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Laccase catalysed conjugation of catechin with poly(allylamine): Solvent effect

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ABSTRACT

The *Trametes versicolor* laccase catalysed the synthesis of poly(allylamine) catechin conjugate by conjugation of catechin with poly(allylamine) was studied in 11 different solvents in order to deduce the solvent effect through an attempt to correlate the initial reaction rate with solvent properties such as hydrophobicity ($\log P$), water solubility ($\log S_w$), electron pair acceptance (E_T^N) and donation abilities (D_N^N), polarisibility and dielectric constant. The initial rate was found to exhibit reasonable correlation with $\log P$, $\log S_w$, $E_T^N + D_N^N$, polarisibility and dielectric constant. The probable explanation for the deviation has been put forward based on established hypothesis. The study revealed, in general that polar solvents favour the initial reaction rate. The organic solvent interferes neither with the laccase–substrate binding process nor with the catalytic mechanism. The contribution of the substrate–solvent interactions to enzyme kinetics was accounted by replacing the substrate concentration by thermodynamic activities.

1. Introduction

Catechin, a bioflavonoid found in Green Tea leaves has various applications in food and pharmaceutical industries for their valuable properties mostly as antioxidant [1]. In recent years, catechins have been increasingly used as a natural ingredient in foodstuffs and feedstuffs for various purposes due to their antioxidant properties for obtaining certain desired components initially retained in a food matrix. 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 [2]. 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 [3]. From these perspectives enzymatic synthesis of catechin conjugates with poly(allylamine) using laccase have been reported in literature which was the first example of laccase catalysed synthesis of conjugate of flavonoid into a poly(allylamine) by oxidative coupling [4]. The conjugate of poly(allylamine)–catechin offers improved physiological properties compared to those of unconjugated catechin.

The potential advantages of enzyme catalysis are low energy requirement, enhanced selectivity and quality of the product. However, solvent selection is a very important factor for the successful application of enzymes in organic reaction systems. The effect of organic solvents on the stability and catalytic activity of laccases depends on the source of the enzyme and purification processes [5–7]. The addition of water miscible organic solvents to the aqueous medium of enzymatic reactions usually reduces the observed reaction rate. In most of the cases, the solvent may directly interact with the enzyme, thereby changing their protein structure and exchanging water molecules in the active center, causing irreversible inactivation of the enzymes [8–11]. However, in all cases the addition of organic solvents affects various physicochemical properties of the medium of enzymatic reaction such as its hydrophobicity, dielectric constant, pH, varying the chemical potentials of all reactants present in the solutions and the free energy of binding substrate binding by the enzyme, even when the observed maximum reaction rates remain practically not affected. We have been studying kinetics and mechanism of lipase catalysed esterification and transesterificataion reactions with emphasis on the pertinent solvent properties with the reaction rates [12–15]. In spite of the importance of laccase catalysis in organic solvents, systematic study of the effect of solvents and their properties, on reaction rate has not been reported in the literature. A part of our research was for the development of a manufacturing process for the extraction of tea polyphenolic derivatives from tea leaves of Assam, India, and their stabilisation in different storage conditions. We have studied the conjugation reaction of catechin with poly(allylamine) in the presence of different forms of laccase (free, immobilized and cross-linked enzyme crystals). In this article, we present a comprehensive study on solvent effect and kinetics of conjugation reaction of catechin with poly (allylamine) using *Trametes versicolor* laccase as a catalyst which is very

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Table 1
Pertinent properties of the solvents used for the study[a].

Sl. No.	Solvent	log *P*	log S_w	E_T^N	D_N^N	Dielectric constant	Polarizability (in units of 10^4)
1	Methanol	−0.74	Miscible	–	–	32.50	–
2	Ethanol	−0.32	Miscible	–	–	24.60	–
3	Acetonitrile	−0.33	Miscible	–	–	37.50	6.33
4	Acetone	−0.23	Miscible	–	–	20.70	4.40
5	Ethyl acetate	0.70	0.21	–	–	6.00	9.70
6	Ethyl ether	0.85	−0.24	0.12	0.49	4.34	10.20
7	Chloroform	2.00	−1.12	0.26	0.10	4.81	9.50
8	Tolune	2.50	−1.80	0.09	0.00	2.38	11.80
9	Carbon tetrachloride	3.00	−1.93	0.09	0.00	2.24	11.20
10	Cyclohexane	3.20	−2.25	–	–	2.00	11.00
11	Hexane	3.50	−2.39	0.07	0.00	1.88	11.90

[a] log *P* is the logarithm partition coefficients in the octanol–water system. log S_w is the logarithm of the saturated solubility of water in the solvent on molar basis, E_T^N is the normalized electron pair acceptance index and D_N^N is the normalized Gutmann donor number. Sources of data: Ref. [12,13,15,37].

much important for scaling up the reaction from process point of view.

2. Materials and methods

2.1. Chemicals

Laccase from *T. versicolor* (E.C. 1.10.3.2), (0.96 U/mg), was procured from Fluka and (+)-catechin, poly (allylamine) (mol. wt. 65,000) were procured from Sigma Aldrich, USA. The solvents used for this study have been given in Table 1, wherein the values of different properties of the solvents have also been mentioned. The solvents were procured from CDH Pvt. Ltd., New Delhi, India. Organic solvents were purified by distillation and trace amount of water was removed by using 3 Å molecular sieve.

2.2. Preparation of catechin conjugate

Synthesis of catechin conjugate of poly (allylamine) was performed according to the method described by Chung et al. using laccase derived from *T. versicolor* [4]. The experiments on solvent effect were carried out under the optimum reaction conditions with 10 mM catechin and 100 mM poly (allylamine) dissolved in a solution of 70% aqueous organic solvent in the presence of 2.5 mg/ml of free laccase from *T. versicolor*. The reactions were carried out in a 50-ml round bottom flask by mixing the reaction substrate at a rotating speed of 150 rpm at 35 °C for a period of 6 h. Aliquots of the samples were withdrawn at a regular interval of time and analysed by UV–vis spectrophotometer. A typical UV–vis spectrum of the reaction mixture is shown in Fig. 1. Peak at 280 nm is for catechin from which concentration of catechin was measured and the concentration of poly (allylamine) was assayed by material balance. There was a characteristic peak at 430 nm due to the formation of the desired conjugate with the structure of Michael type adduct and/or Schiff base as shown in Scheme 1, which was not observed in the laccase catalysed coupling of catechin under the similar reaction conditions. A similar peak was observed in the catechin conjugation on Chitosan [16] or polyhedral oligomeric silsesquioxane [17].

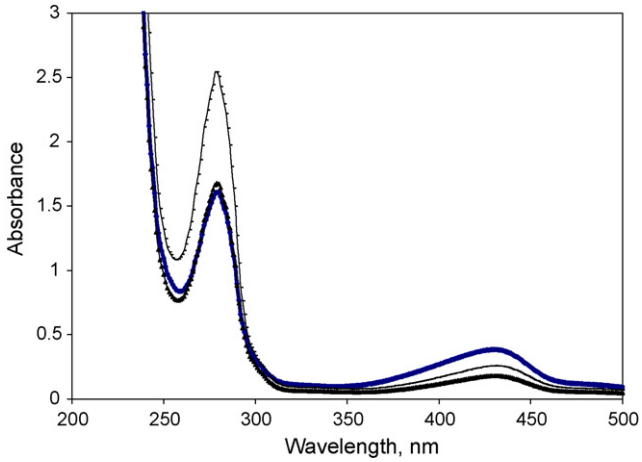


Fig. 1. Typical UV–vis spectrum of conjugation reaction of catechin and paa catalysed by laccase from *Trametes versicolor*.

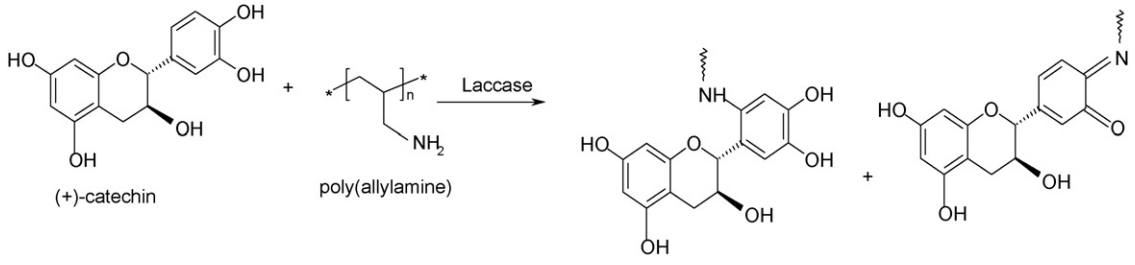
The initial rate was determined by using the equation:

$$r = \frac{S_i - S_t}{tw} \quad (1)$$

where S_i is the initial amount of substrate in moles, S_t is the amount (mM) remaining after time *t* (minutes) of reaction during which the profile corresponding to the first 10% conversion below which the profiles were found linear and *w* is the weight of laccase. The rate was expressed as the amount of substrate converted per unit time and weight of catalyst (mM hr^{-1} g^{-1}). All the experiments were carried out in duplicate and the reproducibility was found to be ±10%.

3. Results and discussion

The results of the present investigation have been interpreted in terms of correlation obtained among various solvent properties and initial reaction rate.



Scheme 1.

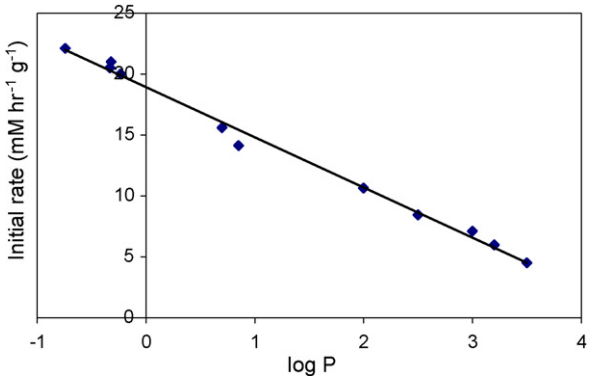


Fig. 2. Initial rate as a function of solvent hydrophobicity. The reaction mixture consists of [catechin] = 10 mM, [paa] = 100 mM, laccase = 2.5 mg/mL.

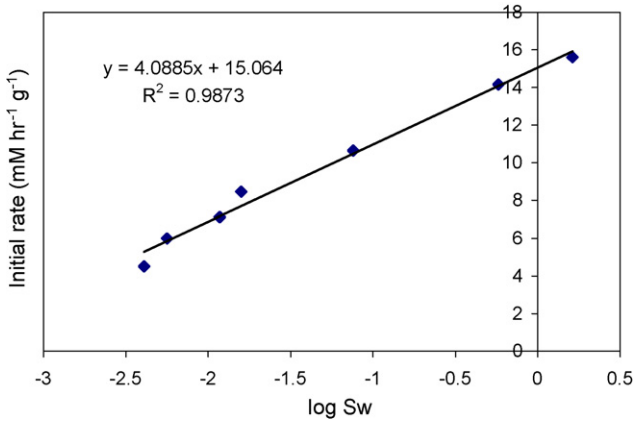


Fig. 3. Initial rate as a function of water solubility of solvents. The reaction mixture was same as for Fig. 1.

3.1. Effect of solvent hydrophobicity

The solvents have been selected on the basis of their solvent hydrophobicity ($\log P$) values as shown in the Table 1. The $\log P$ values have been proposed as a quantitative measure of solvent polarity and activity and stability of the enzyme has been reported to be optimal in the range lying between −0.74 and 3.50 [18–20]. The relationship between initial rate and solvent hydrophobicity is shown in Fig. 2 from which it is apparent that the initial rate decreases almost linearly with increasing $\log P$ values. The correlation between initial rate and $\log P$ can be represented by the following equation:

$$r = -4.12(\log P) + 18.95 \quad (2)$$

with a correlation coefficient of 0.99 which may be considered highly significant. Our finding is identical to that obtained for some other enzyme catalysed reactions [21]. However, in some cases the rate increases with the increase of $\log P$ with a plateau in a s-shaped curve for several other enzyme catalysed reactions [12,22–24]. In the present reaction, less hydrophobic methanol exhibits the highest initial rate probably due to preferential partitioning behaviour of the substrate between the reaction medium and the active site of laccase [25]. This partitioning is likely to diminish as the substrate and solvent hydrophobicities increase [26]. It is also reported that the catalytic efficiency of some enzymes decreases with increase in substrate hydrophobicity [13] and a linear free energy relationships exist between the catalytic efficiency of both substrate and solvent hydrophobicities. It is also noted that, hydrophobic solvents may not be easily accessible to the relatively polar phase around the hydrolytic enzyme for contact with the catalytic surface. There is another effect called product solvation in which product formed in polar solvent is expected to be highly solvated. This can result in highest rate of conjugation reaction. Thus, in our present study the observed variation of initial rate with hydrophobicity is considered to be reasonable.

3.2. Water solubility

The water solubility of the solvent ($\log S_w$) has been recognized as the most useful parameter of the solvent polarity for correlating the rates of enzyme catalysed reactions [27,28]. The relation of initial rate with water solubility for the conjugation reaction is shown in Fig. 3. The relationship could be deduced as

$$r = 4.08(\log S_w) + 15.06 \quad (3)$$

with a correlation coefficient of 0.98, and may also be considered significant. From the figure, it is seen that solvents with higher water solubility favours the conjugation reaction. The increase of initial rate with the increase of water solubility of the solvent is similar to those reported for the lipase catalysed transesterification of 2-o-benzylglycerol with vinyl acetate [13].

3.3. Effect of electron pair donor and acceptor index

For predicting the performance of the reaction media using polarity as the criteria, there is also other fundamental basis, which seems to rely on the donor acceptor interactions of the solvent including hydrogen bonding capability. Solvation of water requires both donation and acceptance of hydrogen bonds (or electron pair) or other dipole–dipole interactions. Accordingly, an attempt has been made to correlate the initial rate with the sum of the normalized electron pair acceptance index (E_T^N) and Gutmann's donor number (D_N^N) and the correlation is as shown in Fig. 4.

The correlation for the present system seems to be rather weak in comparison to that reported by Valivety et al. [29] who established a good correlation between water solubility and ($E_T^N + D_N^N$) of several organic solvents for which these values are available. It is also deduced that the hydrogen bond donation and accepting capacity of the solvent determines both water solubility and equilibrium of the reaction in that solvent. The observed trends on increase of initial rate with $E_T^N + D_N^N$ seem to be reasonable and may be explained from the solvation effect of the catechin. The differential solvation, which would effect the equilibrium position, involves additional acceptor and donor interactions. Solvent capable of either or both of these interactions would favour conjugation, esterification and hydrolysis reactions. The correlation of conjuga-

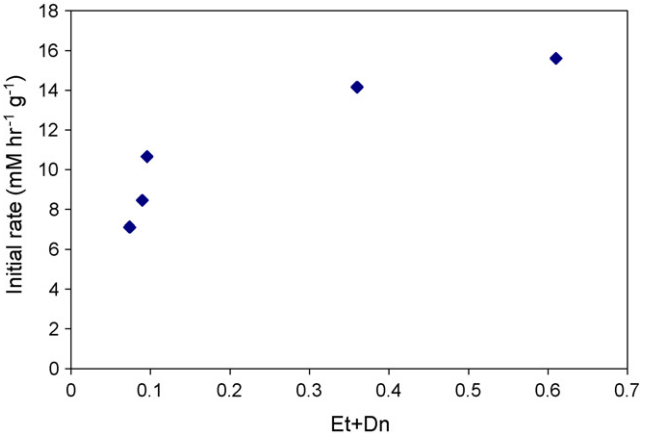


Fig. 4. Initial rate as a function of $E_T^N + D_N^N$.

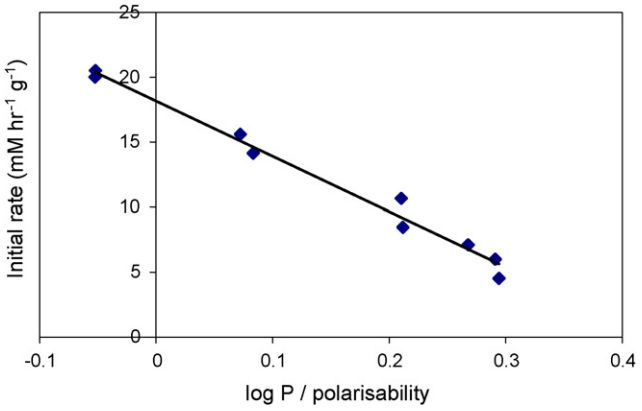


Fig. 5. Initial rate as a function of log *P*/polarizability.

tion rate with log S_w and $E_T^N + D_N^N$ perhaps indicates the role of the bulk behaviour of the solvent and functional group with specific interaction often referred to as chemical effects by the liquid state theories as suggested also for some esterification reactions [13,29].

3.4. Effect of solvent polarizability and dielectric constant

Solvent polarizability represents the ability of a solvent to stabilize the charge of a dipole in solution by virtue of its dielectric constant. Since it is a function of dielectric constant and refractive index, which can be easily measured, their values are known for almost all the solvents. Though, we have attempted to correlate the initial rate with polarizability for better understanding of all the important solvent properties on reaction rates, but no such good correlation was obtained. When polarizability combines with log *P* and expressed in terms of log *P* divided by polarizability, a reasonable good correlation was observed as shown in Fig. 5, indicated the important role of solvent hydrophobicity on initial rate. Similar correlation was observed for other reaction also [13,29].

Since dielectric constant is a function of polarizability, an attempt has also been made to deduce a correlation of initial rate with dielectric constant as an independent parameter and the correlation is shown in Fig. 6. It is apparent that the initial rate increases with the increase of dielectric constant values of the solvent and it does not represent a statistically sound correlation but the observed trends appear to be reasonable.

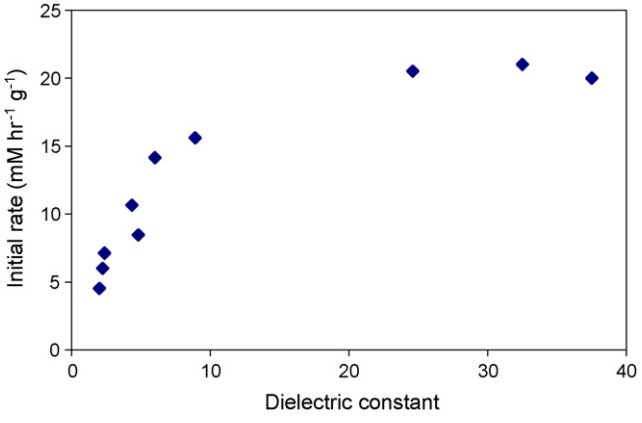


Fig. 6. Initial rate as a function of dielectric constant. The reaction mixture was same as for Fig. 1.

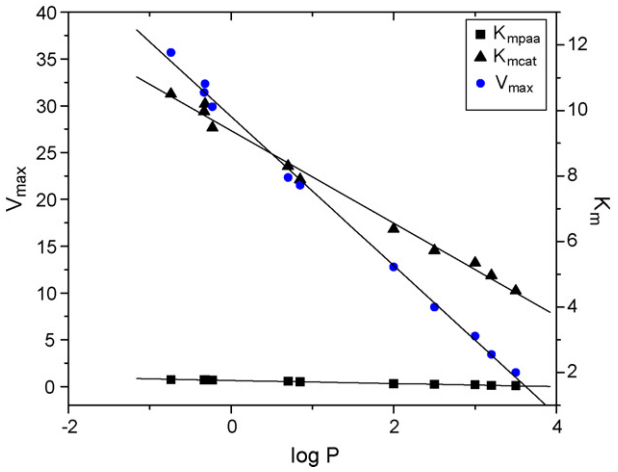


Fig. 7. K_m and V_{max} as a function of log *P*.

3.5. Laccase activity

The relationship between enzyme activity and substrate partitioning on solvation has been used to analyse solvent effect on enzyme catalysed reaction system [30]. Different solvents would exhibit different abilities to solvate the substrate and thus, may influence the thermodynamic activity of the substrate, the measured enzyme activity and partition coefficients of substrates as well as the products [31,32]. The implication is that solvent selection for biocatalysis would depend on the substrate and catalyst type [33].

Enzymatic reactions of lipases and laccase are known to act via Ping-Pong-Bi-Bi mechanism [12,15,34,35] and the model equation is

$$\frac{V}{V_{max}} = \frac{[paa][cat]}{K_{m(paa)}[cat](1 + [paa]/k_i) + K_{m(cat)}[paa] + [paa][cat]} \quad (4)$$

where [paa] and [cat] represent the initial molar concentration of poly(allylamine) and catechin respectively, V_{max} is the maximum reaction rate (mmol/min g), $K_{m(paa)}$ and $K_{m(cat)}$ are the respective affinity constants (mM), k_i is the inhibition constant of polyallylamine (mM). The values of V_{max}, $K_{m(paa)}$, $K_{m(cat)}$ and k_i were measured by numerical parameter identification using the Gauss Newton Algorithm of error minimization which is in the range of ±2% for all the solvents studied in this work and may be considered reasonable. It was found that *T. versicolor* laccase exhibit high affinity for oxidation reactions of different phenolic substrates [36]. In order to assess the effect of solvents, the V_{max} and K_m values of the above have been evaluated and plotted against log *P* values and are shown in Fig. 7. The apparent kinetic parameters for one substrate depend on the thermodynamic activity of the other substrate in support of which specificity constant k_{sp} of laccase is calculated by the equation $k_{sp} = V_{max}/K_m$ and k_{sp}^{int} is calculated by the equation $k_{sp}^{int} = k_{sp}/\gamma$ where γ is the activity coefficient and determined by ASOG method [12]. Statistically significant correlations are obtained as represented by the following empirical equations:

$$K_m = -2.27(\log P) + 9.81 \quad (5)$$

$$V_{max} = -4.94(\log P) + 27.34 \quad (6)$$

with correlation coefficients 0.99 and 0.99 for K_m and V_{max}, respectively. This finally provides an idea about the affinity of laccase, however, the measurements were necessarily carried out at optimum catechin concentration so as to eliminate the inhibition effect and keep specificity constant (V_{max}/K_m) essentially unaltered. The linear correlation between the kinetic parameters and log *P* may

Table 2
Specific constants ($k_{sp} = V_{max}/K_m$) of *Trametes versicolor* laccase in various solvents for conjugation reaction of catechin and poly(allylamine)[a].

Solvent	$k_{sp(cat)}$ (mM)	$^{Int}k_{sp(cat)}$ (mM)	$k_{sp(paa)}$ (mM)	$^{Int}k_{sp(paa)}$ (mM)	k_i (mM)
Methanol	2.66	5.37	41.66	84.35	10.43
Ethanol	2.79	5.65	41.32	86.02	9.98
Acetonitrile	2.78	5.64	40.70	82.41	8.21
Acetone	2.73	5.54	38.95	79.88	8.11
Ethyl acetate	2.96	5.99	40.74	82.48	8.02
Ethyl ether	2.86	5.80	42.31	85.66	7.95
Chloroform	3.22	6.52	53.91	109.13	7.55
Tolune	3.64	7.37	61.91	125.33	7.32
Carbon tetrachloride	4.24	8.59	70.64	143.00	6.85
Cyclohexane	4.64	9.41	105.13	212.82	5.96
Hexane	5.11	10.36	108.72	220.08	5.13

[a] The parameters were obtained from the experimental data at constant catechin concentrations in Fig. 6. The ordinary and intrinsic specificity constants are related to each other via $k_{sp} = \gamma \times {}^{Int}k_{sp}$, where γ is the activity coefficient determined by ASOG method.

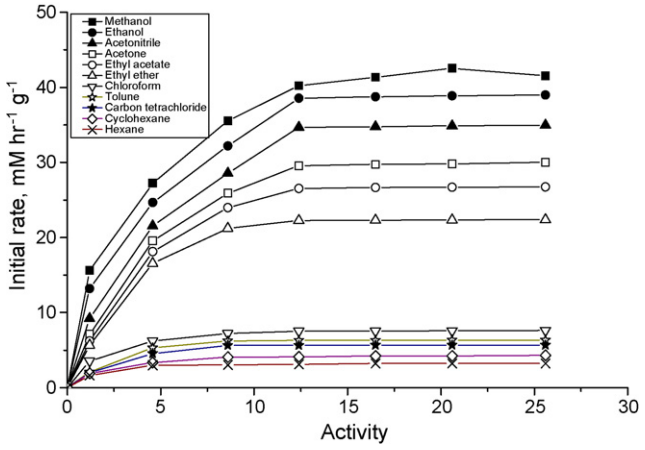


Fig. 8. Initial rate versus activity of laccase.

be considered phenomenologically consistent with the rate versus log *P* correlation shown in Fig. 2.

Evaluation of solvent effect on the performance of an enzyme may also rely on correction of the kinetic parameters for substrate–solvent interactions with a concomitant comparison of their values. The contribution of solvent–solvent interaction to enzyme kinetics can be accounted for by replacing the substrate concentration with the thermodynamic activity in the rate equation. This is considered valid when the organic solvents are assumed to have no interference with the binding process. The initial rate of conjugation reaction of catechin and poly(allylamine) was found to be highest in aqueous methanol and lowest in hexane. However the nature of variation of initial rate versus activity for all the solvent systems appears to be identical as shown in Fig. 8.

It is believed that the only surface of the enzyme participates in catalysis. Since the nature of the solvent and the substrate could affect the available catalytic surface may contribute to variation in reaction rates. The specificity constant ($k_{sp} = V_{max}/K_m$) values were determined by nonlinear regression of data presented in Fig. 7 through the use of Eq. (4) are shown in Table 2. Neglecting the values obtained for cyclohexane and hexane with solvents and comparing the values obtained for other solvent systems, it may be inferred that the variation is less in both the intrinsic and apparent values. Similar effect of solvents on catalytic activity of enzymes has been reported for other hydrolysis and esterification reactions [12,38].

4. Conclusion

The effect of solvent on conjugation of catechin and poly(allylamine) has been studied in dispersed system of *T. versicolor* laccase. The initial reaction rates were correlated well with hydrophobicity and water solubility of the solvents whereas for the other solvent properties the variation of initial rates may be considered as reasonable. Polar solvent is most favoured for the conjugation reaction of catechin and poly (allylamine). The organic solvent interferes neither with the laccase–substrate binding process nor with the catalytic mechanism. The contribution of the substrate–solvent interactions to enzyme kinetics was accounted by replacing the substrate concentration by thermodynamic activities.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cej.2009.07.047.

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