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Amino acid induced hyper activation of laccase and its application in dye degradation

Shamraja S. Nadar, Virendra K. Rathod*

Department of Chemical Engineering, Institute of Chemical Technology, Matunga (E), Mumbai, 400019, India

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ABSTRACT

The catalytic efficiency of enzyme is mainly dependent on its structural conformation and its stability. In this work, laccase, the copper containing enzyme was stabilized with the help of 20 different natural amino acids by non-covalent interactions. It was seen that the positively charged amino acids *viz* arginine, histidine and lysine induced the favourable conformation changes in laccase which led to the enhancement in catalytic activity. Further, the thermal stability of laccase-amino acid conjugates was tested in terms of the deactivation energy (E_d) and half-life ($t_{1/2}$) which showed superior thermal stability in the temperature range of 45–65 °C with respect to free form. In Michaelis-Menten kinetics studies, positively charged amino acid conjugates exhibited higher V_{max} than free laccase. To understand the mechanism of binding of amino acids to laccase and thereby induced conformational changes in laccase, FT-IR data analysis tools and intrinsic fluorescence spectroscopy method were used. At the end, laccase-amino acid conjugates have been employed for enzymatic decolourization of dye solutions and the decolourization was found to be enhanced as compared to that by unconjugated form.

1. Introduction

Enzymes are recognized as one of the key drivers in various sectors such as chemical, biotechnological and allied industrial sectors due to their high selectivity, high efficiency and specificity which make the processes green (Nadar et al., 2017). Enzymes are becoming a vital tool to develop novel and sustainable manufacturing processes for several industries, and hence garnered the focus of numerous researchers to utilize them. However, one of the major barriers to use enzymes as a catalyst is their insufficient stability (*i.e.* narrow pH range, low thermal stability) under processing and operational conditions which limits the applicability in various biotransformation processes (Sheldon and van Pelt, 2013).

From the past decade, the research trend has been focussed on enzyme stability in order to get full potential as a catalyst to extend the enzyme applications. Many different approaches such as protein engineering (by genetic engineering)(Ó'Fágáin, 2003), chemical modification (such as glutaraldehyde) (Barbosa et al., 2014), use of stabilizing agent (sugar, surfactant etc.)(Wang et al., 2011) and immobilization have been used till date in order to get stable active enzyme structure (Jadhav et al., 2014). Mostly, enzyme stabilization by covalent or non-covalent interaction is a simple, efficient and widely used strategy to significantly enhance the kinetic stability of enzymes (Bommarius and Paye, 2013; Deepankumar et al., 2017; Stepankova et al., 2013). Covalent grafting of enzyme with stabilizing agent is one such approach which has been shown to have a significant effect on the thermal and pH stability of enzymes. However, covalent conjugation needs laborious procedures to chemically modify the polymers, for instance, oxidation to get aldehyde groups, as per specific requirements (Muley et al., 2017; Rodrigues et al., 2011).

Enzymes can form non-covalent conjugates with several sugars, polysaccharides and some synthetic polymers under biocompatible conditions through mild interactions such as hydrophobic interactions, steric exclusion, Van der Waals interactions and hydrogen bonds. It has a pivotal role in stabilization of active structure of enzyme (Balcão and Vila, 2015; Iyer and Ananthanarayan, 2008). Pazhang and co-workers used trehalose (as a sugar stabilizer) for stabilization of structure and enzymatic activity of thermolysin. The interactions between sugar moieties and proteins are not as strong as covalent interactions but are able to stabilize enzymes under operational conditions (Pazhang et al., 2006). Ritter and co-helper used PEG stabilization approach (PEGylization) for glucose oxidase which exhibited improved thermal stability. The use of polymers to modify an enzyme offers some advantages such as water holding capacity which keeps the enzyme in active form. Those poly-functional polymers are suitable to give multiple intramolecular or intersubunit interactions which might help in enzyme

* Corresponding author.
E-mail address: vk.rathod@ictmumbai.edu.in (V.K. Rathod).

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stabilization (Ritter et al., 2014). Mazzaferro and co-workers found that polyethyleneimine (M_W, 2000 Da, PEI) improved the melting temperature of lactate dehydrogenase (LDH) due to stabilization by intermolecular forces and formation of protein–PEI complex. Muley and co-authors used four different kinds of polysaccharides for non-covalent conjugation of cutinase derived from *Fusarium sp.* ICT SAC1. They showed that conjugation of polysaccharide to cutinase can cause structural changes which may be responsible for increased thermal and pH stability (Muley et al., 2017). However, the macromolecular nature of polysaccharide offers crowding effect during the stabilizing enzyme which ultimately resulted into decrease in catalytic activity (Poggi and Slade, 2015). To overcome this, Hakiminia and group proposed amino acid based stabilization of lipase. They found that proline had exhibited a number of positive effects on the structure and activity of lipase (Hakiminia et al., 2013).

Laccase (EC 1.10.3.2) has attracted many researchers due to its ability to oxidize broad spectra of substrate. Laccase has been employed as a promising bioremediation solution to protect eco-system from damage caused by industrial effluents (Goncalves et al., 2015). In order to attempt new strategies to increase stability and rigidity of the enzyme without disrupting its active structure, in present work, we have employed 20 different amino acids as enzyme stabilizer. The small size and different functional group of amino acids not only stabilize the active structure of enzyme but also avoid steric hindrances. Further, the thermal stability of laccase-amino acid conjugates was tested and expressed in terms of deactivation energy (E_d) and half-life ($t_{1/2}$) in the temperature range of 45–65 °C. Michaelis-Menten kinetics parameters such as K_m and V_{max} were determined for each combination of laccase and different amino acids. To understand the binding mechanism of amino acids, the structural conformation of laccase was analysed by secondary structure with the help of FT-IR data analysis tools and intrinsic fluorescence spectroscopy method. At the end, free and laccase-amino acid conjugates have been employed for decolorizing congo red and acid violet I dye solutions.

2. Materials and methods

2.1. Materials

Laccase (*Trametes versicolor*, 84 U/mg) was procured from Sigma Aldrich (Bangalore, India). Guaiacol was purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Natural amino acids (Glycine, Alanine, Valine, Leucine, Methionine, Isoleucine, Lysine, Arginine, Histidine, Aspartate, Glutamate, Serine, Threonine, Cysteine, Proline, Asparagine, Glutamine, Phenylalanine, Tyrosine and Tryptophan) were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd. (Mumbai, India). All other chemicals and reagents were of analytical grade and procured from reliable sources.

2.2. Methods

2.2.1. Enzyme activity assay

Laccase activity was determined by oxidation of guaiacol in dynamic spectroscopic assay method which was reported by Baldrian with some modifications. In typical enzyme activity assay, laccase solution (5 mg/mL prepared in buffer) was mixed with guaiacol (5 mM in sodium acetate buffer, 50 mM, pH 5.0) solution. The reaction mixture was kept at 37 °C in a water bath for 5 min. The colourless solution turned into brownish colour due to the formation of coloured compound tetraguaiacol. Finally, the adsorption (optical density) of coloured solution was measured at 450 nm using spectrophotometric method (UV/Vis Jasco V-630, Japan) (Baldrian, 2004). One unit activity (U) of laccase was defined as the amount of the enzyme required for oxidation of one μmole of guaiacol under optimum conditions.

2.2.2. Conjugation of laccase and amino acid

Typically, laccase enzyme solution (5 mg/mL) was prepared in sodium acetate buffer (50 mM, pH 5.0) in a glass beaker. In another glass beaker, glycine (20 mg) was dissolved in same buffer system. The two solutions were then mixed with equal volume, mixed gently and incubated at room temperature (28 ± 2 °C) overnight to interact with each other. Further, these laccase-amino acid solution mixtures were analysed for residual activity (%) which was estimated with respect to the initial activity of the enzyme. The concentration of different amino acids was adjusted according to the same molar amount as of glycine.

The effect of ionic strength (10–60 mM) of acetate buffer (pH 5.0) were investigated in the preparation of laccase-amino acid conjugate.

2.2.3. Thermal stability studies

Thermostability of free laccase and conjugated laccase-amino acid was determined in the temperature range of 45–65 °C. The sample was incubated at known temperature and collected after every 10 min intervals till 60 min. Enzyme activity was determined by standard enzyme activity assay as described above in the section. The inactivation rate constant (k_d) was determined by semi-log plot of residual activity (%) of free and conjugated enzyme against time (min). The half-life ($t_{1/2}$) is the time required to decrease the enzyme activity to half of the native enzyme activity which was evaluated as $0.693/k_d$. Further, linear Arrhenius plot was used to calculate energy required for deactivation (E_d) of the free and conjugated form of enzymes.

2.2.4. Structure analysis of free and conjugated laccase

The intrinsic fluorescence spectroscopy method was used to determine the conformational changes in 3D structure of laccase (enzyme content 5 mg/mL) after conjugating with amino acids in sodium acetate buffer solution. Intrinsic fluorescence spectra of conjugated and free laccase were measured by collecting the emission spectrum with 3 mm path length from 300 to 400 nm in a quartz cuvette (Jasco FP-6500 Spectro-fluorometer, Japan). Tryptophan is excited at 288 nm. Excitation wavelength and emission wavelength was scanned at 5 nm and 1200 nm/s (Nadar and Rathod, 2016).

The fractional changes in secondary structure of free laccase and conjugated laccase was estimated by using FT-IR method according to previous work (Sojitra et al., 2016; Talekar et al., 2014). The FT-IR spectra were recorded from 4000 to 400 cm^{-1} with sample dispersed in the KBr pellets using Shimadzu IRAffinity-1 FT-IR spectrophotometer for free laccase and conjugated laccase-amino acid. The resolution of FT-IR spectra of free and conjugated enzyme was enhanced by taking its secondary derivative of 1700–1600 cm^{-1} (sensitive amide I region). The obtained secondary spectra were made smooth with a 20-point Savitzky-Golay by Essential FT-IR™ V. 3.0. Further, curve fitting of the amide I region was done by Gaussian function multi-peak fitting and area under multicomponent peak was quantified using Origin version 9.0 (Nadar and Rathod, 2017a).

2.2.5. Kinetic parameters

Michaelis Menten kinetic parameters (K_m and V_{max}) of free laccase and laccase-amino acid conjugates were determined by using different concentrations of guaiacol ranging from 1 to 30 mM in sodium acetate buffer (50 mM, pH 5.0). The K_m and V_{max} were calculated by plotting the initial reaction rates corresponding to different substrate concentrations with non-linear regression fitting in the Graph Pad Prism software.

2.2.6. Storage stability studies

Storage stability studies of the free and amino acid conjugated forms were studied by keeping each form of laccase in sodium acetate buffer (10 mM) of pH 5.0 without guaiacol at room temperature (30 ± 2 °C). After 18 days, each conjugated form of enzyme was assayed for residual activity (%) using standard enzyme assay. The residual acidity was determined by considering 100% at zeroth time with respect to its

conjugation.

2.2.7. Dye degradation studies

Congo red (100 ppm) and Acid violet I (10 ppm) dye solutions were prepared in sodium acetate buffer (50 mM pH 5.0). The dye solutions were mixed with free and conjugated form of laccase and incubated at room temperature for complete decolourization. The control sample was maintained without addition of enzyme solution. Decolourization was determined by measuring absorbance at 495 nm for congo red and 520 nm for Acid violet I using spectrophotometric method (Jadhav and Singhal, 2013). The decolourization (%) of dye was determined with respect to buffer.

3. Results and discussion

3.1. Effect of laccase-amino acid conjugation on activity recovery

The enzyme stabilization is an important tool to improve application in many different sectors. In general, the extent of non-covalent interactions rely on the type of amino acids *i.e.* side group of amino acids and the solution composition (e.g. pH and its ionic strength) which ultimately affect the catalytic structure of enzyme and its activity. Classically, amino acids are grouped into three categories based on the propensity of the side chain to be in contact with a polar solvent; hydrophobic (non-polar and non-polar aromatic), polar neutral, and polar charged (positive and negative). The allied differences in activity recoveries with different amino acids can stem from the different modes of interactions/bonding (hydrophobic or electrostatic), leading to the formation of the intramolecular domains which contribute to the structural integrity and stability of the protein (Bhaskara and Srinivasan, 2011). From Fig. 1, it can be observed that, the charged amino acids play an important role in manipulation of enzymatic structure which was reflected in its activity recoveries. Positively charged polar amino acids (*i.e.* arginine, histidine and lysine) helped to enhance the enzyme activity up to 128.3% (Fig. 1 blue). The charge based interaction between amino acid and the active side of laccase led to the favourable conformational changes thereby enhancing its activity. In particular case of histidine-laccase conjugation, the highest enzymatic activity was observed as compared to other amino acid-laccase conjugation. This might be due to the favourable hydrogen and π-π bonding between imidazole residue (present as a side chain in histidine) and active sites of laccase. Additionally, ring flipping mechanism of the imidazole side chain acts as a vehicle for proton transfer (Rebek, 1990). On the contrary, negatively charged amino acids showed lowering of laccase activity after conjugation (Fig. 1 yellow). The polar but neutral amino acids exhibited the activity recovery in between 78 and 92% (Fig. 1 red). This can be attributed to the hydrophobic interaction between enzyme and amino acids which might disrupt/interfere with native conformation of enzyme. Tyrosine was insoluble in aqueous buffer (pH 5) and could not participate in interaction with enzyme. Further, cysteine showed complete inhibition of activity after non-covalent conjugation with laccase (Dhawan and Kuhad, 2002). In case of non-polar amino acids, increasing side chain hydrophobicity (Ala, Met, Val, Leu and Ile) resulted in an increase in the enzyme activity ranging from 52.9 to 90.3% (Fig. 1 green). Also, phenylalanine and tryptophan with aromatic side chains, showed activity recovery of 80.75 and 72.45%, respectively (Fig. 1 purple). Similarly, Hakiminia and co-workers found that the proline induced positive effects on the structure and activity of lipase derived from *Pseudomonas fluorescens* after non-covalent interactions (Hakiminia et al., 2013).

The ionic strength of buffer system plays an important role in the non-covalent interaction studies. Laccase was incubated in different molar concentrations of buffer in the presence of amino acids. The relative activity of conjugates with respect to ionic strength of buffer is presented in Fig. 1b. The most active laccase conjugation was observed at 40 mM acetate buffer. The change in activity recovery might be due to the influence on the net charge and variation in the hydrogen bonds and electrostatic interactions in the enzyme structure (Muley et al., 2017).

3.2. Thermal stability study

The thermostability of enzyme was expressed in terms of deactivation rate constants (k_d) and half-life ($t_{1/2}$). It was determined separately by incubating each conjugated form in acetate buffer (pH 5.0) at different temperatures (45 °C, 55 °C and 65 °C) and the residual activity (%) was assayed after equal interval of 10 min till 1 h (Fig. 2a–d and S1a-o). The k_d and $t_{1/2}$ are summarized for free laccase and its conjugates with different amino acids in Table 1 and S1. After conjugation of laccase with different amino acids, the k_d values were lower than that of free laccase at temperatures in the range of 45–65 °C. Also, the half-life of conjugated laccase was increased by 1.5–2 folds as compared to the free form. Amino acids can prevent enzyme aggregation at higher temperature because of the self-associated inter-molecular interaction and hydrophobic stacking with hydrophobic region of the enzyme. This manifested that interaction between amino acids and enzymes helped to maintain active tertiary structure under thermal treatment of enzyme (Gil and Schrum, 2013; Hamada et al., 2009).

Further, the deactivation energy (E_d) is another mode of expression of thermal stability which was determined by using linear fit of the Arrhenius plot (Fig. 2e and S2a-d). The higher E_d is implies more thermally stable enzyme. The deactivation energy for denaturation of laccase-positively charged amino acid conjugates and free form was 47.01 (average deactivation energy of Lys, Arg and His conjugates) and 38.25 kJ mol^{-1}, respectively. These results imply that conjugation of enzymes with amino acids provides more pronounced protection from thermal denaturation due to non-covalent interactions such as electrostatic interaction, hydrogen bonding and hydrophobic interactions, hence it requires much more energy to break down this active conformation over free form (Kawata and Ogino, 2010; Talekar et al., 2014).

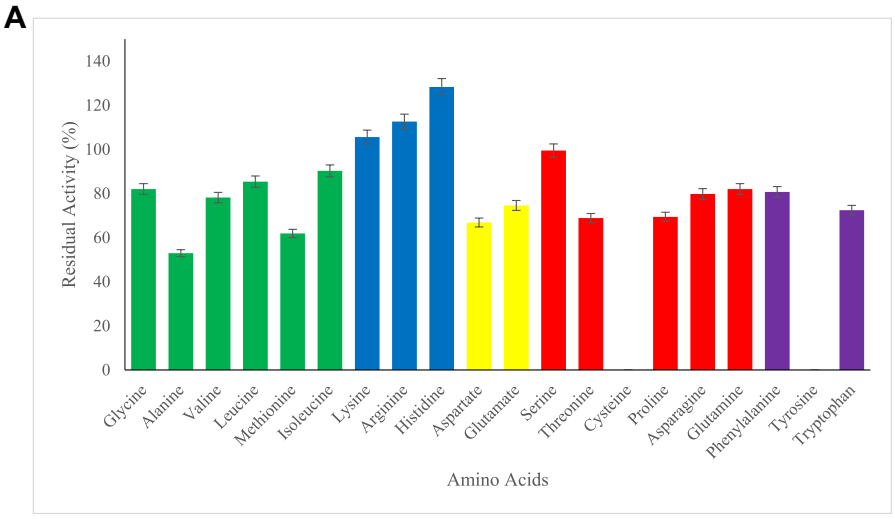
3.3. Kinetic parameters

Kinetic parameters (V_{max} and K_m) of free and conjugated form of laccase were determined by plotting initial reaction rates for each form by varying amounts of guaiacol substrate in the range of 1–30 mM. As shown in Table 2 and S2, K_m values are lower for positively charged amino acids conjugated with laccase. This indicated increased substrate affinity after conjugation of laccase with positively charged amino acids as against free enzyme. Contrarily, K_m values increased for other amino acids after non-covalent interactions. A slight increase in K_m values might be due to the interference of amino acids and structural changes caused by amino acids. These results show that the positively charged amino acids induce the favourable changes in laccase resulting into improved affinity towards substrate.

The V_{max} values were found to be higher for His, Lys and Arg conjugates as compared to free forms and other types of amino acids, on the basis of hydrolysis of guicol. It indicates that the rate of oxidation improved after non-covalent conjugation with positively charged amino acids. This could be possibly explained as the incorporation of positively charged amino acids at various catalytic domains resulting into conformational changes in enzyme, thereby enhancing the catalytic efficiency (Teipel and Koshland, 1971).

3.4. Structural analysis of free and conjugated laccase

Enzyme conformation plays a critical role in determining both the catalytic efficiency and catalytic stability. Any change in the structural conformation is reflected in the enzyme activity. Here, the changes in structural confirmation were investigated by using intrinsic



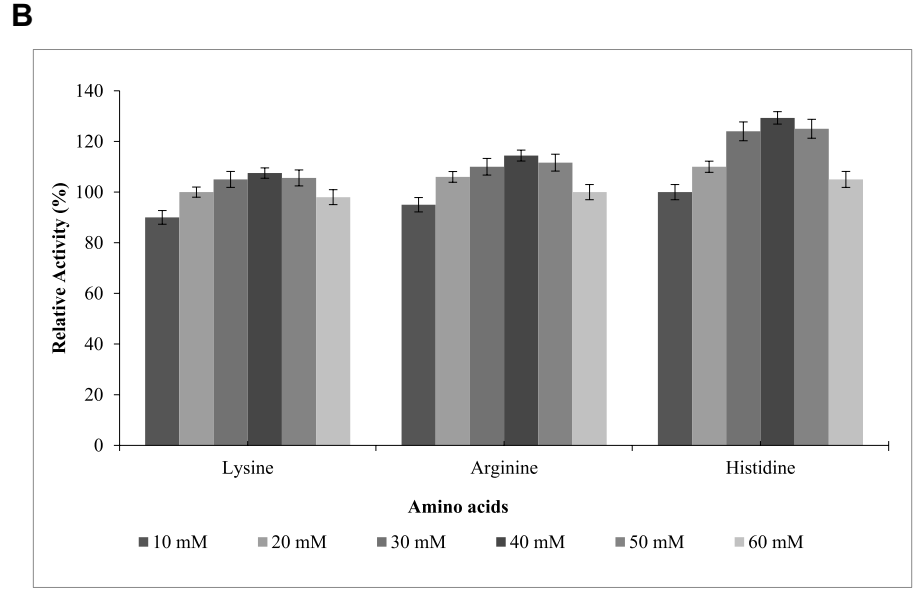


Fig. 1. Residual activity of amino acid-laccase conjugates with respect to free form (100% residual activity is corresponding to 420U). (a) Structure of 20 natural amino acids grouped according to nature of side chain: non-polar (■, green), polar positively charged (■, blue), polar negatively charged (■, yellow), polar non-charged (■, red) and aromatic (■, purple). (b) Effect of ionic strength of acetate buffer (pH 5.0). The measurements were performed in triplicate and the error bar represents the percentage error. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

fluorescence method and FT-IR data analysis tools (Nadar and Rathod, 2017b).

The fluorescence nature of protein is primarily attributed to its amino acid residues, especially Tryptophan (Try), as it is sensitive to the micro-changes in the environment (Zeinabad et al., 2016). Different amino acids interact with enzyme differently due to variation in R group and different nature of amino acids. This could possibly be the reason for unfolding/reshuffling of native structure of enzyme which leads to changes in location of amino acids especially, Try side chains. For the laccase and conjugated laccase with amino acids, the changes in side chain of Try were monitored by the protein fluorescence emission spectrum (Figs. S3a–c). The maximum fluorescence emission wavelength was 325 nm. The fluorescence intensity of conjugated laccase decreased, without showing red or blue shift as related to that of free laccase. This clearly confirmed that the non-covalent interaction with positively charged amino acids exposed the number of Try on laccase surface with respect to control, indicating amino acid induced conformational changes in the laccase.

FT-IR spectroscopy is considered as a useful data tool to investigate conformational changes in enzyme after conjugation with different amino acids which provide useful information regarding the binding mechanism of amino acids to laccase. The amide I band ($1700–1600\ cm^{-1}$) is the most sensitive spectral region of protein structural components, hence considered for the determination of secondary structure using FT-IR spectroscopy data tool. However, due to the close proximity of bands and extensive overlapping of structural component bands, the secondary derivative of amide I band was used to get improved resolution and identify component peak frequencies. Then, the peak areas under multicomponent of amide I bands were quantified using multi-peak fitting using Gaussian function (Nadar and Rathod, 2018).

The second derivative FT-IR spectra for free laccase and conjugated laccase were obtained (Figure S4a-s). The relative contents of α-helix structure ($1650-1658\ cm^{-1}$) (blue), β-sheet structure ($1610-1640\ cm^{-1}$) (red), β-turn structure ($1660-1700\ cm^{-1}$) (yellow) and random coil structure ($1640-1650\ cm^{-1}$) (green) based on the modelled multi-component peak areas were calculated according to the previous literature using software (see Fig. 3) (Nadar and Rathod, 2016). The changes in secondary structures were due to different non-covalent interactions induced on the side group of amino acids. Amino acids might have changed the enzyme hydration shell which could have resulted in the changes in hydrogen donor/acceptor characteristics, salt bridges, molecular geometric pattern and loss or gain of electrostatic repulsions that can interfere with correct enzyme folding, catalytic

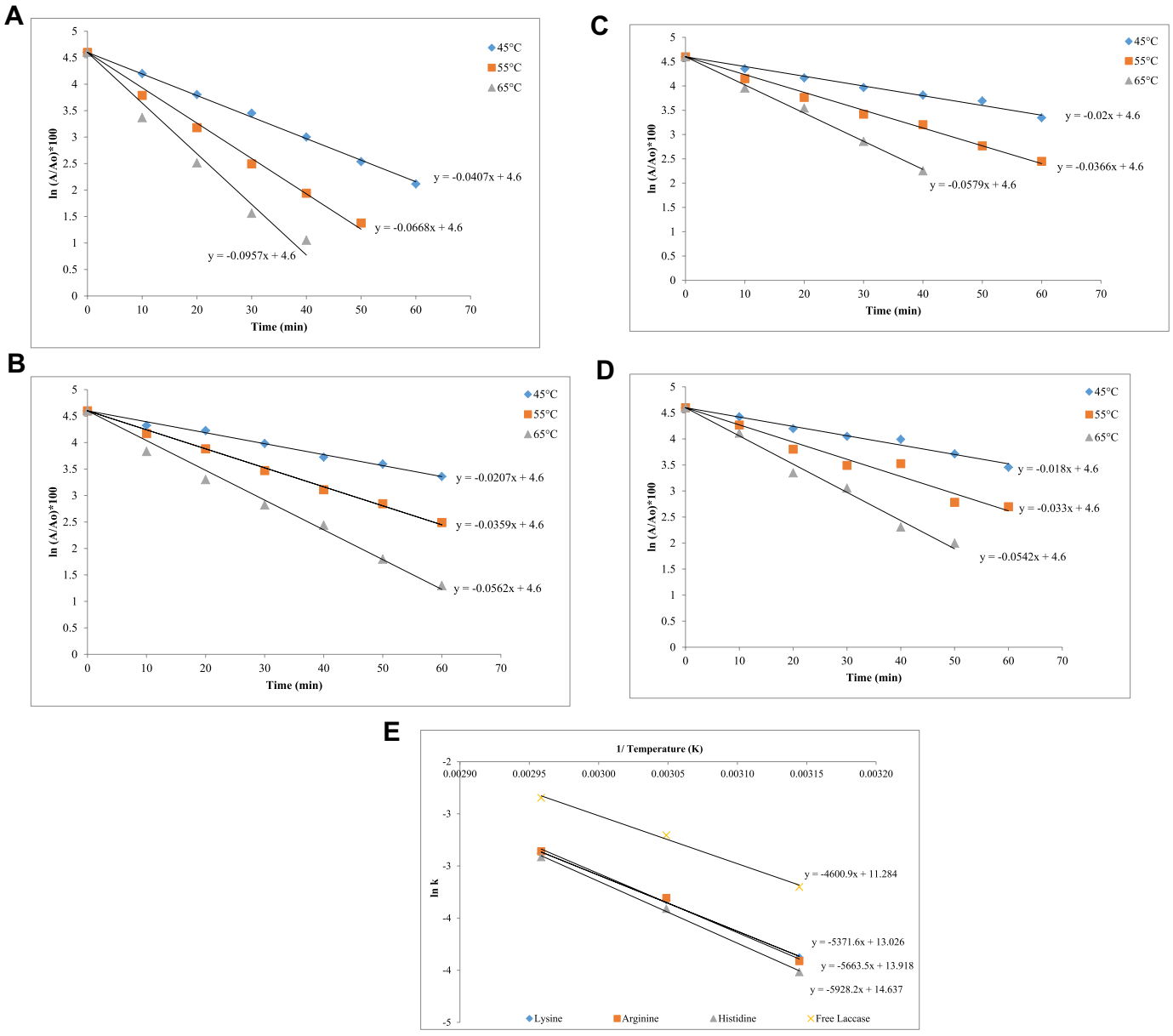


Fig. 2. Thermal kinetics profile of free laccase (a), Lysine-laccase conjugate (b), Arginine-laccase conjugate (c) and Histidine-laccase conjugate (d). Arrhenius plot for inactivation of free and immobilized form (e).

Table 1
Kinetics of thermal deactivation parameter of free and conjugated form of laccase.

Forms	k_d (min^{-1})			$t_{1/2}$ (min)			E_d (kJ mol^{-1})
	45 °C	55 °C	65 °C	45 °C	55 °C	65 °C	45–65 °C
Free	0.0407	0.0668	0.0957	17.03	10.374	7.24	38.25
Lysine	0.0207	0.0359	0.0562	33.47	19.30	12.33	44.65
Arginine	0.0200	0.0366	0.0579	34.65	18.93	11.96	47.08
Histidine	0.0180	0.033	0.0584	38.50	21.0	11.87	49.29

activity and structural stability. The fractional changes in secondary structure of free and conjugated laccase are given in Table 3 and S3. It was seen that the % fractions of α-helix and β-turns reduced after conjugation in case of positively charged amino acids, while the % fractions of β-sheets and random coils increased with respect to the free enzyme. These changes induced structural transformations, in laccase after conjugation which could have affected the active site of the enzyme, thereby, significantly improving its stability and catalytic activity.

Table 2
Michaelis–Menten kinetic parameters of free and conjugated form of laccase.

Forms	K_m (mg/mL)	V_{max} (μmol/min)
Free laccase	0.781 ± 0.019	5.321 ± 0.296
Lysine	0.768 ± 0.023	5.563 ± 0.321
Arginine	0.745 ± 0.032	5.788 ± 0372
Histidine	0.729 ± 0.029	5.982 ± 0.282

3.5. Storage stability studies

Storage stability studies of the free laccase and non-covalently conjugated laccase were calculated by incubating them at R.T. (30 ± 2 °C) in sodium acetate buffer for 18 days. The residual activity after 18 days of storage is shown in Fig. 4. The conjugated laccase

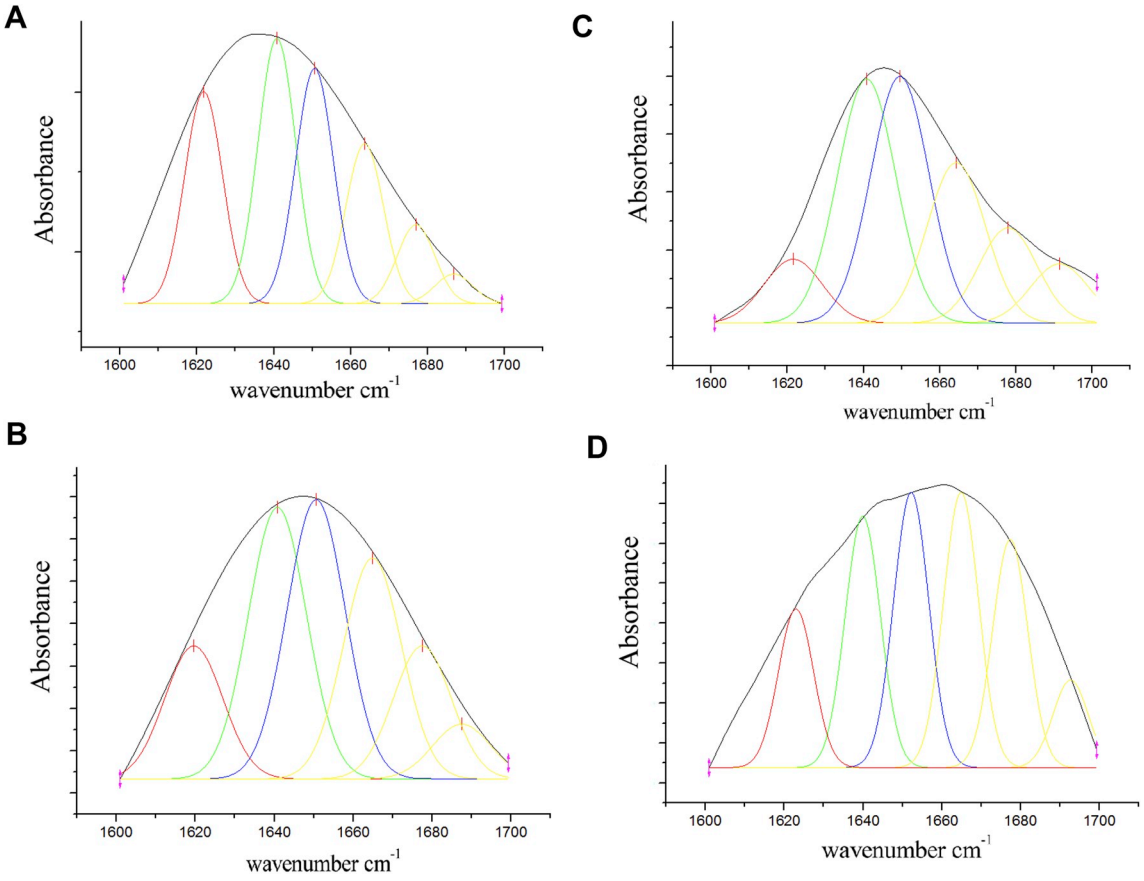


Fig. 3. Secondary structural changes in free laccase (a), Lysine-laccase conjugate (b), Arginine-laccase conjugate (c) and Histidine-laccase conjugate. The structural contents, β-sheet structure (■, red), random coil structure (■, green), α-helix (■, blue) structure and β-turn structure (■, yellow) based on the modelled multi-component peak area were determined by Gaussian multicomponent fitting. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Fractions of secondary structures for free laccase and laccase conjugates.

Form	α-helix (%)	β-sheet (%)	β-turn (%)	Random coils (%)
Free laccase	14.96	11.59	50.34	23.11
Lysine	11.15	17.24	43.48	28.13
Arginine	10.32	14.19	45.15	30.34
Histidine	12.76	18.24	41.87	27.13

showed higher storage stability as compared to the free laccase. The improved stability was attributed the tertiary structure of conjugate formed due to amino acid and enzyme bridging interactions. This prevents possible distortion effects on the active sites of enzyme caused by the buffer solution (Nadar et al., 2018).

3.6. Application of laccase-amino acid conjugate for dye degradation

Textile effluent containing Acid violet I and congo red dyes were completely decolorized using both free and conjugated laccase after 6 and10 h incubation respectively. The decolourization done by using free and conjugated laccase is shown in Fig. 5a and b. It was observed that the positively charged amino acid conjugated with laccase showed faster degradation of dye as compared to other amino acid conjugates. On the other hand, free form showed lower degradation of dye which might be due to lower stability. These results are in accordance with the reported literature for decolourization of textile effluent containing Remazol brilliant blue R dye using gum Arabic conjugated laccase (Chauhan et al., 2017; Jadhav et al., 2014).

4. Conclusion

Laccase was successfully stabilized by conjugating it with 20 different amino acids via non-covalent interactions. The thermal kinetic parameters *i.e.* deactivation rate constants (k_d) and half-life ($t_{1/2}$) and deactivation energy (E_d) revealed improvement in thermal stability of conjugated laccase than the free form. Further, the storage stability of conjugated laccase was found to be 1.5–2 fold higher than the free form after 18 days of incubation. There were prominent changes in the structural conformation of laccase before and after conjugation with different amino acids which were analysed by FT-IR data analysis tools and intrinsic fluorescence analysis. At the end, non-covalently stabilized laccase was successfully used for the degradation of dyes. The non-covalent conjugation process is a single-step, simpler, economical, and facile enzyme stabilization strategy which could be utilized for enzyme applications in thermal processes. The developed conjugate can be potentially utilized for several biocatalytic and biotransformation reactions in different industrial sectors.

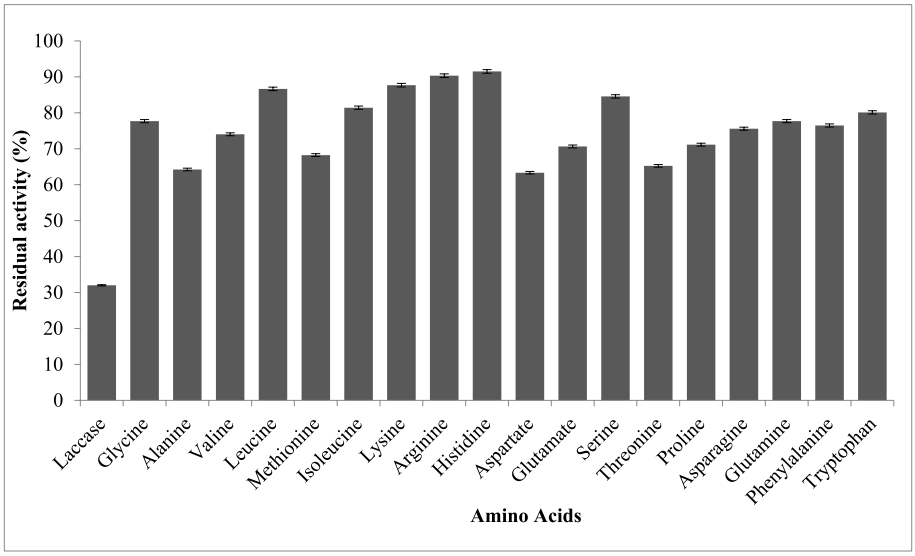


Fig. 4. Storage stability studies of free and amino acid-laccase conjugates at 30 °C in sodium phosphate buffer (pH 7, 100 mM). The experiments were done in triplicate and the error bar represents the percentage error in each set of readings.

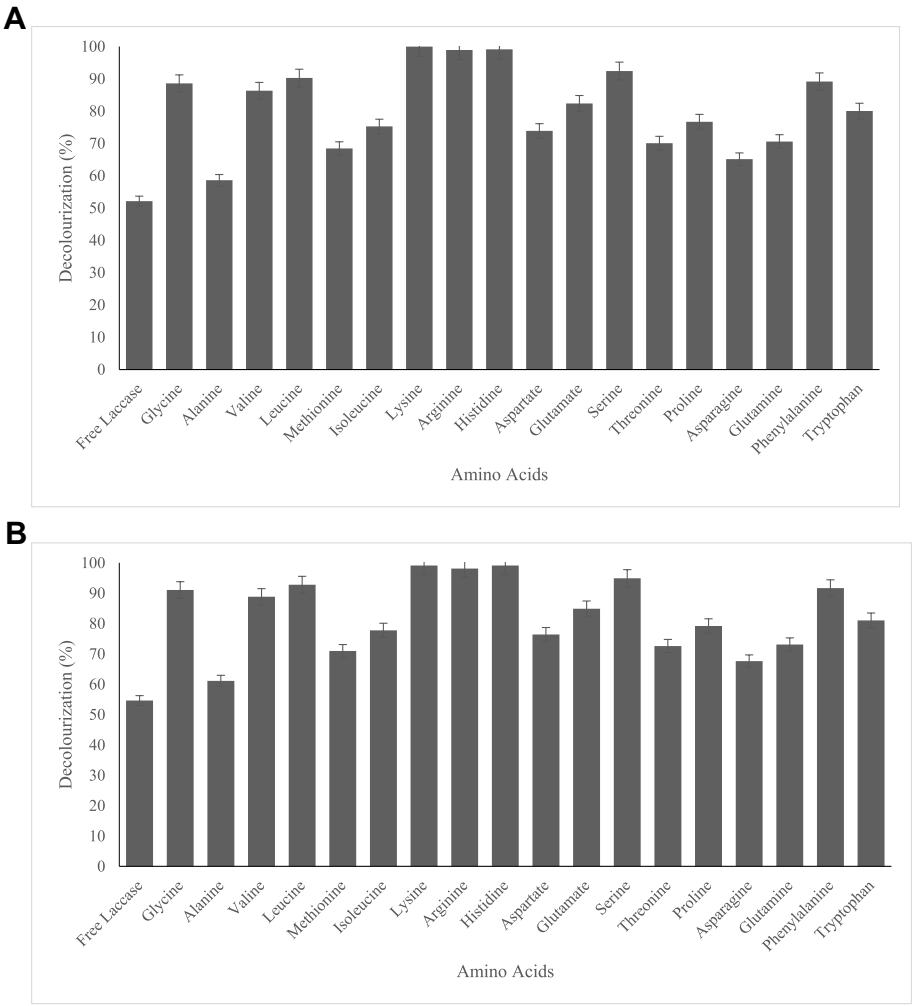


Fig. 5. The percentage decolourization of (a) Congo red (100 ppm) after 10 h and (b) Acid violet I (10 ppm) after 6 h by using different amino acid-laccase conjugates. The measurements were performed in triplicate and the error bar represents the percentage error. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101064.

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