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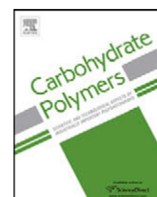

Aqueous state laccase thermostabilization using carbohydrate polymers: Effect on toxicity assessment of azo dye

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ABSTRACT

Thermostability of laccase from *Pleurotus florida* was studied at different temperatures (50–65 °C) using various polysaccharide additives (Guar Gum, starch, agarose and agar). The stability of laccase was enhanced with Guar Gum (0.3%), starch (8%), agarose (0.4%) and agar (0.4%). As revealed by PAGE, among two isoenzymes (L_1 and L_2) of laccase, improvement of enzyme stability observed in the presence of Guar Gum at 60 °C was proved by the appearance of L_2 isoenzyme. The analysis of Arrhenius plot for stabilized laccase revealed the break point shifted to 60 °C with activation energy of 2.41 $KJmol^{-1}$ protected by additive Guar Gum whereas other additives, the break point shifted to 52.5 °C. Stabilized laccase was used for decolorization of azo dye (reactive blue 198). FTIR analysis was performed to confirm the decolorization of dye by laccase. *Allium cepa* toxicity test was performed which indicate that root growth inhibition was dye concentration dependent.

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1. Introduction

Laccases are either mono or multimeric copper-containing oxidases that catalyze the one-electron oxidation of a vast amount of phenolic substrates. Molecular oxygen serves as the terminal electron acceptor and thus reduced to two molecules of water (Ducros et al., 1998). Laccases have widespread applications, many of which have been patented, ranging from effluent decoloration and detoxification to pulp bleaching, removal of phenolics from wines and dye transfer blocking functions in detergents, washing powders and degradation of lignin (Bourbonnais & Paice, 1990; Yaver, Berka, Brown, & Xu, 2001). Although laccase had a wide range of applications in many fields, the long term storage is a major problem. Enzymes undergo a variety of denaturation reactions during production, storage and application in industry. Denaturation is the unfolding of the enzyme tertiary structure to a disordered polypeptide in which key residues are no longer aligned closely enough for continued participation in functional or structure stabilizing interactions (Fagain, 1995).

Stability studies are indispensable during the entire formulation development process. Stability studies on the final product are conducted to define the optimal storage conditions and expiration date. The current stability requirement by the Food and Drug Administration (FDA, 2007) for a pharmaceutical product is less than 10% deterioration after storage for 2 years under the specified storage conditions. The stability studies are often conducted under accelerated (stressed) conditions to accelerate protein formulation development. These stressed conditions include high temperature, high humidity, intensive lighting, extreme pH and increased air–water interfaces induced by vortexing or shaking. Protein-stabilizing chemicals can be broadly divided into the following types: sugars and polyols, amino acids, amines, salts, polymers, and surfactants. Increase in the stability of enzymes under harsh pH and temperature conditions by the addition of salts, polyols, sugars and others has been previously reported for diverse enzyme systems (Costa et al., 2002; Lozano, Combes, & Iborra, 1994). The stability result obtained at high temperatures does not necessarily reflect or predict what happens under real-time conditions. Protein unfolding temperatures are usually in the range of 40–80 °C. This is due to the multiple and changing protein degradation pathways at different temperatures (Yoshioka, Aso, Izutsu, & Terao, 1994). The chemical additives referred as most proteins need a stabilizer in the liquid state for long term storage; these stabilizers are formulation excipients (Allison et al., 1998). Many of these excipients have been long recognized as compatible osmolytes in many different species producing laccase (Yancey, Clark, Hand, Bowlus, & Somero, 1982).

Arrhenius plot are often used to analyze the effect of temperature on the rate of chemical reactions. An Arrhenius plot displays the logarithm of kinetic constants (ln(k), ordinate axis) plotted against inverse temperature ($1/T$, abscissa). For a single rate lim-

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ited thermally activated process, an Arrhenius plot gives a linear or non linear graph, from which the activation energy and the pre-exponential factor can both be determined (Arrhenius, 1889). Observation of the root tip system therefore constitutes a rapid and sensitive method for environmental monitoring (Majer, Grummt, Uhi, & Knasmuller, 2005). Cytotoxicity and environmental have been assessed by the *in vivo* onion (*Allium cepa*) roots tip cell test, which is known to give similar results to in vitro animal cytotoxicity tests (Vincentini, Comparoto, Teixeira, & Mantovani, 2001; Teixeira, Comparoto, Mantovani, & Vicentini, 2003).

In this context, the aim of this study was: (i) to stabilize the laccase enzyme in the soluble state using additives at different temperature (ii) to assess the thermal inactivation pattern of laccase from *Pleurotus florida* (iii) to determine the break point of laccase in free form and with additives by Arrhenius plot (iv) to decolorize the azo dye (Reactive blue 198) and characterise by FTIR and (v) to assess the toxicity of Reactive blue 198 in presence and absence of additive.

2. Materials and methods

2.1. Microorganism and culture conditions

P. florida (NCIM 1243) was purchased from National Collection of Industrial Microorganism, National Chemical laboratory, Pune, India. The organism was maintained through fortnightly transfer at 30 °C on potato dextrose agar. Culture medium consists of 1 g of glucose; 1 g of malt extract; 0.5 g of potassium nitrate; 10 ml of salt solution was made upto 100 ml with distilled water. Salt solution 100 ml containing: 0.2 g of potassium dihydrogen phosphate; 0.5 g of magnesium sulphate; 0.1 g of calcium chloride and 0.5 g of potassium chloride. After two days of cultivation at 30 °C under agitation (120 rpm), 50 ml of the culture was used to inoculate 500 ml culture medium and the fungus was further grown at 30 °C under agitation (120 rpm). Media were sterilized by autoclaving at 121 °C for 15 min at 15 lbs. After 8 days the cells were removed by filtration through filter paper (Whatmann no. 1). The clear supernatant was stored at 4 °C and used for purification (Palonen, Saloheimo, Viikari, & Kruus, 2003).

2.2. Laccase Purification

The method for laccase purification was adapted from a protocol described by Das et al. (Das, Chakraborty, & Mukherjee, 2001) with slight modification. The culture supernatant was clarified by centrifugation at 21,000 × *g* for 15 min. The supernatant was then fractionated with ammonium sulphate at 40–80% saturation and centrifuged at 21,000 × *g* for 30 min at 4 °C. The supernatant was discarded, pellet was resuspended with Buffer A (10 mM of sodium acetate buffer [pH 5.6]) and dialyzed (membrane molecular weight cut off 10,000 Da) at 4 °C. The dialysate was loaded onto a pre-equilibrated DEAE-Sephacel anion exchange chromatography column, the column was washed with the Buffer A until the A280 reading was less than 0.02. Bound protein was eluted with a linear gradient of KCl (0–1 M KCl) at a flow rate of 1 ml min^{-1}; the eluted fractions were assayed for laccase activity. Active fractions were dialyzed against Buffer B (100 mM sodium acetate buffer, pH 5.6). After 12 h, dialysed fractions were subjected to sephadex G 50 gel filtration. The fractions containing laccase isoenzyme was eluted at a flow rate of 4 ml h^{-1}. The active fractions containing laccase was pooled and further used for the stabilization studies.

2.3. Laccase assay

Laccase (EC 1.10.3.2) activity was measured at 30 °C using 20 mM ABTS as the substrate (Wolfenden & Wilson, 1982). The assay mixture (1 ml) contained 780 μl of distilled water, 20 μl of ABTS from stock (20 mM), 150 μl of sodium acetate buffer (pH-5.6) and 50 μl of enzyme. The absorbance increase of assay mixture was monitored at 420 nm (ε_{420} = 36.0 mM^{-1} cm^{-1}). The enzyme activities were expressed as international units (U), defined as the amount of enzyme needed to produce 1 μmol product min^{-1} at 30 °C and presented as Ug^{-1} dry substrate. Protein was determined by the method of Lowry et al. (Lowry, Rosebrough, Farr, & Randall, 1951) with bovine serum albumin as the standard.

2.4. Stability assays

Stability assays were carried out in 100 mM sodium acetate buffer (pH 5.6). Sealed tubes containing 0.5 U of laccase in a total volume of 2 ml were incubated at 50, 55, 60, and 65 °C, respectively, and the activity was measured periodically. The effects of various polysaccharide additives after 24 h at pH 5.6 were tested at the following concentrations: Guar Gum (0.5%, 0.1%, 0.15%, 0.2% and 0.25%), starch (4%, 5%, 6%, 7% and 8%), agarose (0.1%, 0.2%, 0.25%, 0.3% and 0.4%) and agar (0.1%, 0.2%, 0.25%, 0.3% and 0.4%). All chemicals used are commercially available and were used without further purification.

2.5. Kinetic analysis

To determine the kinetic parameters of stabilized laccase enzyme using Guar Gum, activity assays was performed for ABTS at 60 °C (pH 5.6). The data was plotted by using the Michaelis–Menten equation to obtain K_m, V_{max} and K_{cat} with the molar extinction coefficient of ε_{420} = 36.0 mM^{-1} cm^{-1}.

2.6. Non denaturing polyacrylamide gel (PAGE)

Native PAGE (12%) was performed to determine the stability of laccase and their thermal inactivation pattern. Partially purified extract of laccase enzyme was used as control. The thermal treatment of laccase was carried out at 60 °C in presence and in absence of Guar Gum. The thermalstability of laccase was visualized by incubating the gel for 3 min at 37 °C in the presence of 0.5 mM ABTS in sodium acetate buffer pH 5.6.

2.7. Arrhenius plot

The stabilization of laccase enzyme by polysaccharide additives followed the model of first order exponential decay (Mutafov, Avramova, Stefanova, & Angelova, 2006), $C_t = C_0 e^{-kt}$, where *k* stands for the stabilization rate (h^{-1}); C_0 and C_t (mg l^{-1}) are the concentrations of additives at the zero time and at time *t* (h), respectively. For $t > 0$ the stabilization rate, *k*, was evaluated as:

$$k = -\ln \frac{(C_t - C_0)}{t} \quad (1)$$

$$\ln k = \ln A - \left(\frac{E}{R}\right)\frac{1}{T} \quad (2)$$

where *k* is the rate constant of a reaction at temperature *T* kelvin. The units of *k* will depend upon the order of the reaction; *A* is a constant for a particular reaction with the same units as *k*; *E* is the Arrhenius activation energy (in J mol^{-1}) of the reaction. This is a constant and does not vary with temperature; *R* is the gas constant (8.3145 J mol^{-1} K^{-1}). ln means log to the base *e*. A plot of ln *k* against 1/*T* follows the form "$y = mx$" and should be a straight line. The slope (gradient) of the line is equal to *ER* from which *E* may be calculated.

The normal logarithms of the evaluated mean values of the stabilization rates, ln *k*, were plotted against the corresponding absolute temperature (1/*T*) and it is used for localization of the break points on the Arrhenius plot for laccase enzyme.

2.8. Inhibitory studies of stabilized laccase

The effects of sodium azide, EDTA, and thioglycolic acid (0.01–10, 1–10 and 0.1–1 mM, respectively) on laccase activity were determined after 15 min of preincubation of the enzyme with the various inhibitors before and after stabilization using Guar Gum at 60 °C.

2.9. Dye decolorization by stabilized laccase

To find out the effect of laccase with Guar Gum (0.25%) on Reactive blue 198 (RB 198) decolorization. The reaction mixture was incubated at 60 °C and it contains, 50 mg l^{-1} dye concentrations, 10 U ml^{-1} partially purified laccase in 100 mM sodium acetate buffer (pH 5.6) in a total volume of 1 ml in an eppendorf tube.

2.9.1. Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared spectroscopy analysis was used to examine the surface functional groups that are involved in decolorization of the RB 198 dye using stabilized laccase. FTIR analysis was carried out using Perkin Elmer Spectrophotometer and changes in % transmission at different wavelengths were observed. The spectra were collected within a scanning range of 4000–400 cm^{-1}.

2.9.2. *A. cepa* assay

The *A. cepa* test provides a rapid screening procedure for chemicals and environmental agents which may represent environmental hazards. Root growth inhibition assay was performed as a 96 h semi-static exposure test (Mamta Kumari, Mukherjee, & Chandrasekaran, 2009). Healthy equal sizes of common onions were obtained from Salem local market of Tamil Nadu, India. The dried outer scales were carefully removed leaving the ring of root primodial intact. *A. cepa* was exposed for 96 h to different dilutions of the azo dye in presence and absence of Guar Gum as follows:

RB 198 dye : 0, 100, 200, 300, 400 and 500 ppm

Each concentration was set up in 3 replicates. The base of each onion bulbs was grown on different concentration of environmental agents inside a 30 ml beaker and placed away from sunlight for 4 days after that the root length was measured. Growth inhibition was estimated as EC_{50} (the effective concentration of a chemical producing 50% of the total effect).

3. Results and discussion

3.1. Effect of additives on enzyme stability

Laccase has a wide range of applications in many fields, the long term storage is a major problem. The thermolabile laccase was stabilized under in vitro conditions using various polysaccharide additives. Fig. 1 shows the stability of partially purified laccase (Control) which decreases with increasing temperature. The loss of enzyme activity was prevented at high temperature using stabilizers. Most proteins need a stabilizer for long term storage, these stabilizers are formulation excipients, which are also referred to as chemical additives (Allison et al., 1998).

The laccase enzyme was partially purified from the culture filtrate of *P. florida* as summarized in materials and methods. The resultant partial purified laccase was purified to 6.4 fold, with a yield of 36% and the specific activity of 52.6 U/mg of protein. In the present work various polysaccharide additives were added to increase the stability of laccase in the liquid state focusing on protein stabilization. The stabilization experiments were performed in conditions wherein the polysaccharide additives are in solution state. The additives added to the laccase at different temperatures

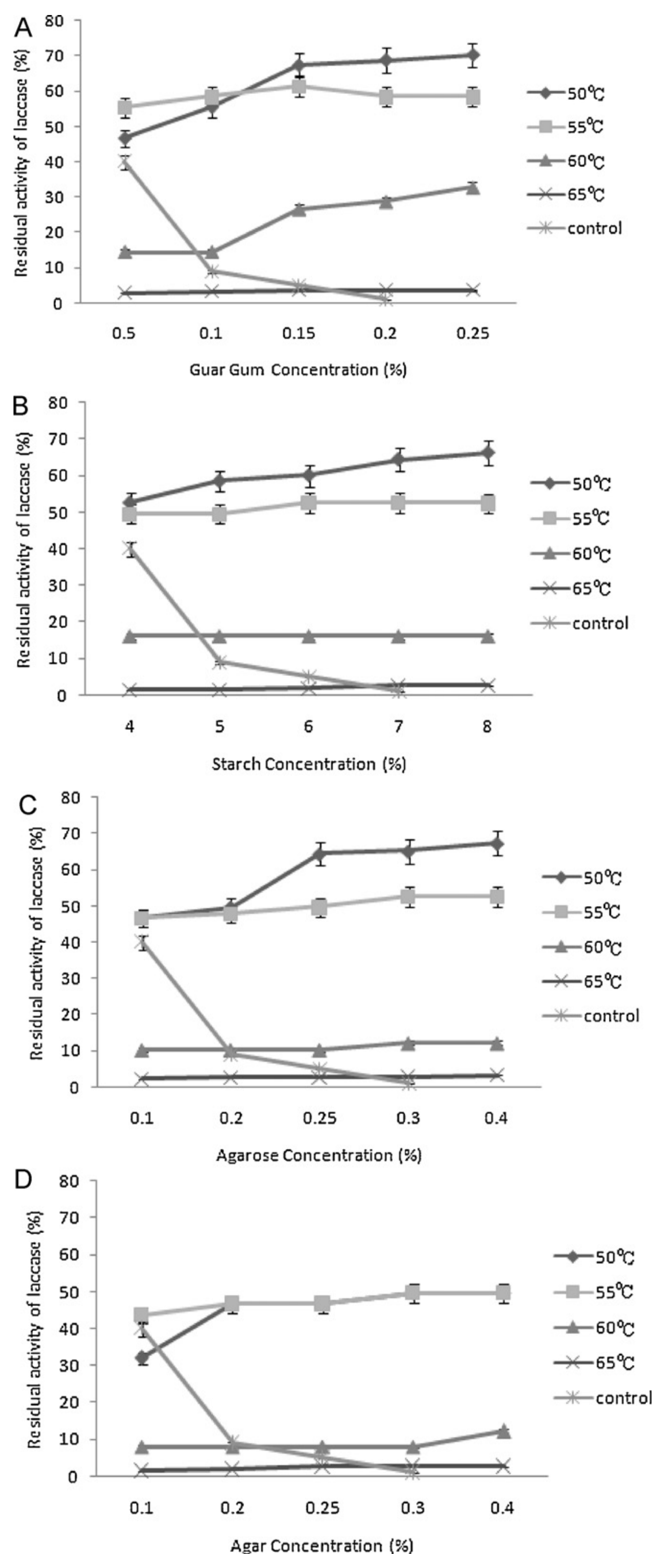

Fig. 1. Stabilization of laccase enzyme using Guar Gum (A), starch (B) and agar (C) agarose (D) at different concentration after 24 h.

(50 °C, 55 °C, 60 °C and 65 °C), increased the enzymatic activity when compared to the control. The increase of additives concentration was generally associated with an increase in laccase activity (Fig. 1A–D). Fig. 1 demonstrates the ability of Guar Gum, starch, agar and agarose in converting thermolabile *P. florida* laccase into a thermostable one. The major reasons for the observed thermostability may be due to gelling nature of the polysaccharide additives.

The observed enhanced stability at 50–65 °C in response to both Guar Gum and starch might be useful for wide range of industrial applications.

Protein stabilization by low molecular weight solutes is a widely used strategy. The increase in thermal stability of enzymes may attain through compounds like sugars (Baptista, Cabral, & Melo, 2000), polyols (Costa et al., 2002) and salts (Baptista et al., 2000). In particular, the role of polyols in enzyme stabilization was identified to be a water-structure maker, which depresses the hydration of enzyme and hence its denaturation (Costa et al., 2002; Lozano et al., 1994). To utilize partially purified laccases more efficiently for the biotechnological applications it is necessary to better understand the properties of this enzyme at a physicochemical and kinetic level, rather than the requirement of large amounts of enzyme production. The stability of partially purified proteins seems to be dependent upon various extrinsic factors namely, pH, buffer and temperature; these in turn, may influence the modulation of enzymes by affecting intrinsic mechanisms.

The stabilization of multimeric enzymes by polymers had been used to prevent sub-unit dissociation in ribonuclease (Fernandez-Lafuente, 2009). *P. florida*, partially purified laccase stabilization activity was much more stable than other purified fungal laccases, indicating an advantage from the standpoint of the liquid state stability. For example laccase of *Pleurotus sajor-caju* losses enzyme activity drastically after pre-incubation at 50–65 °C (Lo, Ho, & Buswell, 2001; Murugesan et al., 2006); *Pycnoporus sanguineus* shows a rapid loss of activity at temperatures above 35 °C and 40 °C (Pointing, Jones, & Vrijmoed, 2000); and laccase of *Agaricus blazei* at 40 °C retained 50% of activity after 25 min but only 10% after 120 min (Ullrich, Huong, & Dung, 2005). An increase of additives (Veratryl alcohol, trehalose, glycerol, mannitol, gluteraldehyde, $CuSO_4$ and 1-HBT) concentration was associated with a decrease in laccase activity from *Fomes sclerodermeus* laccase except $CuSO_4$ and mannitol (Papinutti, Pedro Dimitriu, & Flavia Forchiassin, 2008). Palvannan et al. (Palvannan, Thangavelu, Jayapragasam, & Boopathy, 1998) reported that thermostabilization of aspartyl protease from *Rhizomucor pusillus* was attained by sugars like trehalose, sucrose, sorbitol, etc. Polysaccharide like modified starch was used to stabilize enzymes like trypsin (Fernandez, Villalonga, Cao, Alex, & Villalonga, 2004). To the best of our knowledge this is the first report demonstrating the ability of polysaccharide additives to stabilize the laccase efficiently in solution state. Studies in the area of stabilization of industrially important enzymes using polysaccharides as additives are limited.

3.2. Kinetics of laccase at stabilized condition

The determination of kinetic activity in the stabilized laccase by Guar Gum against ABTS was performed at 60 °C. The V_{max} (1.5 μmol min^{-1} mg^{-1}) and K_{cat} (8.6 s^{-1}) values of the enzyme stabilized by Guar Gum were improved when compared to the control (V_{max} – 0.097 μmol min^{-1} mg^{-1} and K_{cat} – 0.5 s^{-1}). The kinetic constant of stabilized laccase shows higher catalytic efficiency than control. This result indicates the confirmation of laccase thermostabilization using additive (Guar Gum).

3.3. Pattern of isoenzyme stability

The PAGE for different thermal treatments of partially purified enzyme suggested that the isoenzyme forms of laccase display distinct thermal sensitivity patterns, which could account for the gradual loss of activity of the enzyme toward the higher temperature incubation. *P. florida* laccase has two isoenzymes L1 and L2 (Fig. 2, lane 1). L1 was stable whereas L2 disappeared at 40 °C (Fig. 2, lane2). Fig. 2, lane 3 shows the pattern of laccase isoenzyme stability using additive (Guar Gum). The effect of additives on the

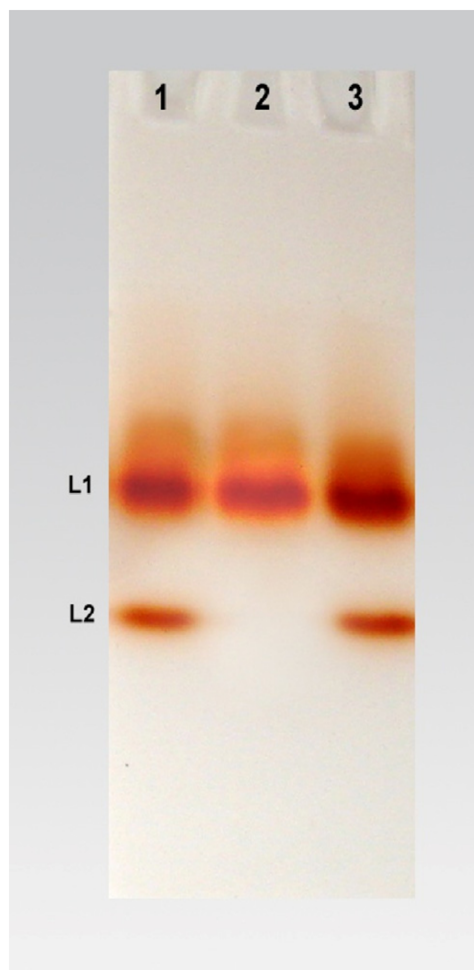

Fig. 2. *P. florida* laccase isoenzymes and its stabilization efficiency. Partially purified laccase (lane 1), heat treated laccase at 60 °C (lane 2) and laccase incubated with Guar Gum at 60 °C (lane 3) for 24 h, the fractions concentrated 10-fold, and applied to Native PAGE.

isoenzyme stability indicates that additives obviously increased the laccase stability and this may be due to the appearance of L2 in the PAGE. This fact could explain the drastic change in enzyme activity of laccase isoenzymes. Leandro Papinutti et al. reports that *Fomes sclerodermeus* L2 was the most resistant, while L1 were almost completely inactivated after 6 h at 50 °C (Papinutti et al., 2008).

3.4. Arrhenius plot

It is generally recognized that the enzymatic reactions are background of all living processes. Therefore, it is not surprising that there is a vast majority of studies using the Arrhenius equation for interpretation of numerous indices associated with different physiological functions of various classes of organisms. Most of these works analyze the subject either in the narrow temperature ranges having predominantly practical significance or study the stabilization of enzyme by extremely complex consortia using additives to raise the activity of enzyme (Papinutti et al., 2008). This is probably the reason why no one of them gives any evidence for the existence of break points on the Arrhenius plot. The stabilization rate of laccase as revealed by Arrhenius plot was shown in (Table 1).

Fig. 3A–D shows the Arrhenius plot for laccase using various additives. Table 1 shows the difference in activation energies (Ea) among the various additives (Guar Gum, starch, agarose and agar) when compared to control. The plot depicts two distinct lines with different slopes (non-linear or two part linear). The non-linear

Table 1
Results of the two-part linear regression analysis from the experimental data.

Name	Enzyme	Guar Gum	Starch	Agarose	Agar
Distribution of the experimental points	Three points to the left, three points to the right	Four points to the left, two points to the right	Four points to the left, two points to the right	Four points to the left, two points to the right	Four points to the left, two points to the right
Temperature range (°C)	55–65	50–65	50–65	50–65	50–65
E_a (kJ mol^{-1})	1.10	2.41	1.64	1.41	1.37
A	5.14	8.84	9.42	7.80	6.86
r^2	0.96	0.98	0.96	0.78	0.68
Right part					
Temperature range (°C)	20–50	20–40	20–40	20–40	20–40
E_a (kJ mol^{-1})	0.98	4.74	7.39	2.72	2.51
A	3.14×10^5	2.58×10^{20}	4×10^{28}	5.20×10^{12}	8.77×10^{11}
r^2	0.96	1.0	1.0	1.0	1.0
Break point temperature (°C)	40	60	52.5	50	52.5

regression analysis was performed. Nonlinear Arrhenius plots have been reported for many enzymes. This nonlinearity can appear as a curvature (Allen, Blum, Cunningham, Tu, & Hofman, 1990), a break (Massey, Curti, & Ganther, 1966) or a jump (Moosavi-Nejad, Rezaei-Tavirani, Padiglia, Floris, & Moosavi-Movahedi, 2001). In some cases an enzyme may show different forms of the Arrhenius plot with different substrates (Allen et al., 1990). The break in the Arrhenius plot for laccase in the absence of additives (control) was 40 °C. Addition of Guar Gum to laccase shifted the break point to 60 °C. There are two main reasons possible for a nonlinear Arrhenius plot (Massey et al., 1966; Biosca, Travers, & Barman, 1983). First there may be a change in the rate-limiting step of enzymatic reaction. Enzyme reaction pathways consist of steps, each described by rate constants and characteristic energies of activation. As the temperature is varied, a change in the rate-limiting step can occur. The nonlinearity may be due to purely kinetic phenomena without any changes in the conformation of the enzyme's active site (Allen et al., 1990). Another reason is a temperature-induced conformational change in a soluble enzyme or phase change in a membrane-associated enzyme, or both. For the enzyme laccase it

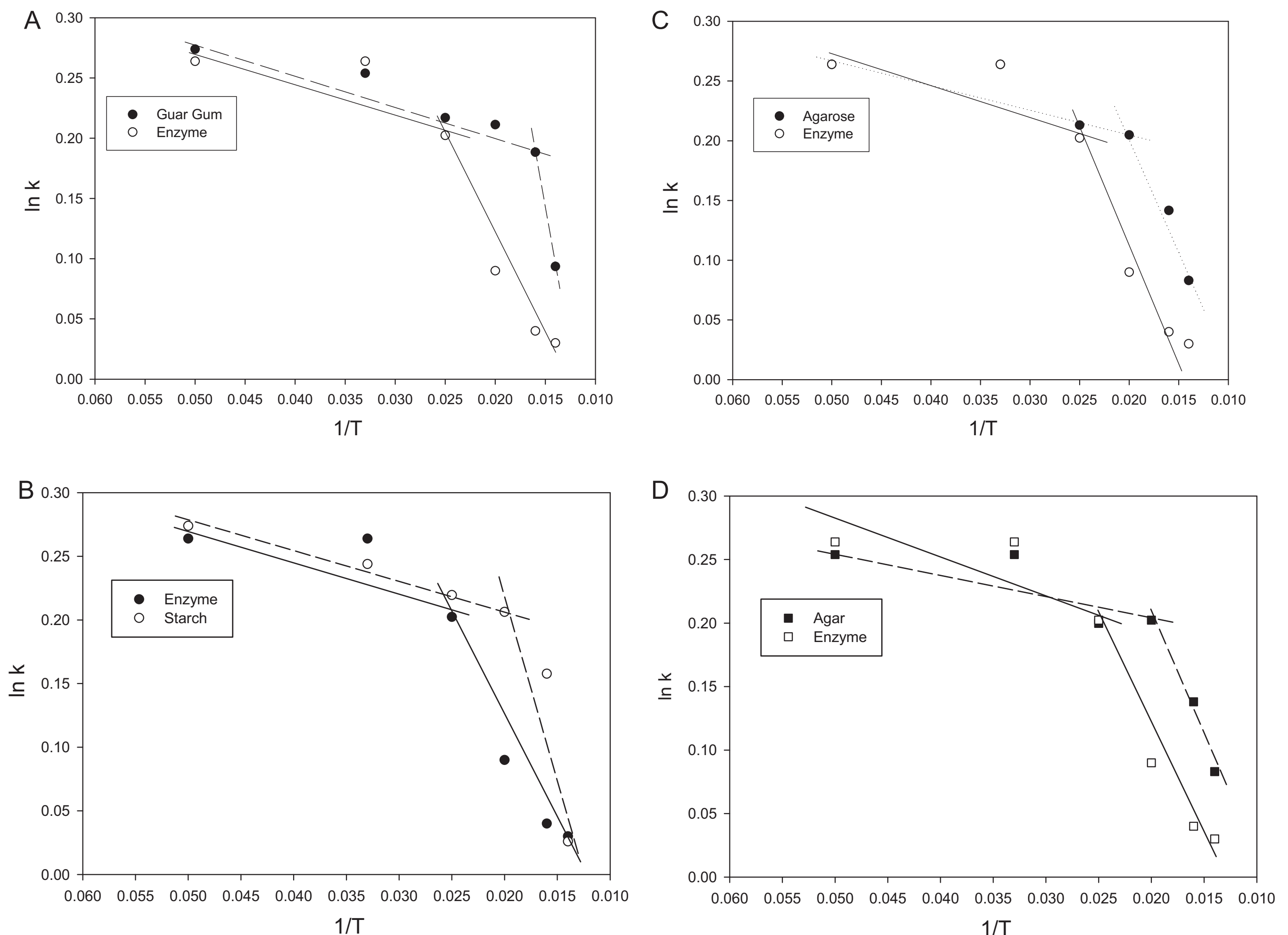

Fig. 3. Break point determination by Arrhenius plot for laccase stabilization by Guar Gum (A) and Starch (B) Agarose (C) and Agar (D).

Table 2
Inhibitory studies for stabilized and unstabilized laccase.

Inhibitor	Concentration (mM)	% Inhibition for stabilized laccase[a]	% Inhibition for unstabilized laccase[a]
Sodium azide	0.01	12	100
	0.05	80	100
	0.1	93	100
	10	96	100
EDTA	1	30	100
	2	34	100
	5	63	100
	10	90	100
Thioglycolic acid	0.1	91	100
	0.2	94	100
	0.5	95	100
	1	100	100

[a] All values are the means of triplicate measurements, and the coefficients of variation were less than 5%.

is clear from the Arrhenius plot the break point has been moved by 20 °C that is in the range of 40–60 °C, the activation energy is obviously higher (Table 1), although in both ranges the activity increases with temperature by using additives in liquid state. On the other hand, the sudden alteration of the activation energy as a response of temperature change is a well known phenomenon in microbial physiology. The reasons for the sudden change of the activation energy are still unclear.

Angelova et al. (Angelova, Avramova, Stefanova, & Mutafov, 2008) reported the same kind of two-part linear Arrhenius plot for azo bond reduction by microorganisms. According to them the two-part linear Arrhenius plot favoured bottle neck hypothesis for the temperature dependent decolorization of azo bond reduction. Our results also follow a similar pattern of increase in activation energy with a two-part linear Arrhenius plot. Hence our pattern of temperature dependent stabilization also follows the bottle neck reaction, however, the mechanism for such a hypothesis cannot be explicitly confirmed. Ceuterick et al. reported that the change of bottle neck reaction may depend on the thermal characteristic of the particular enzyme but in the extremely complex intracellular organization it may depend also on the properties of membrane structures supporting or surrounding this particular enzyme which are themselves temperature dependent (Ceuterick, Peeters, & Heremans, 1978). Based on the experimental results of our present investigation we incline to support the bottle neck hypothesis. Thus this finding will be used to understand the temperature effect of different enzymes used in various industries.

3.5. Inhibition studies of stabilized laccase

The stabilized laccase was less inhibited by Cu-chelating agents, when compared to the unstabilized one (Table 2). The order of inhibition was thioglycolic acid > sodium azide > EDTA. The stabilized laccase was completely inhibited at higher concentration of inhibitors. This may be due to increased enzymatic activity at stabilized condition.

3.6. Effect of Guar Gum vs. temperature on dye decolorization

The influence of temperature on dye decolorization in presence and absence of Guar Gum was evaluated by incubating the reaction mixture at various temperatures. The result clearly demonstrated that decolorization increased with increase in temperature up to 60 °C, beyond which decolorization decreased sharply for reactive blue 198. Fig. 4 shows the Guar Gum which increases the decolorization rate up to 43% when compared to the control (2%) at 60 °C. At 70 °C, the decolorization decreased to 10% from the maximum.

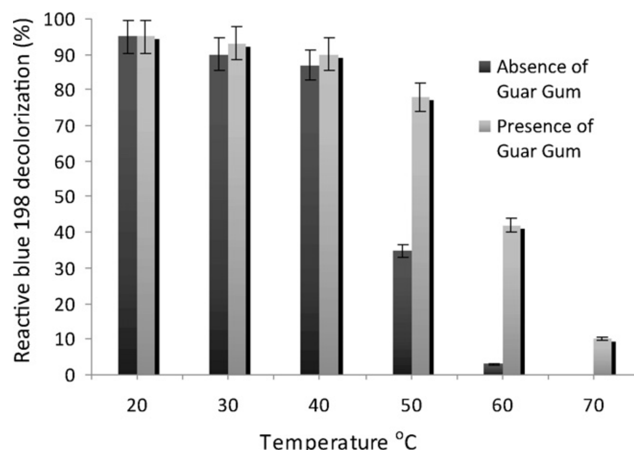

Fig. 4. Effect of temperature on reactive blue 198 decolorization by laccase in presence and absence of Guar Gum.

This result clearly indicates the role of Guar Gum in stabilizing the laccase enzyme there by playing a critical role in reactive blue 198 decolorization.

3.7. FTIR characterisation

The FTIR spectra of RB 198 decolorization treated with laccase in presence and absence of Guar Gum were shown in Fig. 5A–C. The untreated dye shows peak at 3918 cm^{-1}, 3431 cm^{-1}. This may be due to O–H, N–H stretch representing the presence of hydroxyl bond and heteroaromatic group. After the laccase treatment in absence of Guar Gum, the peak had shifted to the wave number 3405 cm^{-1} suggesting that there is N–H stretching and in presence of Guar Gum the two peaks were newly formed at 3359 and 3500 cm^{-1}. This may be due to the formation of primary amines and secondary amines. In RB 198 the peaks are seen at 2389 cm^{-1} and 2077 cm^{-1}, this may be due to N–H stretching vibrations. After laccase treatment in presence and absence of Guar Gum single peak was observed at 2083 cm^{-1} and 2081 cm^{-1}, hence these corresponding groups may be involved in the decolorization process by assymetrical NH^3 bending vibration and the torsional oscillation of the NH^3 group. In untreated dye (RB 198) the peaks are found at 1637 cm^{-1}, 1413 cm^{-1} 1386 cm^{-1} and 1120 cm^{-1} which may be due to C–O–C (isopropylether), C=O stretching and C–O bending vibrations. After laccase treatment in absence of Guar Gum there was a significant change observed suggesting that peaks are found at 1638 cm^{-1}, 1413 cm^{-1}, 1277 cm^{-1}, 1119 cm^{-1}, 1018 cm^{-1} due to isopropylether, C=O stretching and phenol groups are involved in decolorization process. In addition peaks at 1413 cm^{-1}, 1277 cm^{-1} and 1018 cm^{-1} in RB 198 disappeared after laccase treatment with Guar Gum due to methylene twisting and wagging vibrations, C–O, O–H plane bending vibration and C=O stretching, hence these corresponding peaks in RB 198 might be involved in the decolorization process. In untreated RB 198 shows peak at 659 cm^{-1}. This peak had shifted to 661 cm^{-1} and 658 cm^{-1} in presence and absence of Guar Gum due to out of plane bending in the bonded O–H group.

3.8. Toxicity assessment using allium test

The results in Fig. 6 showed the Root growth inhibitory effects of decolorized RB 198 dye by laccase at 60 °C in presence and absence of Guar Gum on *A. cepa*. The estimated EC_{50} (concentration of a chemical producing 50% of the total effect) of *A. cepa* exposed to presence and absence of Guar Gum was 300 and 180 ppm, respectively (Fig. 6). Very little growth was observed

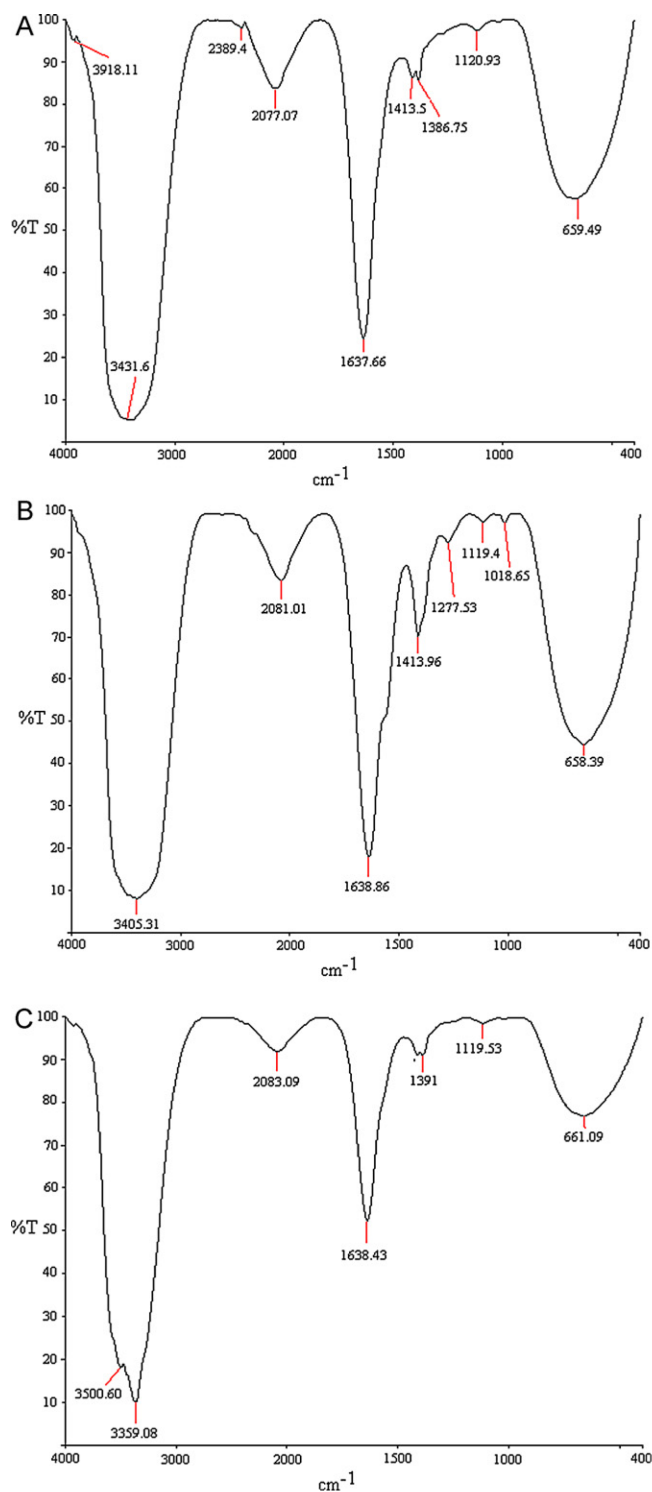

Fig. 5. The FTIR spectra of reactive blue 198 untreated (A), laccase treated in absence of Guar Gum (B) and laccase treated in presence of Guar Gum (C).

in the *A. cepa* exposed to RB 198 dye concentration greater than 300 ppm. The results obtained from stabilized laccase using Guar Gum revealed a concentration of dye dependent decrease in root length, as the concentration increases from 100 to 500 ppm the root length significantly decreased ($p < 0.05$) when compared with the control. This result was similar to the other reports (Radic et al., 2010; Olusegun Samuel, Fidelia Osuala, & Peter Odeigah, 2010).

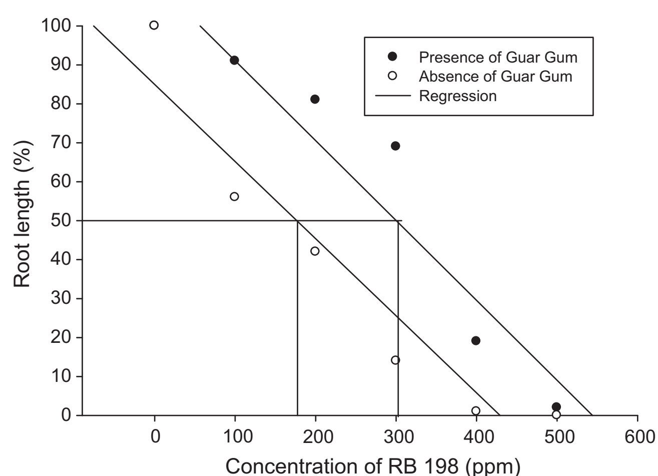

Fig. 6. Growth inhibition of *Allium cepa* roots exposed to RB 198 dye in presence and absence of Guar Gum.

4. Conclusion

Results of the present investigations indicate that the stabilization of laccase using Guar Gum in liquid state provide certain advantages as compared to starch, agarose and agar. We hope that this observation could contribute to elucidation of the mechanisms of temperature effect on stabilization of laccase having both scientific and practical impact. The stabilization of enzyme by Guar Gum gave higher activity, improvement of break point to higher temperature and showed better thermal stability. Decolorization and toxicity assessment of azo dye using stabilized laccase providing information on the need for environmental managers. This will enable proper biochemical analysis of industrial effluent in order to identify the constituent that is really toxic and its prompt removal from the effluent before discharge.

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