilization.

Effect of Reaction Time on Activity. The relative activity of immobilized enzyme increased with increasing time up to 30 min (Figure 5), and then decreased since the amount of substrate decreased due to enzymatic reaction. According to the data, 30 min was accepted as optimal interaction time and applied for all runs.

Repeated Use and Storage Stability

Reusability is highly important due to cost considerations. Following six-repeated use, the relative activity of immobilized enzyme only decreased 82.5% of its original activity [Figure 6(a)]. The storage period for immobilized enzyme was longer than that of free one. 72% of original activity was maintained for immobilized enzyme after four weeks. Only 24% of original activity was retained for free enzyme in the same storage period,

proving that immobilization process increased the storage stability of the enzyme [Figure 6(b)].

LC-MS/MS Analysis

To test the potential application of laccase immobilized cryogels, ABTS and DNS degradation by immobilized enzyme was examined (Figures 7 and 8). The degradation products were analyzed by LC-MS/MS measurements. MS spectra were firstly recorded for standard DNS and ABTS solutions (Figures SF-2 and SF-3, Supporting Information). For standard ABTS solution, the retention time was 2.75 min for positive ion scanning while no response for negative ion scanning was recorded. Conversely, DNS gave a response for negative ion scanning with a retention time of 8.5 min. Therefore, positive ion scanning in case of ABTS was applied whereas negative ion scanning was used in case of DNS solution. When MS spectrum of DNS solution treated with immobilized enzyme was examined, peak 1 having the same retention time and the same mass/charge ratio with standard solution belonged to ABTS. The peaks 2, 3, 4, and 5 proved the degradation products of DNS having mass/charge ratios as 184.20, 200.25, 225.25, and 228.30, respectively. These peaks belonged to the release of CO_2, the release of aldehyde, formation of quinone and protonation of structure. The negative ion scanning chromatogram of DNS treated with immobilized laccase proved the decrease in amount of DNS as well.

When positive ion chromatograms of DNS treated with free and immobilized enzymes were compared, the resulting peaks were the same. The same result was valid for MS spectra and negative ion chromatograms of both enzymes used for DNS treatment, which means that both free and immobilized enzymes have similar activities on the substrate molecules and converted them into same final products as expected. As a result, the immobilization procedure did not affect the enzyme activity meanwhile enhancing properties such as pH, temperature dependences, reusability, storage stability, etc. of enzyme molecules.

Negative ion chromatograms and MS spectra of ABTS and DNS before and after enzymatic treatment were also considered to compare efficiencies of both enzymatic systems (Figure 9). As given in figure, ABTS has no chromatogram in negative ion radiation meanwhile DNS has a peak including five different MS products at which have m/z ratios as 91.15, 183, 226.9, 228, and 229. After treatment with immobilized laccase, the peaks at 183 and 229 m/z ratio disappeared meanwhile the intensities of the peaks at 91.15 and 227 m/z increased from 0.1613 to 0.7097 and from 1.9355 to 2.00, respectively. Also, the intensity of the peak at 228 m/z decreased from 0.6290 to 0.1839. In case of free enzyme used, the peaks at 183 and 229 m/z ratio also disappeared. The intensities of the peaks at 91.15, 227, and 228 m/z changed to 0.7419, 2.0645, and 0.1710, respectively. The disappearance of the peak at 183 m/z is the most promising evidence for enzymatic conversion of substrate, DNS molecules. This peak depends on the CO_2H and/or NO_2 cleavage from basic structure. But, we have to focus on the peak at 91 m/z which is the smallest molecule retained in order to compare the efficiencies of free and immobilized laccases. The intensity increased 4.3999-folds for immobilized laccase whereas that increased 4.5995-folds for free laccase, which indicates free enzyme has more activity (104.5%) on the substrate. The results correlated with the results obtained and discussed during optimization studies and positive ion treatments when LCMSMS measurements. But, the recovery and reusability features of immobilized enzymes make them more useful, efficient, and cost-friendly alternative for enzymatic treatment of wastewater samples.

CONCLUSION

Herein, we have reported glycidyl methacrylate based cryogel for enzyme immobilization and its performance for biotreatment of a model phenol compound in water. Glycidyl methacrylate was chosen to omit the activation step for enzyme immobilization while cryogel form was preferred due to structural properties and excellent flow dynamics. After optimization of conditions for both enzyme immobilization and enzymatic oxidation of model substrate (ABTS), the cryogels were treated for oxidative removal of a model phenol compound from water. The cryogels proposed could be classified as a promising alternative for enzyme immobilization and biological wastewater treatment because enzyme activity and stability were protected meanwhile storage and temperature stabilities were improved.

REFERENCES

1. Michalowicz, J.; Duda, W. *Polish J. Environ. Stud.* **2007**, *16*, 347.
2. van Schie, P. M.; Young, L. Y. *Appl. Environ. Microbiol.* **1998**, *64*, 2432.
3. Miodrag, S.; Lidija, N. *Work. Living Environ. Protect.* **2004**, *2*, 345.
4. Loncar, N.; Bozic, N.; Andelkovic, I.; Milovanovic, A.; Dojnov, B.; Vujcic, M.; Roglic, G.; Vujcic, Z. *J. Serbian Chem. Soc.* **2011**, *76*, 513.
5. Stanisavljevic, M.; Nedic, L. *Work Living Environ Protect.* **2004**, *2*, 345.
6. Sukan, A.; Sargin, S. *J. Biomater. Nanobiotechnol.* **2013**, *4*, 300.
7. Narayan, A. V.; Pushpa, A. *J. Environ. Res. Develop.* **2012**, *7*, 724.
8. Singh, N.; Singh, J. *Prep. Biochem. Biotechnol.* **2002**, *32*, 127.
9. Strong, P. J.; Claus, H. *Crit. Rev. Environ. Sci. Technol.* **2011**, *41*, 373.
10. Kunamneni, A.; Plou, F. J.; Ballesteros, A.; Alcalde, M. *Recent Pat. Biotechnol.* **2008**, *2*, 10.
11. Yarapolov, A. I.; Skorobogatko, O. V.; Varfolomeyev, S. D. *Appl. Biochem. Biotechnol.* **1994**, *49*, 257.
12. Khan, A. A.; Alzohairy, M. A. *Res. J. Biol. Sci.* **2010**, *5*, 565.
13. Gnanasalomi, V. D. V.; Gnanados, J. J. *Asian J. Exp. Biol. Sci.* **2013**, *4*, 581.
14. Thomas, I.; Hausmann, K.; Schlesser, D.; Schulze, A. *Molecules* **2014**, *19*, 11860.
15. Fernandez-Fernandez, M.; Sanroman, M. A.; Moldes, D. *Biotechnol. Adv.* **2013**, *31*, 1808.

16. DiCosimo, R.; McAuliffe, J.; Poulose, A. J.; Bohlmann, G. *Chem. Soc. Rev.* **2013**, *42*, 6437.
17. Duran, N.; Rosa, M. A.; D'annibale, A.; Gianfreda, L. *Enzyme Microb. Technol.* **2002**, *31*, 907.
18. Madhavi, V.; Lele, S. S. *Bioresources* **2009**, *4*, 1694.
19. Zhang, J.; Xu, Z.; Chen, H.; Zong, Y. *Biochem. Eng. J.* **2009**, *45*, 54.
20. Davis, S.; Burns, R. G. *Appl. Microbiol. Biotechnol.* **1992**, *37*, 474.
21. Arica, M. Y.; Altıntas, B.; Bayramoglu, G. *Bioresour. Technol.* **2009**, *100*, 665.
22. Kunamneni, A.; Ghazi, I.; Camarero, S.; Ballesteros, A.; Plou, F. J.; Alcalde, M. *Process Biochem.* **2008**, *43*, 169.
23. Gomez, E.; Bastida, J.; Gomez, J. L.; Bodalo, A.; Hidalgo, A. M.; Gomez, M. *Enzyme Microb. Technol.* **2006**, *39*, 1016.
24. Niladevi, K. N.; Prema, P. *World J. Microbiol. Biotechnol.* **2008**, *24*, 1215.
25. Liu, Y.; Zeng, Z.; Zeng, G.; Tang, L.; Pang, Y.; Li, Z.; Liu, C.; Lei, X.; Wu, M.; Ren, P.; Liu, Z.; Chen, M.; Xie, G. *Bioresour. Technol.* **2012**, *115*, 21.
26. Hu, X.; Zhao, X.; Hwang, H. M. *Chemosphere* **2007**, *66*, 1618.
27. Uygun, M. *J. Chem.* **2013**, *2013*, 1.
28. Qiu, H.; Xu, C.; Huang, X.; Ding, Y.; Qu, Y.; Gao, P. *J. Phys. Chem.* **2008**, *112*, 14781.
29. Stanescu, M. D.; Fogorasi, M.; Shaskolskiy, B. L.; Gavrilas, S.; Lozinsky, V. I. *Appl. Biochem. Biotechnol.* **2010**, *160*, 1947.
30. Plieva, F. M.; Galaev, I. Y.; Noppe, W.; Mattiasson, B. *Trend Microbiol.* **2008**, *16*, 543.
31. Lozinsky,V. I.; Plieva, F. M.; Galaev, Y. I.; Mattiasson, B. *Bioseparation* **2006**, *10*, 163.
32. Whitaker, J. R. In Handbook of Food Enzymology; Whitaker, J. R.; Voragen, A. G. J.; Wong, D. W. S., Eds.; Marcel Dekker: New York, **2003**; Chapter 4, pp 31–65.
33. Liu, Z. F.; Zeng, G. M.; Zhong, H.; Yuan, X. Y.; Fu, H. Y.; Zhon, M. F.; Mav, H.; Li, J. B. *World J. Microbiol. Biotechnol.* **2012**, *28*, 175.
34. Heinzkill, M.; Bech, L.; Halkier, T.; Schneider, P.; Anke, T. *Appl. Environ. Microbiol.* **1998**, *64*, 1601.
35. Dehghunifard, E.; Jafari, A. J.; Kalantary, R. R.; Mahvi, A. H.; Faramarzi, M. A.; Esrafili, A. *Iranian J. Environ. Health Sci. Eng.* **2013**, *10*, 1.
36. Asadgol, Z.; Forootanfar, H.; Rezaei, S.; Mahvi, A. H.; Faramarzi, M. A. *J. Environ. Health Sci. Eng.* **2014**, *12*, 93.
37. Xu, F. Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis Bioseparation, Wiley: New York, **1999**; pp 1545–1554.
38. Kim, Y.; Nicell, J. A. *Bioresour. Technol.* **2006**, *97*, 1431.
39. Kurniawati, S.; Nicell, J. A. *Bioresour. Technol.* **2008**, *99*, 7825.
40. Singh, G.; Bhalla, A.; Capalash, N.; Sharma, P. *Indian J. Sci. Technol.* **2010**, *3*, 48.