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Preparation and characterization of cross-linked enzyme crystals of laccase

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Abstract

Laccase from *Trametes versicolor* was crystallized using ammonium sulphate and the resultant crystals on cross-linking with glutaraldehyde produced insoluble and catalytically active enzyme. These cross-linked enzyme crystals (CLEC) of laccase had improved thermal stability (four-fold) than the native enzyme. The half-life of CLEC laccase at 60 °C was 123 min compared to 24 min for the soluble enzyme. The kinetics of oxidation reactions catalyzed by CLEC laccase was studied using various substrates 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid) (ABTS), guaiacol, catechol, pyrogallol, syringaldazine and catechin. ABTS was found to be the best substrate for CLEC laccase (K_m = 0.859 mM) and had a catalytic efficiency ($k_{cat}/K_m = 3.73 \times 10^3$) higher than the other substrates. The CLEC laccase showed lower specific activity, V_{max} and k_{cat} values than the native enzyme for all the substrate studied and this may be due to the partial inactivation of laccase crystals by glutaraldehyde, and also the diffusion limitation of the substrate through the channels in the cross-linked crystal structure of laccase enzyme. CLEC laccase had a higher activity in non-polar organic solvents like hexane, toluene, isooctane and cyclohexane. The preparation and characterization of CLEC laccase is reported for the first time.

Keywords: Laccase; CLEC; Thermal stability; Kinetics; Crystallization; Cross-linking; Organic solvent

1. Introduction

Enzyme stabilization [1] is one of the major challenges in the biocatalytic process optimization. Enzymes have to be used at higher temperatures, shear rate and organic solvent environments for the production [2] of pharmaceuticals, agro chemicals, consumer care products, etc. Immobilization of enzymes is one of the techniques used by the industries to bring down the cost of the process by reusing [3] the enzymes. However, immobilization has few disadvantages such as dilution of catalyst, instability in higher temperatures and low activity loading [4] on the support (0.1–10%, w/w). Cross-linked enzyme crystals (CLEC) technology [5] is one of the most exciting developments in the area of biocatalysis.

Cross-linking of enzyme crystals brings about both stabilization and immobilization of enzyme without dilution of activity [6]. Cross-linked enzyme crystals are prepared by controlled precipitation of enzymes into microcrystals followed by cross-linking using bifunctional reagents to form strong covalent bond between free amino acid groups in the enzyme molecules [7]. In a CLEC, the lattice interactions in the enzyme crystal when fixed by inter- and intramolecular chemical cross-links provide additional physical and thermal stability [8]. In an immobilized enzyme, the enzyme is linked by point attachment to a two-dimensional solid surface, but a protein in cross-linked crystal is stabilized by links in all three-dimensional structure. Hence, CLECs are highly active, recyclable and having good mechanical stability. Cross-linked enzyme crystals have additional advantage of good stability under solvent, shear stress, temperature and storage conditions [9].

Laccase [10] from *Trametes versicolor* (E.C.1.10.3.2), one of the polyphenol oxidase (*p*-diphenol oxidase), belongs to the family of blue multi-copper oxidase of low specificity. Laccase catalyzes the reduction of molecular oxygen to H_2O and does not require H_2O_2 as cosubstrate. The active site of laccase consists of four copper ions involved in a coordinated oxygen reduction. Copper sites have been historically divided into three classes: copper type 1 (T1) or blue copper, type 2 (T2) or normal copper

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and type 3 (T3) or coupled binuclear copper center based on their spectroscopic features, which reflect the geometric and electron structure of the active site. This enzyme couples the oxidation of the substrate, which takes place at type 1 copper site with the reduction of dioxygen to water taking place at the types 2 and 3 trinuclear copper cluster site [11]. Laccase can catalyze the oxidization of various polyphenols, methoxy phenols and diamines but not tyrosine. Laccase is being used in biosensor [12], biofuel cell [13] and in biotransformations [14] applications. Development of cross-linked crystals of laccase will pave way to its enormous applications in biocatalytic process.

2. Experimental

2.1. Materials

Laccase from *T. versicolor* (E.C.1.10.3.2) (18,830 IU/mg of powder, ABTS units) was purchased from Fluka and glutaraldehyde, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid) (ABTS), catechol, catechin, pyrogallol and guaiacol were purchased from Sigma (St. Louis, USA). Ammonium sulphate (Enzyme grade) was purchased from SISCO Laboratories, India. All other reagents used were of analytical grade.

2.2. Crystallization of laccase

Laccase was crystallized by batch method. Fifty milligrams of crude laccase was dissolved in 3 ml of sodium acetate buffer, 0.1 M at pH 5.6. Ammonium sulphate (75% saturation) was added in small portions over 3 h by stirring at 5 ± 1 °C. Then, the super saturated solution was kept at this temperature undisturbed for 20 h. The crystals formed were separated by centrifugation at 2000 rpm for 8 min and washed with isopropanol to remove excess $(NH_4)_2SO_4$.

2.3. Cross-linking of laccase crystals

Twenty milligrams of laccase crystals was cross-linked using 2 ml of glutaraldehyde solution at different concentrations [0.5–3.0%, v/v] in isopropanol for 20 min at 25 °C. After cross-linking, it was washed three times with 0.1 M sodium acetate buffer at pH 5.6 to remove excess glutaraldehyde and stored in the same buffer at pH 5.6.

2.4. Enzyme assay measurement

The laccase assay [15,16] was done with 0.1 mg of enzyme in a reaction medium containing the substrate in 0.1 M sodium acetate buffer at pH 5.0 and the color of the oxidation products was read at the appropriate wavelength in a spectrophotometer (Shimadzu, UV 2100). The oxidation product of ABTS was read at 420 nm (ε_{max} = 36,000 M^{-1} cm^{-1}), of guaiacol was read at 470 nm (ε_{max} = 26,600 M^{-1} cm^{-1}), of syringaldazine in 10% DMF was read at 530 nm (ε_{max} = 65,000 M^{-1} cm^{-1}), of catechol was read at 450 nm (ε_{max} = 2211 M^{-1} cm^{-1}), of pyrogallol was read at 450 nm (ε_{max} = 4400 M^{-1} cm^{-1}) and that of catechin in 10% methanol was read at 390 nm (ε_{max} = 4019 M^{-1} cm^{-1}). The unit of enzyme activity was expressed in units/mg of enzyme protein.

2.5. Assay of CLEC laccase

The assay mixture containing the substrates in 0.1 M sodium acetate buffer, pH 5.0, 1 mg CLEC laccase was added and stirred continuously for 1 min, and then the increase in absorbance was monitored at definite time intervals.

2.6. Determination of physical characteristics

2.6.1. Density

Density of the CLEC laccase was obtained using a helium Auto pycnometer (Micromeritics model 1320).

2.6.2. Surface area

Surface area was measured by the Brunauer, Emmett and Teller (BET) technique using a Zetasizer (Melvern, UK) in which liquid nitrogen was the adsorbent.

2.6.3. Crystal morphology

The crystal morphology was observed under a scanning electron microscope (JEOL, Japan) at 10 kV accelerating voltage, after sputtering with gold.

2.7. Kinetics of thermal inactivation

Kinetics of thermal inactivation of CLEC and soluble laccase were studied at different temperatures between 40 ± 0.5 °C and 70 ± 0.5 °C in a shaking water bath (Julabo). One milligram of CLEC/native enzyme was added to 2.0 ml of 0.1 M acetate buffer, pH 5.0, and kept in a constant temperature bath at desired temperature. After each half an hour interval, the assay of the biocatalyst was carried out by rapidly cooling the reaction mixture to room temperature.

The thermal inactivation constant was determined from the Arrhenius equation [17].

$$K = A \exp\left(-\frac{E_a}{RT}\right)$$

where E_a is the energy of activation and R is the universal gas constant (8.314×10^{-3} kJ $kmol^{-1}$ K^{-1}).

A plot of log % residual activity versus time was drawn at different temperatures, the slope gave the values of inactivation rate constant k_r [18].

2.8. Activation energy calculation

The thermal stability of CLEC and soluble laccase was determined by the inactivation rate constant (k_r) as a function of temperature, in the range 40–70 °C, the temperature dependence of k_r was analyzed from Arrhenius plot ($\ln k_r$ versus $1/T$). The activation energy (E_a) was obtained from the slope of the plot.

Half-life of CLEC and soluble laccase was calculated from the quadratic equation for each temperature.

2.9. Enzyme kinetics

Kinetics of soluble enzyme as well as CLEC was done at 0.1 M sodium acetate buffer, pH 5.0, by varying the substrate concentration, while keeping the concentration of biocatalyst constant (1612 IU/mg of CLEC laccase). Using Enzyme Kinetics Software "Kinetics Pro" (add on module of Sigma plot), the Michaelis–Menten constant (K_m) and velocity maximum V_{max} were calculated from Hanes plot (a plot of [S] versus [S]/[V]). From those values, k_{cat} and catalytic efficiency k_{cat}/K_m values were calculated.

2.10. Organic solvent stability

Organic solvent stability of the CLEC laccase was done by incubating 1 mg of CLEC in various organic solvents and organic solvent–water (50%, 75% and 100%) mixtures for 30 h. The organic solvents were chosen according to the hydrophobicity, dielectric constants and log P values. After the incubation period, the assay of CLEC laccase was done by the procedure mentioned earlier. The activity was compared with the original activity and the activity relation was calculated.

3. Results and discussions

Laccase enzyme was crystallized by batch crystallization method and the crystals obtained were of 2–25 μM in size (Fig. 1). The general properties of CLEC laccase are given in Table 1. A glutaraldehyde concentration of 0.5–1.0% (v/v) did not produce good cross-linked crystals and the crystals got dissolved in the assay mixture. The best glutaraldehyde concentration was found to be 1.5% for cross-linking and further increase in the concentration of glutaraldehyde reduced the catalytic activity of the crystal (Fig. 2).

3.1. Thermal stability of CLEC laccase

Thermal stability of CLEC laccase was improved by four times after the cross-linking. According to earlier reports, the thermal stability of CLECs can be two or three orders of magnitude greater than that of the soluble enzyme. The crystalline

Fig. 1. Scanning electron microscopic view of CLEC laccase.

Table 1
General properties of CLEC laccase

Property	CLEC laccase	Soluble laccase
pH optimum	5.5	3.0
Thermal stability	Up to 60 °C	Up to 50 °C
Specific activity	1612 IU/mg crystal	18830 IU/mg protein
K_m (ABTS) (mM)	0.8595	0.141
V_{max} (ABTS) (μmol/min/mg)	2873.2	21155.5
k_{cat} (ABTS) (S^{-1})	3.21	23.62
k_{cat}/K_m (ABTS) (M^{-1} S^{-1})	3.73×10^3	1.67×10^5
Crystal size (m^2/g)	2–25	
Surface area (m^2/g)	2.456	

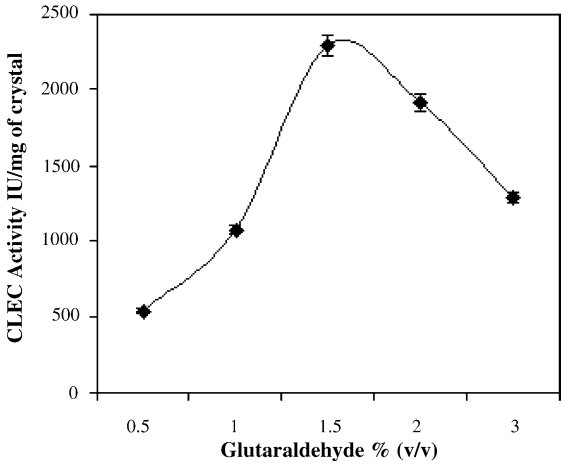

Fig. 2. Cross-linked crystals activity of laccase at different glutaraldehyde concentrations.

enzyme maintains its native conformation at elevated temperature and having lower tendency to aggregate. This is because in CLECs, the enzyme molecules are symmetrically arranged and hence their native conformation is stabilized. When an enzyme forms a crystal, a very large number of stabilizing contacts are formed between individual enzyme molecules [19]. Energy must be put into the system in order to disrupt these new contacts, so that additional energy is required to break the covalent cross-links before the CLEC begins to dissolve and then denature. The stabilization is also due to the lattice contacts in the crystal rather than from chemical cross-links. The relative activity of CLEC laccase increased above 400% after 90 min incubation in 40 °C (Fig. 3). After that, the activity was decreasing and the half-life of CLEC laccase at 40 °C was found to be 558 min (Table 2). At 50 °C, the activity increased to 300% after 60 min of incubation, and then it started decreasing. The half-life of CLEC laccase was found to be 453 min at 50 °C

Table 2
Half-life of soluble and CLEC laccase at various temperatures

Temperature (°C)	CLEC (min)	Soluble laccase (min)
40	558	304
50	453	112
55	229	55
60	123	24
70	25	6

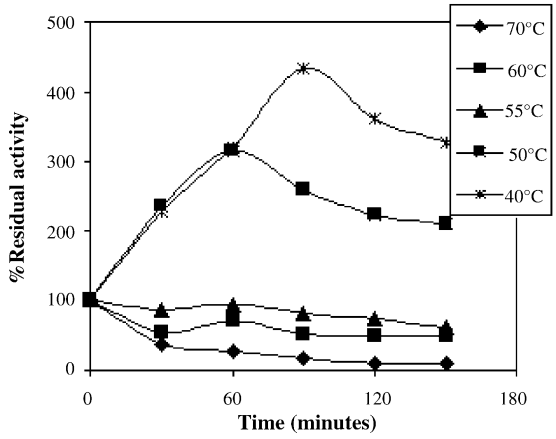

Fig. 3. Thermal activation/inactivation of CLEC laccase at different temperatures.

and 229 min at 55 °C. The thermal stability started decreasing above 55 °C. The half-life of CLEC laccase was 123 min at 60 °C and 25 min at 70 °C, whereas native laccase had a half-life of only 24 min at 60 °C and 6 min at 70 °C. The relative activity of native enzyme at 40 °C increased to 150% after 1 h of incubation (Fig. 4). Above 40 °C, the activity started decreasing when the incubation time increases. The activation energy (E_a) of CLEC and native laccase were found to be $-31.59\,kJ\,kmol^{-1}\,K^{-1}$ and $-55.04\,kJ\,kmol^{-1}\,K^{-1}$. CLEC laccase had activation energy of $14.45\,kJ\,kmol^{-1}\,K^{-1}$ higher than that of native enzyme. Inactivation rate constant k_r was determined from the slope of the plot log % residual activity versus time of incubation (Fig. 5). Arrhenius plot of thermal inactivation (Fig. 6) showed that native enzyme had a steep inactivation curve from 70 °C to 40 °C. However, in CLEC laccase, there was a steep inactivation from 70 °C to 60 °C after that there is little decrease in thermal stability, and as such the CLEC laccase does not obey Arrhenius equation and the E_a obtained is not the true one (because the slope was not accurate). Native laccase had thermal stability and half-life four times lower than that of CLEC. The increased thermal stability

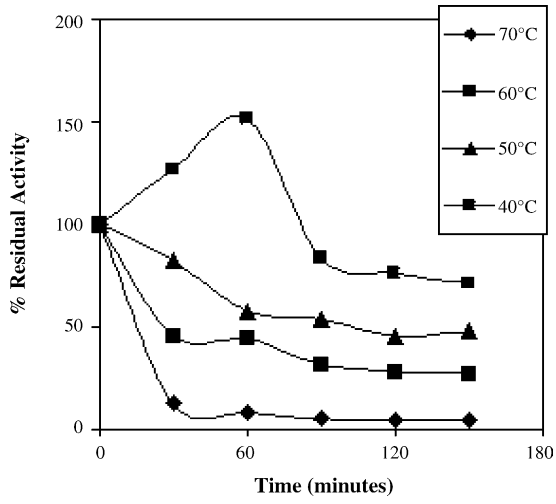

Fig. 4. Thermal activation/inactivation of soluble laccase at different temperatures.

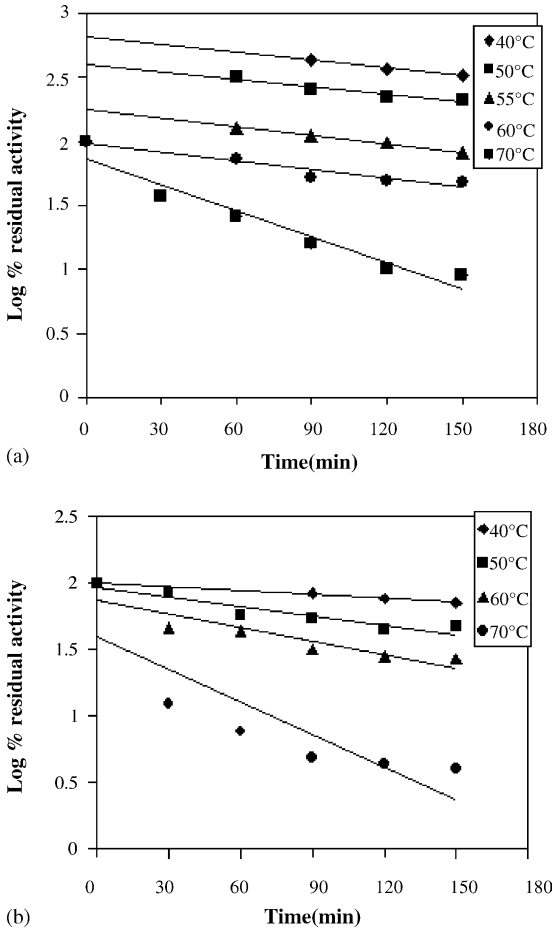

Fig. 5. Determination of k_r at different temperatures: (a) CLEC laccase and (b) soluble laccase.

may be due to the pre-ordered arrangement of the molecules by inter- and intramolecular cross-links between the crystals, and hence the rigidity of the three-dimensional arrangement of molecules in the CLEC [20]. The increased thermal stability of

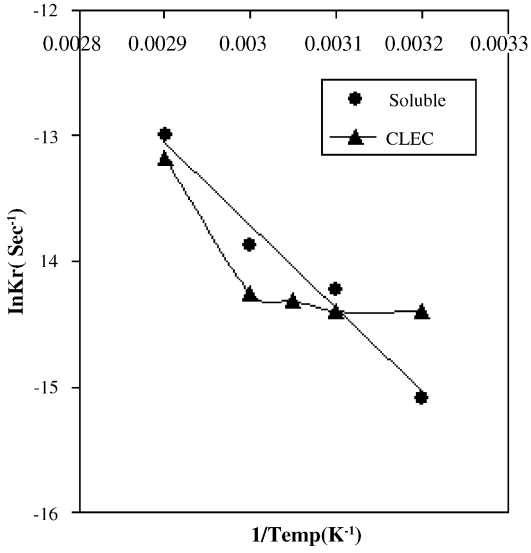

Fig. 6. Arrhenius plot for the thermal inactivation of soluble and CLEC laccase.

Table 3
Kinetic constants for the oxidation of various substrates by soluble laccase

Substrate	V_{max} (μmol/min/mg)	k_{cat} (S^{-1})	K_m (mM)	k_{cat}/K_m ($S^{-1}\,M^{-1}$)
ABTS	21155.5	23.62	0.141	1.67×10^5
Catechol	5699.9	6.36	0.539	1.18×10^4
Pyrogallol	2067.0	2.31	0.149	1.54×10^4
Guaiacol	5014.0	5.60	0.623	0.90×10^4
Syringaldazine	8084.6	9.03	0.741	1.22×10^4
Catechin	21490.8	23.99	0.778	3.08×10^4

Table 4
Kinetic constants for the oxidation of various substrates by CLEC laccase

Substrate	V_{max} (μmol/min/mg)	k_{cat} (S^{-1})	K_m (mM)	k_{cat}/K_m ($S^{-1}\,M^{-1}$)
ABTS	2873.2	3.210	0.859	3.73×10^3
Catechol	787.1	0.879	2.717	0.32×10^3
Pyrogallol	487.2	0.544	1.059	0.51×10^3
Guaiacol	72.7	0.081	1.113	0.07×10^3
Syringaldazine	39.4	0.044	1.684	0.03×10^3
Catechin	1876.0	2.094	2.084	1.00×10^3

CLEC laccase offers major advantages to the organic chemist, to perform the laccase catalyzed reactions at higher temperature thereby increasing the reaction rate.

3.2. Kinetic studies of CLEC laccase

The Michaelis–Menten constant (K_m) and velocity maximum V_{max} were calculated from Hanes–Woolf plot using Kinetics Pro software (Figs. 7–12 given in supplementary information). The kinetic constants of CLEC as well as native laccase were determined by the oxidation of various substrates such as ABTS, syringaldazine, catechol, catechin, guaiacol and pyrogallol (Tables 3 and 4). The catalytic efficiency of native enzyme is better than CLEC laccase for all the substrate studied. The V_{max} of the CLEC is only 12% of that for the soluble enzyme with the most reactive substrate ABTS. The lower catalytic efficiency of CLEC laccase is due to the partial inactivation of the enzyme crystals by glutaraldehyde during cross-linking and also due to the diffusion limitations in the crystal structure. The diffusional loss is also evidenced by the increase of activity with temperature and the lower % V_{max} lost (23%) with the slower reacting pyrogallol. Inactivation by the high level of covalent cross-linking is also likely to be a major source of inactivation. In *T. versicolor* laccase, type 3 Cu either 1 or 2 is bound to three histidine and oxygen atom, while type 2 copper is coordinated to only two histidines and weakly to a water molecule [21]. It is reported in the previous studies that during crystallization, the enzyme was trapped in a form devoid of the type 2 copper and this depletion of T2 Cu is known to render laccases inactive [22]. Another possibility for the lower catalytic efficiency may be due to the inadequate exposure of the active centers of laccase for substrates, or the channels in the crystal form are too narrow where the substrates or products could not diffuse into it [23], and also due to the rigidity of enzyme molecules after cross-linking will not allow any changes in the conformation of active site in order to accommodate the substrate. Even in the absence of diffusion limitations, as in the case of small substrates the enzyme active centers may be sterically hindered by the neighboring enzyme molecules and thus be inaccessible to the substrate [24]. ABTS was found to be the best substrate for CLEC and native laccase with low K_m values 0.859 and 0.141, respectively. This may be due to the affinity of ABTS, to transfer the charge between Cu(II) (type 2 copper) to S of ABTS [25]. The best catalytic efficiency (k_{cat}/K_m) of native and CLEC of laccase was found to be 1.67×10^5 and 3.73×10^3, respectively, for ABTS oxidation. Catechol and guaiacol showed lower catalytic efficiency (k_{cat}/K_m). Guaiacol forms biphenoquinone after oxidation, and catechol and syringaldazine also form colored quinones, which may also inhibit the enzyme action [26].

3.3. Stability of CLEC laccase in organic solvent

According to Lanne et al., high biocatalytic activities are favored in solvents having log *P* between 2 and 4 [27]. CLEC laccase had higher activity in non-polar organic solvents (Table 5) such as hexane, toluene, isooctane and cyclohexane due to their lower dielectric constant (∼2) and higher log *P* values (2.73–4.0). CLEC had medium activity in organic solvents such as ethyl acetate, chloroform, isopropanol and acetone. Their log *P* values are in between 0.73 and −0.24 and the dielec-

Table 5
Stability of CLEC laccase after incubation with organic solvent–water (50%, 75% and 100%) mixtures for 30 h (activity retention in %)

Solvent	Dielectric constant	log *P*	Polarity	100% solvent	50% solvent	75% solvent
Hexane	1.9	4.0	Non-polar	29.6	83.6	37.5
Toluene	2.4	2.73	Non-polar	50	95	58
Isoocatane	2.1	4.5	Non-polar	42	90	83
Cyclohexane	2.0	3.44	Non-polar	24	68.5	42.5
Ethyl acetate	6.0	0.73	Dipolar aprotic	2.3	52.6	30
Chloroform	4.8	1.97	Non-polar	5.1	52.9	11.7
Isopropanol	19.9	0.05	Polar protic	5.0	40.5	9.5
Acetone	20.7	−0.24	Dipolar aprotic	1.6	50.5	3.5
Ethanol	24.6	−0.30	Polar protic	3.5	10.5	N.D.
Methanol	32.7	−0.74	Polar protic	0	4.3	N.D.
Acetonitrile	37.5	−0.34	Dipolar aprotic	2.14	10.2	N.D.
Water	80.2	–	Polar aprotic	53		

tric constant between 4.8 and 20.7. Solvents such as ethanol, methanol and acetonitrile were found to have very low activity due to their higher dielectric constant. There was no activity detected for CLEC laccase in solvents such as DMSO, THF and 1,4-dioxane. The decrease in activity in these polar solvents is due to the stripping of water from the surface of the enzyme by competing through hydrogen bonds between the protein atoms [28–30]. The increase in stability of CLEC in organic solvents is due to the lattice contacts in the crystal after crystallization. Cross-linking increases the rigidity of the enzyme molecules and hence reduces the unfolding of the three-dimensional structure of the protein by the organic solvents. But the native laccase lost its full activity within 3 h after incubation with different solvents.

4. Conclusions

Cross-linked enzyme crystals of laccase had good thermal stability (four-fold) than native enzyme. CLEC laccase had a lower K_m and k_{cat}/K_m probably due to the change in microenvironment around the active site and any diffusion limitation of the substrate through the channels in the cross-linked crystal structure. ABTS was found to be the best substrate with lower K_m value due to the charge transfer between Cu(II) (type 2 copper) to sulphur atom of ABTS. The CLEC laccase was stable in polar organic solvents such as hexane, toluene, isooctane and cyclohexane. The same CLEC laccase has been used in an ampherometric biosensor for the determination of phenols such as 2-amino phenol, guaiacol, catechol, pyrogallol and catechin in 50–1000 μmol concentration level [31] and continuous biotransformation of pyrogallol to purpurogallin in a Packed Bed Reactor [32].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2005.11.001.

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