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A comparative study of free laccase and laccase immobilized in copper alginate

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Enzyme immobilization has gained considerable attention due to the incredible properties exhibited by the enzymes in immobilized condition. Therefore, in the present study, a comparative analysis of free and immobilized laccase is reported using various substrates. The ideal substrate for immobilization was found to be copper alginate with an immobilization yield of 91.078%. The optimum pH was 4 and 5, respectively, for free and immobilized enzymes while the optimum temperature was found to be 50 and 60°C, respectively. The kinetic parameters V_{max} and K_m were found from the Line weaver-Burk plot and were 48.076 U/mL and 0.480 mM for free enzyme while 55.55 U/mL and 1.277 mM for the immobilized enzyme, respectively. The catalytic efficiency K_{cat} was found as 100.01s^{-1} for free enzyme and 43.5s^{-1} for its immobilized counterpart. Out of the various metal ions used, Co^{2+} was found to enhance the activity of an immobilized enzymes. The storage stability of the enzyme was studied and was found that only 32.44% of initial activity was retained by free enzyme whereas, 70.21% of activity was retained by immobilized enzyme upon storage for four weeks at 4°C. The thermal stability studies shows that the immobilized enzyme retained 32.60% of its initial activity and the free enzyme retained 1.14% of initial activity on exposure to 60°C for 3 h. Finally, the reusability of immobilized laccase beads was evaluated by decolorization of methyl orange for five repeated cycles and a percentage decolorization of 32.04% could be retained at the fifth cycle. This study therefore, suggests copper alginate-immobilized beads to be an effective option for various applications

Keywords: Copper alginate, Immobilization, Laccase, Stability analysis, Storage analysis

It is well known that enzymes have myriad applications ranging from industry to medicine to environment to food. Latest advancements in biotechnology and protein engineering have further led to the development of novel enzymes with enhanced qualities. Though their potential is well exposed and well tapped, certain problems associated with them like the high cost of isolation and purification, instability in organic media, and high temperature impede their utilization extensively. These problems are addressed by immobilizing the enzymes on a suitable support system[1].

Immobilizing enzymes seems to be an effective and acceptable alternative as this setup displayed several advantages such as making enzyme recovery feasible, increase in stability and durability, rapid enzyme separation, tolerance to extreme pH, temperature, and high substrate concentration and reusability[2-4]. Different methodologies exist for immobilization and the most commonly used include surface binding, gel entrapment, covalent linking between the enzyme and matrix, using cross- linkers, entrapment in reverse micelles[5]. The use of biological polymers as a matrix for attachment has added advantages as they are nontoxic, economical, biocompatible, and biodegradable. The active sites of the enzyme are protected from the negative effects[6] of the components of the reaction mixture. Laccases (benzenediol: oxygen oxidoreductase, EC.1.10.3.2) are polyphenol oxidases that catalyze one- electron oxidation of a wide variety of substrates. They are called multi copper oxidases[7] since they contain copper atoms at the catalytic centre. The substrates of laccase include polyphenols, aromatic diamines, phenolic acids, methoxy substituted phenols, and other compounds[8,9]. Laccase catalyzes the oxidation of these substrates coupled with the reduction of molecular oxygen to water. One critical factor of laccase is that it oxidizes both the toxic and nontoxic substrates. Laccases are used in thefood industry, textile industry, wood processing industry, chemical and pharmaceutical industries[10]. This enzyme is highly specific and ecologically sustainable and is found to be a proficient catalyst.

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In this light, the present work was carried out to immobilize laccase on a suitable matrix. Various properties were investigated in this study including the kinetic studies of polymer entrapped laccases with respect to the free counterpart. Also, the storage, thermal, and reusability studies of free and immobilized laccase were conducted to explore the industrial applicability.

Materials and Methods

Chemicals

Solvents, buffers, and other chemicals used in this study namely sodium alginate, gelatin, agar- agar, chitosan, copper alginate, sodium dodecyl sulphate (SDS), ammonium per sulfate (APS), N,N-methylene bisacrylamide, N,N,N,N-tetra methylethylenediamine (TEMED) were procured from Sigma chemicals, India.

Immobilization of laccase on different matrices

Immobilization on calcium alginate

Immobilisation was carried out by the previously reported method of Mdaghri[11]. Enzyme solution (0.1 mg/mL) as added to 3% sodium alginate and mixed thoroughly by gentle stirring for 20 min. The beads were formed by dripping the solution into 100 mL of 0.2 M $CaCl_2$ by means of a hypodermic syringe and needle at room temperature (28°C). The beads were kept for hardening at 4°C for 2 h. After incubation, beads were washed 2-3 times in 0.1 M sodium acetate buffer of pH 4.5. The curing solution and washing solutions were collected to calculate the immobilization efficiency.

$IE\% = (A_o\text{-}A_f)/A_o*100$

A_o and A_f are activities of laccase solution and filtrate, respectively.

Immobilization on gelatin

Gelatin (0.6 g) was dissolved in 10 mL 0.1 M sodium acetate buffer (pH 4.5) by continuous stirring for 5 min at 50°C. The solution was poured into a pre assembled glass plate and cooled to room temperature and thereafter incubated at 4°C for 2 h. The gel was then cut into small pieces and washed with deionized water and stored in the buffer at 4°C. The enzyme activity was determined by standard assay protocols[12].

Immobilization on agar- agar

Agar- agar solution 3%13 (*w/v*) was prepared by mixing 3g of agar- agar in 100 mL 0.1 M sodium acetate buffer (pH 4.5) by mild heating at 50°C. It was then mixed with an equal volume of enzyme solution of concentration 0.1 mg/mL in the ratio 1:1. The mixture was casted by pouring into a pre-assembled glass plate at room temperature. After solidification, the gel was cut into small pieces and washed 2-3 times using deionised water and stored in the buffer at 4°C.

Immobilization on copper alginate

Sodium alginate solution (3%)[14] was mixed with enzyme solution of concentration 0.1 mg/mL in acetate buffer (pH 4.5) and gently stirred for 20 min at room temperature. The mixture was dropped into excess of 0.2 M $CuSO_4$. The beads were kept for hardening at 4°C for 2 h, followed by washing in deionised water for storage in acetate buffer (pH 4.5) at 4°C.

Immobilization on Chitosan

Chitosan (2%) was dissolved in 1% acetic acid solution by mild heating at 50°C with continuous stirring[13]. The solution was then dropped to 1M KOH and continued low rate stirring for 2 h at 30°C for bead hardening. Beads were then washed with deionized water and stored in the buffer. The activation of beads was carried out by incubating it with 1.5% gluteraldehyde at 30°C for 4 h. Activated beads were separated by filtering and washed with deionized water to remove unbound gluteraldehyde. Beads were mixed with 10 mL enzyme solution (0.1 mg/mL) and kept overnight at 4°C for enzyme immobilization. Enzyme immobilized beads were separated and washed and immobilized enzyme activity was determined.

Immobilization using Polyacrylamide

Immobilization of laccase by entrapment in polyacrylamide gel[15] was carried out by mixing freshly prepared 1.67 mL acrylamide and N,N'-methylene bis acrylamide (30:0:8) solution with laccase (3 mL), deionized water (0.445 mL), 10% ammonium persulphate (0.375 mL) and TEMED (0.01 mL). This mixture was poured into a petriplate and allowed to solidify at room temperature for 30 min and then kept for 1 h at 4°C. The gel after solidification was cut into small cubes and washed with sodium acetate buffer (50 mM, pH 4.5) to remove the unbound enzyme.

Guaiacol assay method for laccase

Laccase activity was determined using guaiacol as the substrate[16]. The intense brown colour development due to oxidation of guaiacol by laccase can be correlated to its activity and was read at 450 nm. Guaiacol (2 mM) in sodium acetate buffer (10 mM pH 4.5) was used as substrate. The reaction

mixture contained 3 mL acetate buffer, 1 mL guaiacol and 1 mL enzyme source, and blank contained 1 mL of distilled water instead of enzyme source. The mixture was incubated at 30°C for 15 min and absorbance was read at 450 nm using a UV spectrophotometer. Enzyme activity was expressed as International Units (IU), where 1 IU is defined as the amount of enzyme required to oxidize 1 µM of guaiacol per min. The laccase activity in U/mL is calculated using the extinction coefficient of guaiacol (12,100 M^{-1} cm^{-1}) at 450 nm by the formula: E.A = (A*V) / (t*e*v), where E.A = Enzyme Activity (U/mL), A = Absorbance at 450 nm, V = Total volume of reaction mixture (mL), v = enzyme volume (mL), t = Incubation time (min) and e = Extinction Coefficient M^{-1} cm^{-1}).

Characterization of free and immobilized laccases

Scanning electron microscopy (SEM) analysis of free and laccase immobilized copper alginate beads were carried out to study the morphological features.

Optimization of sodium alginate and $CuSO_4$ concentration on immobilization and determination of loading efficiency

Alginate solutions of different concentrations (1, 1.5, 2, 2.5, 3, 3.5, 4% *w/v*) was prepared and the enzyme was added in the ratio 1:4 (*v/v*). It was mixed well and dropped into 0.2 M $CuSO_4$ solution and kept for 1 h incubation. Beads were collected and washed two times with 0.5% $CuSO_4$ (*w/v*) and three times with buffer. The protein concentration of the filtrate and the activity of the wash liquid were determined. The effect of $CuSO_4$ concentration on immobilization was determined by choosing the optimum alginate concentration and mixing it with the enzyme in the ratio 1:4 (*v/v*). It was then dropped into various concentrations of $CuSO_4$ solution (0.1, 0.15, 0.2, 0.25, 0.3 M). The beads after hardening were collected and washed several times with buffer. The protein concentration of filtrate and activity of the wash liquid was determined. The loading efficiency and immobilization yield[17] were defined as the percentage of total enzyme entrapped and specific activity ratio of entrapped laccase to free laccase, respectively.

$$\text{Loading efficiency } \% = ([C_i V_i - C_f V_f]/C_i)*100$$

where, C_i= initial protein concentration, V_i- initial volume of enzyme solution, C_f= protein concentration in the total filtrate, V_f= total volume of filtrate

Effect of pH and temperature on enzyme activity

To study the effect of pH on the activity of the enzyme, both free and immobilized enzymes were incubated with buffers of pH varying in the range 3.0 to 10.0. Buffers such as glycine-HCl buffer (pH 3), sodium acetate buffer (pH 4 and 5), citrate buffer (pH 6), phosphate buffer (pH 7 and 8), and carbonate bicarbonate buffer (pH 9 and 10) were used in the study. The ratio between enzyme activity at each pH value and the maximum attained activity was used to calculate percentage relative activity. In order to investigate the effect of temperature on the activity of the enzyme, both free and immobilized enzymes were incubated at temperatures varying from 20-80°C to find the optimum temperature by performing a laccase assay.

Determination of kinetic properties of free and immobilized laccase

The effect of substrate (guaiacol) on the activities of both free and immobilized forms were studied by using various concentrations of substrate ranging from 2 mM-16 mM. The kinetic parameters K_m (Michaelis-Menten constant) and V_{max} (maximum reaction rate) of free and immobilized enzymes were studied. K_m and V_{max} values were calculated from the Lineweaver-Burk plot.

Effect of metal ions

In order to study the effect of various metal ions on the activity of laccase, the enzyme was preincubated for 15 min with 1 mM of $MgSO_4$, $MnSO_4$, $FeSO_4$, KCl, NaCl, $CaCl_2$, $BaCl_2$, $ZnSO_4$, $CoCl_2$ and $NiCl_2$ prior to substrate addition. Residual enzyme activity was assayed. Control was maintained without adding metal ions.

Thermal stability analysis

Temperature stability of both free and immobilized laccase were analyzed by heating the enzymes in 50 mM sodium acetate buffer (pH 4.5) for 3 h and the residual activity was measured by guaiacol assay of samples taken at each 30 min interval.

Storage stability analysis

Storage stability of free and immobilized enzyme were determined by incubating the samples in 50 mM sodium acetate buffer (pH 4.5) at 4°C or 28 days. The residual activity was measured by guaiacol assay of samples taken at one- week intervals.

Results and Discussion

Fungal laccases has shown more promise and potential in its immobilized form on a support to its free counterpart as the former exhibited improved thermal and storage stability combined with good

performance for reusability[18,19]. In this light, the present study reports the immobilization of laccase on various support systems and evaluates the various parameters.

Immobilization efficiency

The efficiency of different polymers namely sodium alginate, copper alginate, chitosan, agar agar and polyacrylamide on immobilization of laccase was studied and the percentage immobilization efficiency of each polymer is given in (Fig. 1A).

It was found that the immobilization efficiency was maximum for copper alginate and hence can be considered as a promising substrate for immobilization compared with other polymers. This result is well supported by the study carried out by Phetsom *et al*.[20] who reported copper alginate to be the best substrate for immobilizations. Zhang *et al*.[14] also reports that because of the coupled effect of copper ion activation and copper alginate entrapment, the activity of laccase was found to increase by 3.0 fold. Similarly, copper alginate was found to be a better support/matrix than calcium alginate for laccase from *Tramates villosa*[21]. Another study by Kocaturk and Yagar[22] has shown that polyphenol oxidase immobilization was better with copper alginate beads.

Alginate being a biological polymer is most widely used due to its property of biodegradation and its capacity to form gels. The copper alginate beads used in the present study is given in (Fig. 1B). The better efficiency of alginate may be due to the high affinity[23] of copper to alginates.

Characterization of free and immobilized laccase

Scanning Electron Microscopy (SEM) was performed to understand the surface topology of copper alginate beads before and after the entrapment of laccase. There were alterations in the surface morphology of beads with and without laccase entrapment. After immobilisation the polymer surface was found to be rough as the control bead surface was found to be smooth. That might be due to the entrapped enzyme on the porous surface of polymers. The SEM images of the bead without enzyme are shown in (Fig. 1C) and the enzyme entrapped bead is shown in (Fig. 1D & E).

Optimization of sodium alginate and $CuSO_4$ concentration on immobilization and determination of loading efficiency

In the preliminary work, the immobilization conditions (sodium alginate concentration and copper sulphate concentration) for enzyme entrapment in alginate beads that would affect the immobilization yield were studied. The optimal concentration of sodium alginate as a support matrix is very important for enzyme immobilization and substrate penetration in the porous structure of alginate beads. At a lower

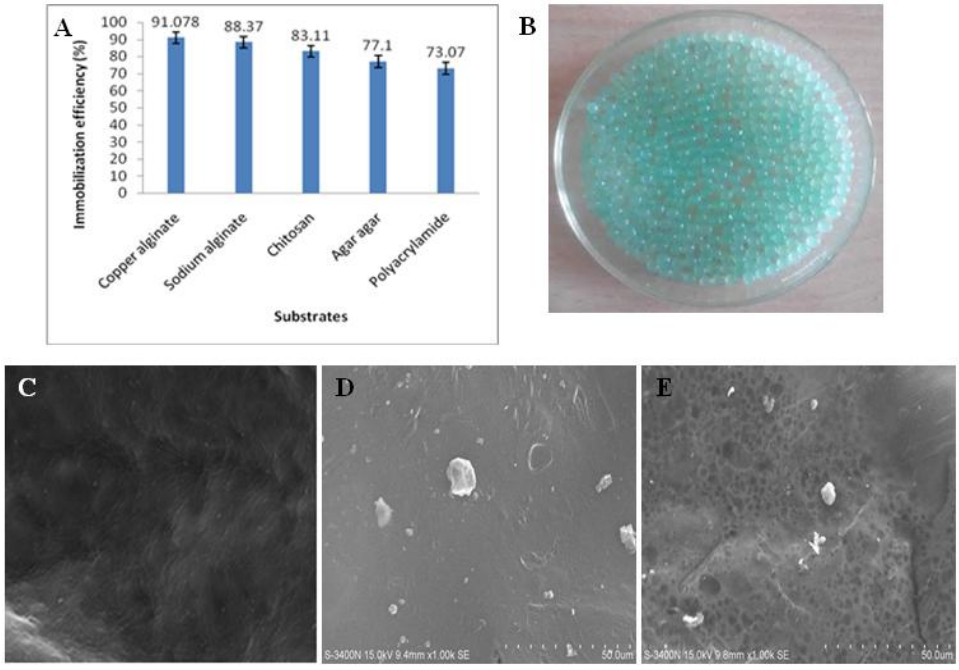

Fig. 1 — (A) Laccase immobilisation efficiency of different substrates; (B) Copper alginate beads; (C) SEM image of copper alginate bead without laccase; (D) and (E) SEM image of enzyme immobilised beads

concentration of sodium alginate the mechanical strength of the bead was found to be very poor. Loading efficiency was found to be maximum (84.25± 2.3%) with 2.5% sodium alginate. However, a gradual decrease in loading efficiency was observed for 3%, 3.5%, and 4% sodium alginate. The optimum concentration of copper sulphate was found as 0.25 M. The loading efficiency increased as the concentration of $CuSO_4$ increased and reached a maximum value of 83.97± 2.188%. It was found that the further increase in copper sulphate concentration cause a decrease in the loading efficiency. The results are shown in (Fig. 2A & B).

This variation in the loading efficiency may be due to the increase in the pore size of the beads. Geethanjali and Subash[24] found that an increase in the concentration of sodium alginate makes the immobilization of enzymes difficult. It may be due to the increase in the degree of cross- linking as the percentage of sodium alginate increases. Sukri and Sakeenah[25] also reported that the loading efficacy of xylanase decreased beyond 3% (*w/v*) of sodium alginate. Based on these results copper alginate was found to be an ideal choice for immobilization.

Effect of pH on enzyme activity

The effect of pH on free and immobilized laccase was studied by carrying out the reaction at a pH range of 3.0 to 10.0 and the results are provided in (Fig. 3). It was observed that the % activity was initially high for the free enzyme than its immobilized counterpart. At pH 4 to 5, the activity was high for free enzyme while the immobilized enzyme showed maximum activity at pH 5. As the pH increased further, there was a significant decrease in the enzyme activity in both conditions indicating that acidic pH was favoring enzyme activity compared to basic pH.

These results are well supported by the report of Bagewadi *et al*.[26] who stated that lower pH was suitable for the activity of laccase isolated from *T harzianum*. Also Mazlan *et al*.[27] reported that the optimum pH for free laccase was found to be 4.0 and immobilized laccase was shifted to pH 5.0. The reduction in the enzyme activity of both the free and immobilized enzyme under increased pH conditions could be because of the deviations in protein folding or may be due to the change in the functional groups[28] located in the active site of the enzyme.

Effect of temperature

The temperature activity profiles of free and immobilized laccase showed 100% relative activities at 50 and 60°C, respectively. Even at highest studied temperature, the enzyme activity of the immobilized enzyme was 35% while the free enzyme showed 25% relative activity (Fig. 4). Similar results were reported by Mdaghri *et al*. (2013)[11] who stated a rise in the optimum temperature by 10°C for lipase in grey mullet and laccase of *Lentinus polychrous* from their free counterpart. Another report by Chaudhary *et al*., (2016)[29] reported that the immobilized laccase

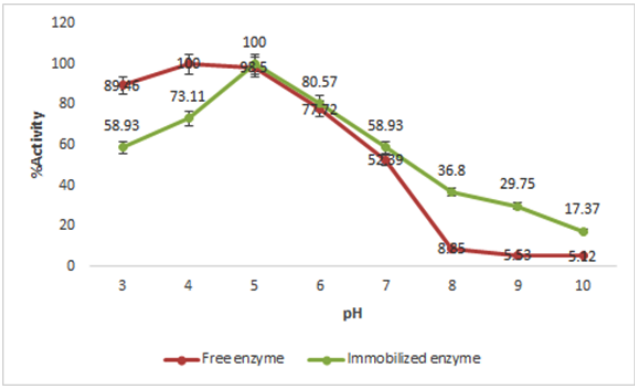

Fig. 3 — Effect of pH on enzyme activity

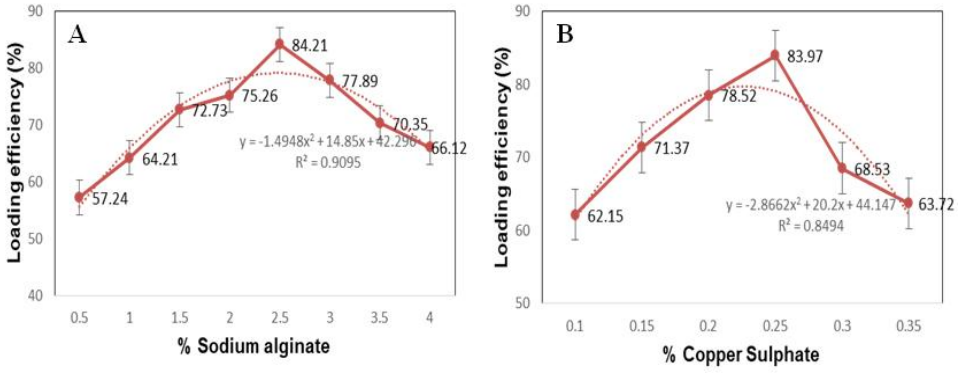

Fig. 2 — Effect of (A) sodium alginate; and (B) copper sulphate on loading efficiency

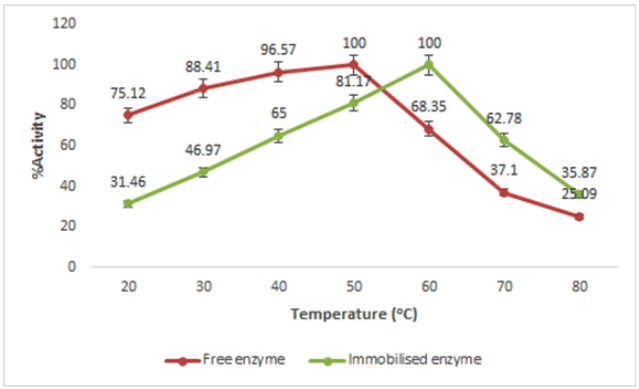

Fig. 4 — Effect of temperature on enzyme activity

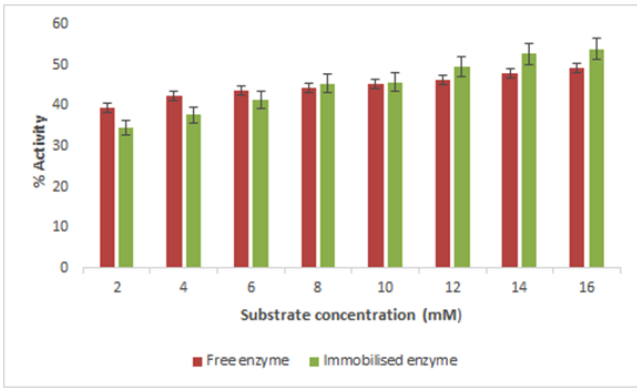

Fig 5 — Effect of substrate concentration on enzyme activity

showed maximum activity at 55°C while free laccase was showing activity at 45°C. This increase in activity of the immobilized enzyme at a higher temperatures may be ascribed to the limitations in the conformational changes after immobilization. It may also be due to the gain in kinetic energy in these molecules that enable them to reach the active site of the immobilized enzyme rapidly which shifts optimum temperature towards higher temperature in the case of immobilized enzymes. Broadening of the optimum temperature range has been observed in various cases due to high intrinsic specific activity (Zuler effect).

Determination of kinetic properties of free and immobilized laccase

In order to study the effect of substrate concentration on activity, free and immobilized laccase was treated with different concentrations of guaiacol (substrate) ranging from 2-16 mM (Fig. 5). The results clearly showed that the activity of the enzyme increased with an increase in substrate concentration. Maximum activity was observed at 10 mM for free enzyme and thereafter remained constant. In the case of the immobilized enzyme, maximum activity was observed at a guaiacol concentration of 14 mM. Chaudhari *et al*., (2016)[29] reported a saturation concentration of laccase with guaiacol as a substrate to be 8 mM or free enzyme and 10 mM after immobilization.

The kinetic constants V_{max} and K_m were determined from the Lineweaver Burk plot. K_m is calculated from the x intercept and V_{max} from the y intercept. Apparent K_m value was found to be increased when compared with that of immobilized enzyme. This shows a slight decrease in the affinity of the enzyme towards the substrate after immobilization. The apparent K_m values were found to be 0.480 mM for free enzyme and 1.277 mM for immobilized enzyme. The decrease in affinity may be due to factors like change in enzyme conformation, steric effect, change in micro environmental conditions *etc*. Shujing *et al*.[30] reports that the laccase isolated from *Mycena purpureo fusca* showed a K_m value of 0.296 mM when ABTS is used as the substrate. The low K_m value shows that the enzyme has more affinity towards ABTS. The maximum velocity of the immobilized enzyme increased when compared to the free counterpart. The V_{max} values were found to be 48.076 U/mL and 55.55 U/mL, respectively, for the free and immobilized enzyme (Fig. 6A & B). 1U (µM/min) is defined as the amount of the enzyme that catalyzes the conversion of one micromole of substrate per minute under the specified conditions of the assay method. Other studies also reported an increase in K_m[31,32], which clearly indicates an apparent low affinity of the enzyme towards its substrate in contrast to the free form. Fatarella *et al*.[33] also reported that the apparent K_m value after immobilization got increased and the V_{max} showed an increase compared to the free enzyme. The ratio of V_{max}/K_m (K_{cat}) defines a measure of catalytic efficiency of an enzyme- substrate pair. K_{cat} was found to be 100.01 s^{-1} and 43.5 s^{-1}, respectively, for free and immobilized enzymes. The ratio K_{cat}/K_m gives an indication of how well an enzyme can differentiate between two different competing substrates. In another study by Sergio *et al*.[34], the laccase isolated from *Pycnoporus sanguineus* showed the K_{cat}/K_m ratio of 9.4 × 106 s^{-1} M^{-1} for ABTS as substrate. In the present study, the ratio K_{cat}/K_m was found to be 208.35 mM^{-1} s^{-1} and 34.064 mM^{-1} s^{-1}, respectively, for free and immobilized laccase and therefore, Guiaiacol was shown to be an efficient substrate.

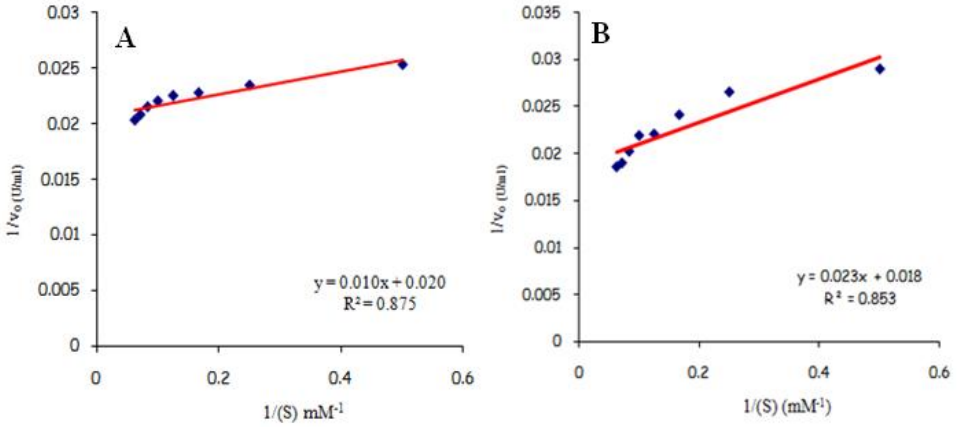

Fig. 6 — Line-weaver Burk plot of (A) free; and (B) immobilised laccase

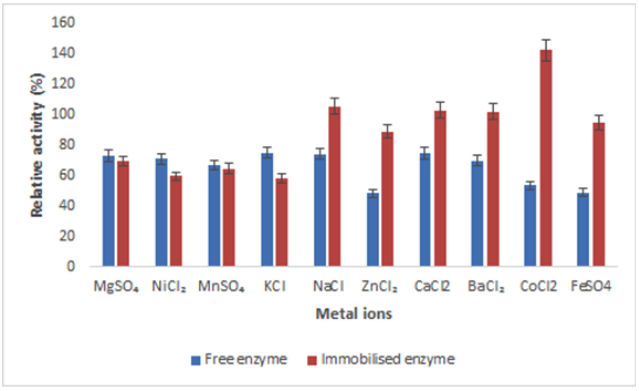

Fig.7 — Effect of metal ions on enzyme activity

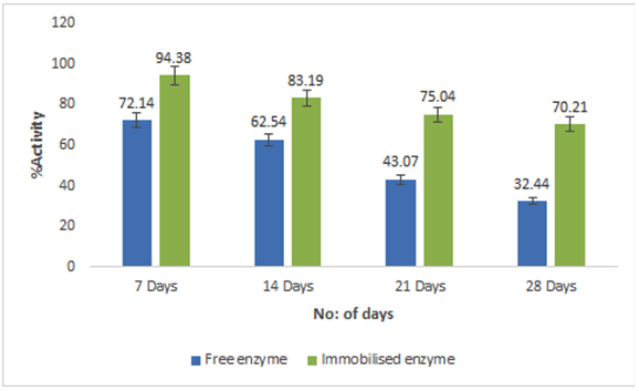

Fig. 8 — Storage stability of the free and immobilized enzyme

Effect of metal ions

It is a well-known fact that some enzymes associate with metals as cofactors that aid in the activity of the enzyme. On the contrary, they may affect the conformation of the enzyme thereby reducing the enzyme activity. The effect of metals is greatly dependent on the nature of metal ions and the type of enzyme involved. Therefore, in this study, an attempt has been made to understand the influence of various metal ions on the activity of free as well as immobilized laccase (Fig. 7). It has been observed that the effect of metals was quite varied in both conditions. The enzyme activity without added metal ions was taken as 100% activity for both free and immobilized laccase. The graph clearly shows the relative activity of both free and immobilized enzymes in the presence of various metal ions. In the presence of $FeSO_4$and $ZnCl_2$, the free enzyme could retain only48.8% of the initial activity, whereas, in the presence of Mg^+, K^+, Na^+, Ca^+ ions the enzyme retained above 70% of initial activity. It was reported that relative activity of laccase from *Nigrospora*[35] was found to be increased by 15% in the presence of Cu^{2+}, 18% in the presence of Na^+. In the case of the immobilized enzymes, the activity was enhanced in the presence of Co^{2+} ions. The relative activity was found reduced in the presence of Ni^{2+}, Mn^{2+}, and K^+ ions.

Storage stability

Owing to the enormous applications of enzymes in various industries, storing the enzyme for long periods with the retention of biological activity is of supreme importance. The stability of free and immobilized laccase during storage was determined by keeping the enzymes at 4°C for four weeks (Fig. 8). The enzyme immobilized on copper alginate showed greater stability than the free laccase enzyme. After the first week, the activity of the free enzyme was 72.14% and 94.38% for immobilized enzyme. The activity was found to be declining with respect to time for both free and immobilized enzymes. After

four weeks of storage at 4°C, the residual activities exhibited by free and immobilized laccase were found to be 32.44% and 70.21%, respectively.

The storage stability is an important criterion for biocatalyst mediated processes because it affects the economics of industrial bioprocesses, which are closely linked to the production cost of the enzyme. Studies carried out by Zhang *et al.*[14] showed that immobilized laccase retained 89% of activity after storage at 4°C for 40 days whereas, free laccase displayed only 48% activity. Lin *et al.*[36] also reported that free laccase activity decreased faster in comparison to laccase immobilized in metal ion chelated magnetic microspheres. Laccases are usually immobilized through adsorption, or covalent linkage to a surface or entrapment. The method of immobilization on any surface is an indispensable factor in its activity as this process should ensure the preservation of the native structure of the enzyme in the appropriate orientation that is responsible for its binding and activity.

Thermal stability

Temperature is one physical parameter that markedly effects the functioning of the enzyme. Higher temperatures lead to loss of structure thereby leading to loss of activity. The stability of an enzyme at higher temperatures is an added advantage to the functionality of the enzyme. In the present investigation, the free and the immobilized enzyme were incubated at 60°C for 3 h to study the effect (Fig. 9). It was observed that as the time of incubation increased, the activity was reduced in both the free and immobilized enzymes. However, the immobilized enzyme retained 89.72% of initial activity after 30 min of incubation and 32.60% activity after 3 h. But free laccase retained only 55.01% of activity after 30 min and 1.14% after 3 h of incubation. These

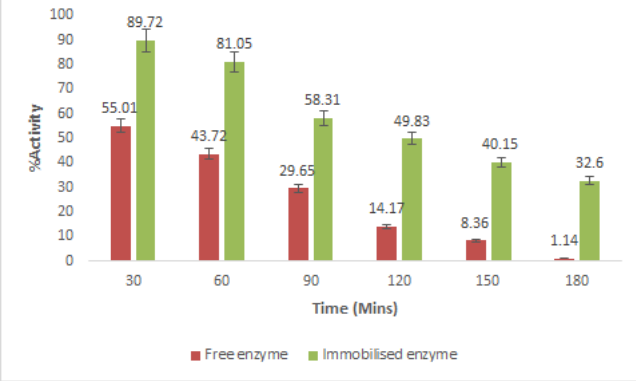

Fig. 9 — Thermal stability analysis

results indicate that immobilization has increased the stability of enzymes at high temperature. The stability of enzyme at high temperatures favors commercial applications of the enzyme

It was suggested that additional bonds established between enzyme and support during immobilization that promoted stabilization of immobilized enzyme[37]. It was reported earlier that immobilized spore laccase[38] were very stable at 60°C. In another study, laccase from *T. versicolor* immobilized on chitosan beads[39] showed more thermal stability than free laccase. The results obtained in this study are well in agreement with the report of Metin[40] as they stated that free laccase retained 39% of original activity at 55°C for 120 min incubation and immobilized laccase retained 72% of its original activity for the same period at 55°C. Another study showed that immobilization was effective in enhancing the stability for a concoction of enzymes protease, amylase, lipase, and cellulose *Aspergillus niger* and *Phanerochaete chrysosporium* using cellulose nanoparticles[41].

Reusability studies

One of the primary objectives of the immobilization of enzymes is reusability. It shows the ideal number of cycles each batch of immobilized enzymes could be used. The reusability data is critical for industries as it influences the cost of production. Every batch of the enzyme has its optimum number of cycles, and if used less will increase the production cost or if used more would decrease the quality of the enzyme. Hence determining reusability is important to assess the retained activity of the enzyme after each cycle of reaction. In the present study, the immobilized enzyme was used for the decolorization of methyl orange (Fig. 10).

It was found that the decolorization of methyl orange was 70.6% after first cycle and reduced to 32.04% at the end of the fifth cycle. Yinghui[42] reported that laccase enzyme immobilized in PVA- alginate could be reused for 10 cycles with a retained activity of 60%. Zou[43] reported that natural nano- structured bacterial cellulose could be used to immobilize laccase and immobilized enzyme proved to be stable and reported that 69% of the original activity after recycling seven times. In another study, Lloret[44] reports that laccase immobilized sol- gel matrix was tested for the continuous elimination of Acid Green 27 dye as a model compound in a packed-bed reactor (PBR). Removals of 70, 58, 57, and 55% were achieved after four consecutive cycles with limited adsorption on the support. A recent study demonstrated

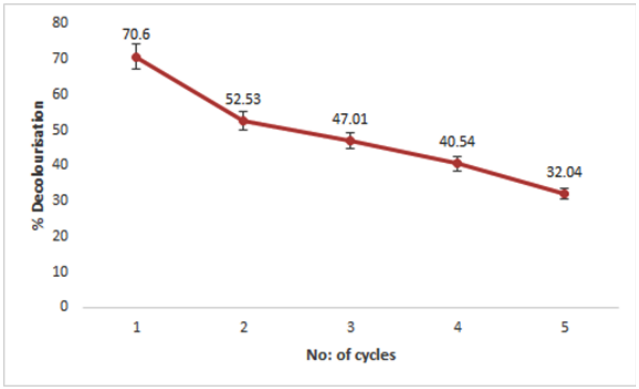

Fig. 10 — Reusability analysis

the immobilization of laccase on nanoparticle- mediated robust physisorption on natural cellulose and also proved their efficacy in reusability.[45]

Conclusion

The efficiency of laccase in industry and environment makes its study interesting. The present investigation was carried out to compare the efficacy of free and immobilized enzymes on various substrates and determine the various parameters affecting the activity of the enzyme. The enzyme was immobilized in copper alginate as it was found that the immobilization yield in copper alginate was comparatively highest compared with other polymers. The immobilized enzyme was found to be temperature tolerant (55°C) compared to its free counterpart. The K_{cat} values were found to be 480.76 U/mg and 555.50 U/mg, respectively, which proves the catalytic efficiency, is more for immobilized enzyme. The immobilized enzyme retained 70.21% of its activity even after storage at 4°C for one month were as the free enzyme could retain only 32.44% of its activity. Also, it was proved that the immobilized enzyme could be used for repeated cycles of application without much loss in activity thereby suggesting the immobilized enzyme as a promising catalyst for industrial applications.

Conflict of interest

All authors declare no conflict of interest.

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