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Kinetics and mechanism on laccase catalyzed synthesis of poly(allylamine)–catechin conjugate

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ABSTRACT

The conjugation reaction of catechin with poly(allylamine) has been studied in aqueous methanol using free, immobilized and cross-linked enzyme crystals (CLEC) of laccase from *Trametes versicolor* with particular emphasis on the effect of pertinent variables and kinetic aspects of the reaction. The stability of the CLEC was better than that of the free and immobilized enzymes for practical application. The kinetics of the conjugation reaction conformed to the so-called Substituted-enzyme mechanism with catechin inhibition. In case of immobilized laccase system marginal diffusional limitation could be inferred from the experimental data. The kinetic parameters $K_{m(paa)}$ and $K_{m(cat)}$ estimated by non-linear regression analysis were found to be 0.75, 1.8967, 2.135 and 11.769, 15.1816, 29.489 for free, immobilized and CLEC laccase, respectively.

1. Introduction

Catechins are the basic structural units of condensed tannins and belong to the class of flavon-3-ols and are found in a wide variety of vegetables, herbs and teas [1,2]. The growing interest of the tea catechins in the recent years is attributable to the evidence of a relationship between tea consumption and prevention of certain forms of human diseases including atherosclerosis, emphysema, ulcerative colitis, diabetes, multiple sclerosis, rheumatoid arthritis, Parkinson's diseases and cancer [3–5]. However, the use of catechins is limited because of their poor water solubility and easy degradability by light irradiation in aqueous solution resulting in rapid browning [6]. Catechin was also reported to have pro-oxidant effect in the aqueous phase and generate reactive oxygen species, such as hydrogen peroxide [7]. Structure–activity relationship studies of flavonoids have shown that the antioxidant activity is attributable to the electron donating ability of the phenolic hydroxyl groups in the structure [8,9]. In contrast, relatively high molecular fractions of tea flavonoids have been reported to exhibit enhanced physiological properties for a relatively longer period in-vivo correlating with no pro-oxidant effect [10]. From these perspectives in recent years, several studies have been conducted on oxidative coupling for catechin derivatives in order to improve both the physiological and pharmacokinetic properties of the catechins [11–13]. Oxidation of catechins for instance has been achieved by using polyphenol oxidase [14], peroxidases or laccase [15,16] or by chemical means [17,18]. Enzymatic synthesis of catechin conjugate with poly(allylamine) using laccase has been reported in literature [19]. The conjugate of poly(allylamine)–catechin offers improved physiological properties compared to those of unconjugated catechin.

Since enzymatic reactions have advantages over chemical synthesis in terms of low energy requirement, high product quality due to stereospecific and regioselective transformation of the substrate, environmental friendliness, etc. We have been studying enzyme catalyzed reactions with emphasis on kinetic and mechanistic aspects [20–24]. There is now a growing interest towards the synthetic derivatives of tea catechins with prolonged antioxidant activity against LDL per oxidation. Successful application of the enzymes requires stabilization of the enzyme, which is often affected by the solvent media. Immobilsation is a commonly used method for enzyme stabilization, storage and reusability. However, CLEC technology appears to be an attractive technological preposition perhaps due to its high activity and stability are superior to both crude and conventionally immobilized enzymes [25]. CLECs are prepared by controlled precipitation of enzymes in a suitable medium into microcrystals followed by cross-linking using bifunctional reagents such as glutaraldehyde to form strong covalent bonds between free amino acid groups in the enzyme molecules [26]. CLEC has additional advantages of good solvent stability, shear stress, temperature and storage conditions [27,28]. In view of this, we attempt to study the kinetics and mechanism of laccase catal-

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ysed conjugation of poly(allylamine) with catechin in dispersed as well as immobilized media. A complementary study of the reaction has also been made using CLEC of laccase.

2. Materials and methods

2.1. Materials

Laccase from *Trametes versicolor* (E.C. 1.10.3.2) (0.96 U/mg) was procured from Fluka. Catechin, poly(allylamine) (mol. wt. 65,000), glutaraldehyde were procured from Sigma–Aldrich, USA. Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium acetate, acetic acid, methanol, acetone, dimethyl sulfoxide, calcium acetate, celite 545 powder were procured from CDH Pvt. Ltd., New Delhi, India. All solvents used for this study were procured from CDH Pvt. Ltd., New Delhi, India and purified by distillation and dried over 3 Å molecular sieves prior to use.

2.2. Preparation of CLEC laccase

Laccase was crystallized by a batch method studied in our previous work [23] wherein one hundred milligrams of crude laccase was dissolved in a 5 ml of 50 millimolar (mM) phosphate buffer (pH 7.0) prepared from potassium dihydrogen phosphate and dipotassium hydrogen phosphate. Then 5 ml of 1.0 M calcium acetate and 5 ml of 30% dimethyl sulfoxide was added and stirred for 4 h at 25 °C. The solution was kept at this temperature for 24 h and then the crystals formed were separated by centrifugation and washed with isopropyl alcohol. The crystals were cross-linked in 5% (v/v) glutaraldehyde solution in 50 mM phosphate buffer of pH 6.5 at 4 °C for 6 h. After cross-linking, the crystals were filtered and washed three times with 20 ml of 0.02 M acetate buffer (pH 4.5) prepared from sodium acetate and acetic acid by repeatedly adding fresh buffer solution to remove excess glutaraldehyde. The cross-linked enzyme crystals were characterized by FTIR (PerkinElmer, system 2000) and stored at room temperature until use.

2.3. Preparation of immobilized laccase

For immobilization of laccase, celite 545 was used as a support. The support powder (2.0 g) was added to 5 ml aqueous solution of laccase containing 60 mg/ml enzyme and stirred with a magnetic stirrer at room temperature for 1 h following a method reported for immobilization of enzymes such as lipase [23]. Then 20 ml of chilled acetone was added and the suspension filtered through a buchner funnel. The immobilized enzyme was washed on the filter paper with another 20 ml of chilled acetone and dried in vacuum dessicator for 4 h. The amount of laccase immobilized on the support was estimated from an analysis of laccase concentration in the aqueous phase with a UV–vis spectrophotometer (Shimadzu, Model 6A) before and after the immobilization and calculated from the equation

$$W = \frac{(C_i - C_f)V}{m_i} \quad (1)$$

where C_i and C_f are the initial and final concentration of laccase in mg/l, V is the volume of laccase solution (l) and m is the weight of adsorbent or immobilized media (g) taken initially. Protein concentration in the aqueous phase was determined by using bovin serum albumin as standard [29]. The enzyme loading was found to be 10.2 mg/g of solid support.

2.4. Preparation of catechin conjugate

Synthesis of catechin conjugate of poly(allylamine) was performed by using laccase as a catalyst derived from *T. versicolor* [19].

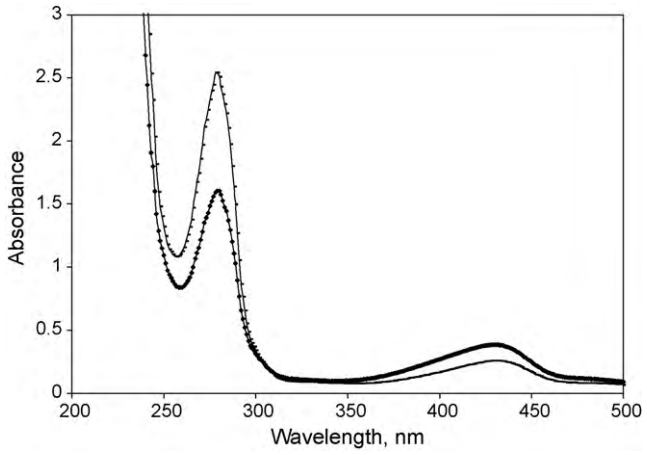
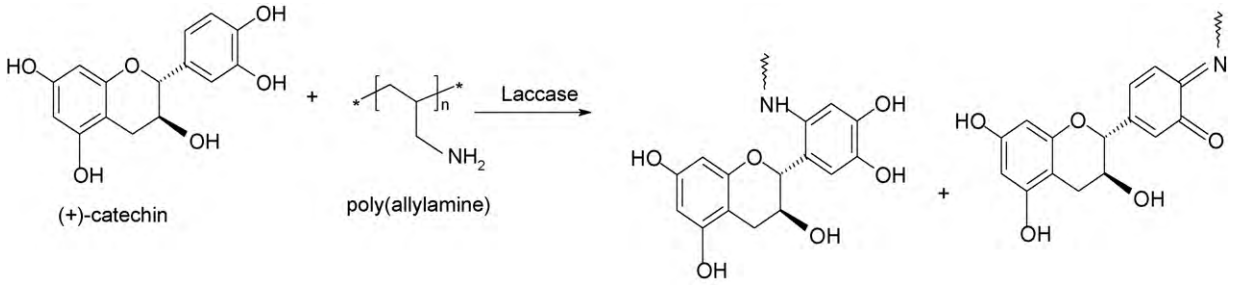


Fig. 1. Typical UV–vis spectra of conjugation reaction of catechin and paa catalysed by laccase from *Tramates versicolor*.

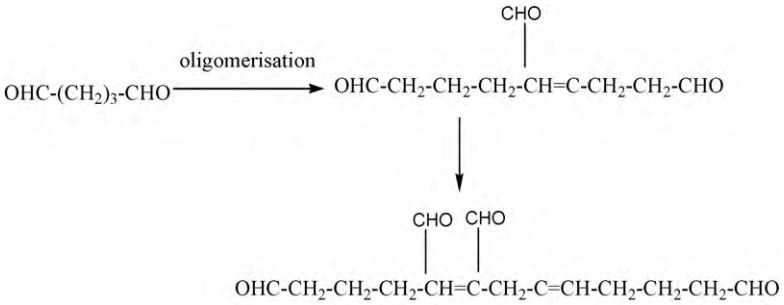
The kinetic experiments were carried out in a 50 ml round bottom flask in which the reaction mixture was agitated vigorously with a magnetic stirrer. The temperature, laccase and substrate concentrations were varied systematically from 35 to 50 °C, 2.5–10 mg/ml and 10–100 mM respectively using 70% aqueous methanol as the solvent [24]. Aliquots of the samples were withdrawn at regular interval of time and analyzed by UV–vis spectrophotometer using a calibration curve at 280 nm wavelengths for catechin concentrations. A typical UV–visible spectra of the reaction mixture is shown in Fig. 1. The concentration of poly(allylamine) was estimated from a material balance based on the UV analysis of feed and product mixture. The UV analysis was carried out in duplicate and the reproducibility was found to be ±2%. The formation of the desired conjugate with the structure of Michel type adduct and/or Schiff base (Scheme 1) was ascertained from a characteristic peak at 430 nm (Fig. 1), which was however not seen in the laccase catalyzed oxidative coupling of catechin under the similar reaction conditions [24]. In case of the catechin conjugate with chitosan [30] or polyhedral oligomeric silsesquioxane [31] an identical peak was observed at the same wavelength. The initial reaction rates were calculated from the conversion versus time profiles corresponding to the first 10% conversion below which the profiles were found to be linear. The rate was expressed as the amount of substrate converted per unit time and weight of catalyst (mM/hr g). All the experiments were carried out in triplicate and the reproducibility was found to be ±2%.

2.5. Determination of laccase stability

For determining the laccase stability the reaction was conducted with free, immobilized and CLEC laccase for a fixed period of time. Preincubation experiments were carried out by incubating the enzyme at a concentration of 2.5 mg/ml with 20 ml of 10 mM catechin in 70% aqueous methanol solvent system and/or otherwise at different temperatures and for a specific duration. After this, 20 ml of 100 mM poly(allylamine) was added to make poly(allylamine)–catechin conjugate and then the reaction was carried out as usual. This was the general procedure followed in all the experiments conducted for stability study at specified substrate concentration and temperature.



Scheme 1. Laccase catalysed conjugation of catechin with poly(allylamine).



Scheme 2. Oligomerisation of glutaraldehyde [27].

3. Results and discussions

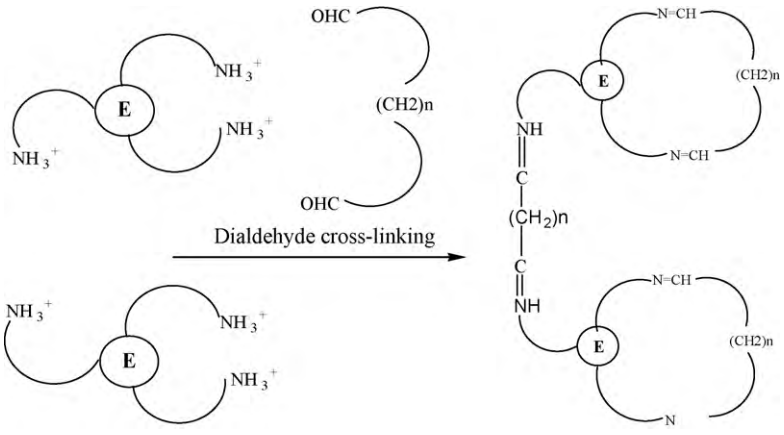
3.1. Characterisation of CLEC laccase

The cross-linking of enzyme with gluteraldehyde involves the reaction between this bifunctional reagent and the residual free amino acid groups of the enzymes. Glutaraldehyde forms a mixture of oligomers of different lengths and structures in aqueous solutions. The long chain of oligomers of glutaradehyde formed in aqueous solutions is given in Scheme 2. Cross-linking with glutaraldehyde forms strong covalent bonds between the amino groups of lysine residues within and between the enzyme molecules (i.e., intra- and intermolecular imine cross-linking) in a crystal lattice as shown in Scheme 3 [27].

The IR spectra for gluteraldehyde, laccase and CLEC laccase before and after cross-linking were recorded. The IR absorption band corresponding to the aldehyde group of gluteraldehyde was observed at 1710.8 cm^{-1} and that of amine group in the protein at 3445.7 cm^{-1}. After cross-linking the IR stretching band for the aldehyde group disappeared and a band for the imine was observed at 1650.3 cm^{-1} attributable to cross-linking of laccase to glutaraldehyle. The SEM photograph of the CLEC laccase is given in Fig. 2 and was taken in a scanning electron microscope (M/S Carl Zeiss, UK, model: LEO 1430 VP) from which it appears that CLEC laccase comprises of crystal structure. The value of particle size estimated from SEM photograph was found to lies in the range of 2–25 μm, which corresponds to the value reported in literature for CLEC laccase cross-linked by glutaraldehyde [26]. The atomic particle size of the CLEC laccase was also analyzed by Laser particle size analyzer (Mastersize 2000 Model, Malvern Instruments Ltd., UK) and the average particle size was found to be 10.47 μm. Apparently the values obtained by the Laser particle size analyzer was taken for interpretation of diffusional effect presented in as subsequent section.

3.2. Effect of enzyme concentration

The effect of enzyme concentration was interpreted from its relationship with initial rate of the reaction. As shown in Fig. 3, the initial rate increases almost linearly with enzyme concentration in



Scheme 3. Schematic representation of inter- and intramolecular imine cross-linking of enzyme crystals through dialdehyde coupling [27].

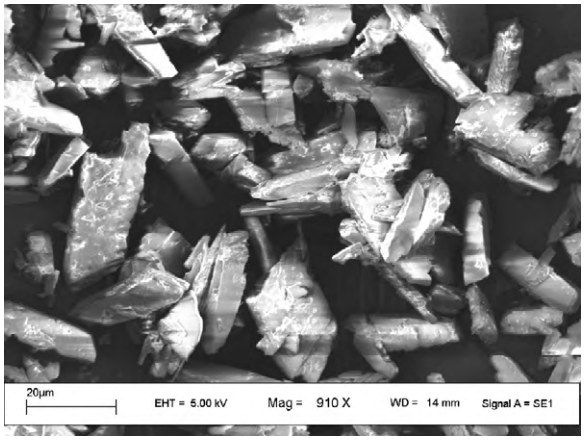


Fig. 2. Scanning electron microscopic view of CLEC laccase.

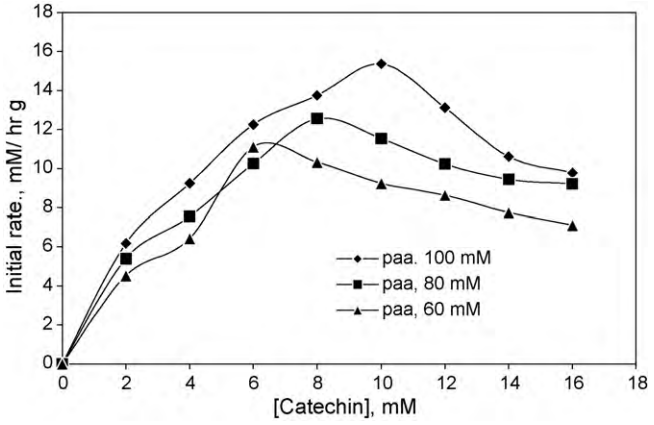


Fig. 4. Initial velocity as a function of catechin concentraton at a fixed concentration of paa. Free laccase = 2.5 mg/ml.

the lower range of laccase concentration and then the rate increases more slowly, reaching an asymptote at a ceratin laccase concentration depending on the reacting system. The higher observed rate for the immobilized laccase may probably attributable to the active form of the amino acids has been adsorbed in the immobilized media. Similar observation also attributable to higher active form of the amino acid crossed linked to gluteraldehyde [25,26] while keeping the amount of active enzyme in the reaction system constant throughout the system. This implies that the reaction is kinetically controlled at low laccase concentrations in agreement with the results for lipase catalyzed esterification [23] and transesterification reactions [21] and laccase catalyzed polymerization of catechol [32]. The observation of relatively slow increase of initial rate at high enzyme concentrations may be attributed to enzyme saturation with the substrate.

3.3. Effect of substrate concentration

The effect of poly(allylamine) concentration was studied by keeping laccase and the catechin concentration constant and the results are presented in terms of variation in initial rate as a function of catechin concentration for various concentrations of poly(allylamine) as shown in Fig. 4. The rate of increase of reaction rate is more pronounced at higher poly(allylamine) concentration and within the range of variables studied here, a catechin to poly(allylamine) molar ratio of 1:10 gives the highest initial reaction rate beyond which there is no further increase in rate. Such an observation may be attributed to the inhibition effect induced by the higher catechin concentration. Thus, optimum catechin: poly(allylamine) ratio may be considered as 1:10.

3.4. Effect of temperature on initial rate

The effect of temperature on the conjugation reaction was studied over the temperature range of 30–70 °C for free, immobilized and CLEC laccase systems under otherwise identical reaction conditions. The initial rate increases almost linearly in the lower range of temperature up to a certain point and thereafter it decreases with increase in temperature as shown in Fig. 5. In case of free laccase the decrease of initial rate is more pronounced as compared to those observed for immobilized and CLEC laccase systems. Enzymes are biocatalysts exhibiting intrinsically lower activation energy specially for free enzyme systems [33]. However, immobilizing the enzyme, diffusion may become rate limiting and the reaction may switch from kinetic to diffusion controlled resulting in a change in the measured apparent E_a. Accordingly, we determined the activation energy for laccase catalyzed conjugation of catechin with poly(allylamine) in free, immobilized and CLEC systems. The activation energy (E_a) was determined by using the Arrhenius rate equation (Fig. 5)

$$k = A\,e^{-E_a/RT} \quad (2)$$

where k is the rate constant (s^{-1}) of the reaction, A is the Arrhenius pre-exponential factor, R is the gas constant (1.987 cal K^{-1} mol^{-1}) and T is the absolute temperature (K). The Arrhenius plots for the conjugation reaction using free, immobilized and CLEC laccase are shown in Fig. 6. The estimated value of the activation energies (E_a)

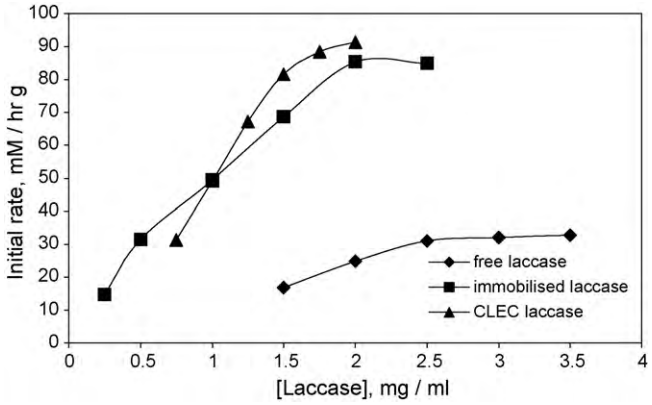


Fig. 3. Effect of free laccase concentration on initial rate of conjugation reaction. The reaction mixture consist of [catechin] = 10 mM, [paa] = 100 mM.

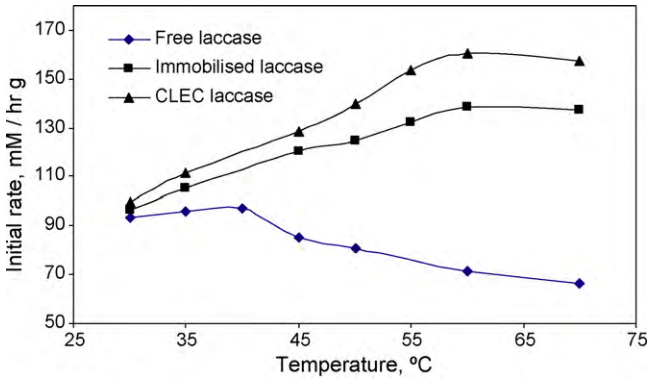


Fig. 5. Effect of temperature on conjugation of catechin with paa. *Reaction conditions*: [paa] = 100 mM; [cat] = 10 mM; [laccase] = 2.5 mg/ml.

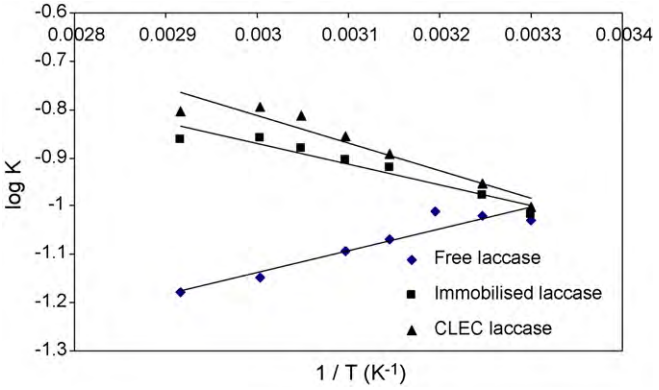


Fig. 6. Activation energy curve based on Fig. 5.

was -20.85×10^3, 19.34×10^3 and 26.05×10^3 J mol^{-1} respectively for free, immobilized and CLEC laccase systems respectively. It is apparent that the value of E_a for free system is lower than those estimated for immobilized and CLEC systems which appears to be in conformity with the rate versus temperature relationship as shown in Fig. 4.

3.5. Reaction mechanism

For detail kinetic study, methanol water mixture has been considered as the solvent system for the entire range of study [24]. A plot of reciprocal of both initial rate and substrate concentration as shown in Fig. 6 has elucidated the reaction mechanism. It is apparent that an increase in poly(allylamine) concentration at constant catechin concentration increases the initial rate. The observation of decrease in initial rate with increase in catechin concentration apparently reflects the catechin inhibition effect. Similar inhibition effect was also observed for transesterification reactions catalysed by immobilized lipase [22]. The plots in Fig. 7 show that this behavior is typical of the so-called Substituted-enzyme mechanism for

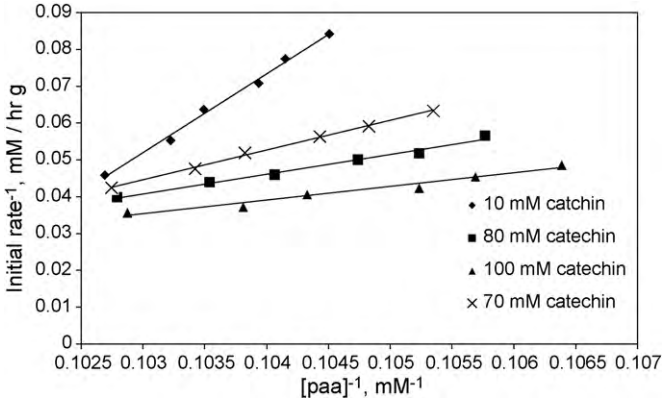


Fig. 7. Reciprocal plot of paa concentration and initial rate at various concentrations of catechin. Free laccase = 2.5 mg/ml, temperature = 35 °C.

which the initial velocity equation is [23]

$$\frac{V}{V_{max}} = \frac{[paa][cat]}{K_{m(paa)}[cat](1 + [paa]/k_i) + K_{m(cat)}[paa] + [paa][cat]} \quad (3)$$

with [paa] and [cat] representing the initial molar concentration of poly(allylamine) and catechin respectively, $K_{m(paa)}$ and $K_{m(cat)}$ are the respective affinity constants, k_i is the inhibition constant of poly(allylamine) and V_{max} is the maximum reaction rate. The kinetic parameters, $K_{m(paa)}$, $K_{m(cat)}$, k_i and V_{max} were estimated from the equation for reaction velocity by numerical parameter identification using the Gaussian–Newton algorithm of error minimization [20] and their values are given in Table 1, wherein the literature values for free, immobilized and CLEC laccase are also given. The kinetic parameters for three enzyme systems reported in Table 1 appear to be in conformity with the rate versus laccase concentration relationship as shown in Fig. 3. The catalytic efficiency of native enzyme is better than CLEC laccase for oxidation of reactive substrate like 2,2′-azinobis(3-ethylbenzo-thiozoline-6-sulfonic acid) (ABTS) having V_{max} 12% that for the soluble enzyme. This may be 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. 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 laccase oxidation of catechol and catechens shows lower catalytic efficiency due to the formation of coloured quinines which may inhibit the enzyme action. This may be the reason for the higher K_m values for the polyphenolic compounds.

It is apparent from the values of these parameters that CLEC laccase is more active than the both free and immobilized laccase. In general, some enzymes on porous particles of discrete sizes exhibit low activity due to mass transfer limitations and interfacial deactivation [35]. In our study, the internal diffusion effect has not been tested in detail due to the unavailability of the catalyst of different sizes, but it may be expected that this effect is also insignificant with the particle size used in the present system. This aspect has been semi quantitatively analyzed from the classical theory of internal diffusion effect in heterogeneous catalysts [36] and by considering two well-known parameters viz. catalytic effectiveness factor (η) and Thiele modulus (Φ_0) which can be defined by the following equation:

$$\eta = \frac{3}{r}\left(\frac{D_e}{k_1 \rho A_g}\right)^{1/2} \quad (4)$$

and

$$\Phi_0 = r\left(\frac{\rho A_g V_m}{D_e[S]}\right) \quad (5)$$

where D_e is diffusivity (cm^2 s^{-1}), ρ is density of particle (g cm^{-3}), r is the radius of the particle (cm), A_g is surface area of the particle (cm^3), k_1 is rate constant (s^{-1}), and [S] is concentration of poly(allylamine) (mM). The values of D_e were estimated from the Wilke–Chang correlation [37]. The values of r, A_g and ρ were determined experimentally and V_m was taken from Table 2. The

Table 1
Comparison of kinetic parameters for free, immobilized and CLEC laccase.

Substrate	Enzyme	System	V_{max} (mmol/ming)	K_m (mM)	K_m (mM)	k_i (mM)	Reference
Catechin	Laccase	Conjugation	31.25	0.75_{PAA}	11.76_{cat}	10.43	This work
Catechin	Immobilized laccase	Conjugation	33.33	1.89_{PAA}	15.18_{cat}	31.61	This work
Catechin	CLEC (laccase)	Conjugation	50.00	2.13_{PAA}	29.48_{cat}	30.42	This work
ABTS	Laccase	Oxidation	5.2	0.26	–	–	[34]
ABTS	Immobilized laccase	Oxidation	3.5	0.16	–	–	[34]
Catechin	Laccase	Oxidation	21.49	0.77	–	–	[26]
Catechin	CLEC (laccase)	Oxidation	18.76	2.08	–	–	[26]

Table 2
Repeated use of free, immobilized and CLEC laccase.

Laccase	No. of use	Concentration of catechin (mM)	Retained activity
Free	First	1.59	100.00
	Second	0.25	0.15
Immobilized	First	2.22	100.00
	Second	2.16	95.09
	Third	1.99	93.94
CLEC	First	2.34	100.00
	Second	2. 29	98.03
	Third	2.24	97.25

Reaction conditions: laccase 2.5 mg/ml; [catechin]: 10 mM; [paa]: 100 mM; temperature: 35 °C; reaction time: 60 min.

numerical values of η and Φ_0 were 0.9987 and 3.2215×10^{-5} respectively, implying marginal internal diffusion effect. These values of η and Φ_0 are consistent with the results obtained for transesterification reactions catalysed by immobilsed laccase from *Pseudomonous cepesia* [22].

3.6. Stability of laccase

The batch stability test experiments were conducted for free, immobilized and CLEC laccase by incubation of the laccase for different times. The initial rates of reaction versus incubation time for the three enzymes are shown in Fig. 8 and it appears that the CLEC exhibits higher stability than immobilized, which is more stable than crude laccase. This may be due to selective cross-linking of the more active form of the laccase with gluteraldehyde [23], thereby imparting higher initial rate. The relatively low stability of immobilized laccase may be due to the weak interactions arising through physical adsorption. The higher reactivity of CLEC may also reflect the higher stability as suggested elsewhere [25].

In order to assayed reusability of the enzyme, the free laccase was recovered from the reaction mixture by lowering the pH of the solution to its isoelectric point (IP). The immobilized and CLEC laccase was filtered off, washed with distilled water, and reused in three successive batches of experiments, and the activity was found to be unaffected even after the third use in both the cases as shown in Table 2.

Stability studies of CLEC laccase in different organic solvents were made on the basis of their solvent hydrophobicity ($\log P$) values and reported by Roy and Abraham [26]. In some other enzyme catalysed reactions, the activity increases with increase of $\log P$ with a plateau in a s-shaped curve [20,38–40]. In our case, less hydrophobic methanol exhibits the highest activity and have been reported in our previous communication [24]. It is also

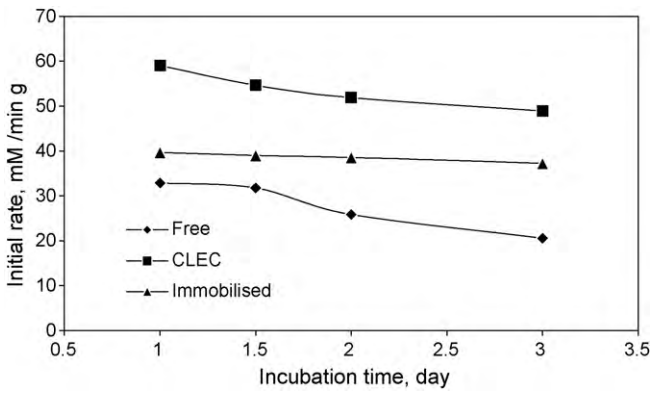


Fig. 8. Initial rate as a function of incubation time.

reported that the catalytic efficiency of some enzymes decreases with increase in substrate hydrophobicity [21] and a linear free energy relationships exist between the catalytic efficiency of both substrate and solvent hydrophobicities. 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 [26], but the native laccase lost its full activity within 3 h after incubation with different solvents. This suggests that the CLEC is the best option for practical applications.

4. Conclusion

The conjugation reaction of catechin with poly(allylamine) catalyzed by free, immobilized and CLEC laccases was affected by reaction conditions such as laccase concentration, temperature, substrate concentration, etc., and the reaction was found to conform to the Substituted-enzyme mechanism. The activity of CLEC laccases was found to be higher than the other forms of the laccase and the activation energy values were found to be in the order of CLEC > Immobilised > free laccase.

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