Environmental Technology & Innovation 20 (2020) 101046

Contents lists available at ScienceDirect

Environmental Technology & Innovation

journal homepage: www.elsevier.com/locate/eti

Combination of thermal and biological treatments for bio-removal and detoxification of some recalcitrant synthetic dyes by betaine-induced thermostabilized laccase

Somayeh Mojtabavi [a,b], Mohammad Reza Khoshayand [a], Mohammad Reza Fazeli [a], Nasrin Samadi [a,*], Mohammad Ali Faramarzi [b,*]

[a] Department of Drug and Food Control, Faculty of Pharmacy, Tehran University of Medical Sciences, P.O. Box 14155-6451, Tehran 1417614411, Iran

[b] Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Tehran University of Medical Sciences, P.O. Box 14155-6451, Tehran 1417614411, Iran

A R T I C L E I N F O

Article history:
Received 21 March 2020
Received in revised form 4 July 2020
Accepted 12 July 2020
Available online 16 July 2020

Keywords:
Laccase
Betaine
Osmolyte
Protein stability
Decolorization

A B S T R A C T

The presence of recalcitrant synthetic dyes in environment is a major concern due to their possible threat on living organisms and aquatic ecosystems. To investigate the potential of combined thermal and enzymatic decolorization, betaine was applied to protect the secondary and tertiary structures as well as the enzymatic activity of laccase under thermal stress. At 60 °C, betaine 1 M significantly enhanced the stability of laccase in addition to keeping its catalytic efficiency ($p < 0.05$). Differential scanning calorimetry showed a 20 °C increase in T_m of laccase in the presence of betaine. Once incubated at 60 °C, betaine exhibited superior protein-stabilizing properties than at 25 °C. Furthermore, fluorescence spectroscopy supported this concept that the overall folded structure of laccase became more rigid in the presence of betaine at 60 °C. Betaine-stabilized laccase was used for combined thermal and enzymatic decolorization of some recalcitrant organic dyes. This osmolyte could improve the enzymatic removal of most dyes at 60 °C. A detoxification study with respect to the inhibition of bacterial growth showed a decrease in microtoxicity of the laccase-treated dye solution in the presence of betaine at 60 °C.

© 2020 Elsevier B.V. All rights reserved.

1. Introduction

Dyes are often discharged in the form of colored wastewater from industries of leather tanning, textile, food, paper, paint, printing, plastics, cosmetics, and pharmaceuticals (Dai et al., 2018). These dyes may build up in the environment, since many wastewater treatment plants allow them to pass through the system practically untreated (Seshadri et al., 1994). The complex chemical structure of most synthetic dyes makes them resistant to degradation (Nejad et al., 2019). Current environmental concern with recalcitrant dyes revolves around the potential carcinogenic health risk presented by these dyes or their intermediate biodegradation products (Seshadri et al., 1994). In general, organic dyes can fade under different conditions such as sweat, water, light, different chemicals e.g. oxidizing agents, and microbial attacks (Dai et al., 2018). Consequently, dyes are barely eliminated using the physiochemical and biological techniques. Still, recent decades

* Corresponding authors.
E-mail addresses: samadin@tums.ac.ir (N. Samadi), faramarz@tums.ac.ir (M.A. Faramarzi).

https://doi.org/10.1016/j.eti.2020.101046
2352-1864/© 2020 Elsevier B.V. All rights reserved.

have witnessed the introduction of diverse physical, chemical, and biological methods to remove dyes from wastewater (Dai et al., 2018; Ahn et al., 2014). Thermal treatment among many is one of the most extensively used methods due to its low cost, simple procedure, and high efficiency (Kumar et al., 2007). Thermal treatment occurs in a water bath based on the fact that extreme temperature acts as an accelerator of oxidation of organic materials (Ahn et al., 2014). It has been reported that by a 2 °C rise in temperature, the oxidation reaction occurs twice faster (Ahn et al., 2014). Today, the use of enzymes is also known as an environmentally friendly procedure for dye removal from wastewater (Pereira et al., 2020). Laccases, as a part of the multi-copper oxidase family, are found in many plants, fungi, and bacteria (Pannu and Kapoor, 2014). The interest in laccases as potential biocatalysts has particularly increased after the discovery of their ability to oxidize organic compounds e.g. chemical dyes (Ashrafi et al., 2013; Younes et al., 2012). In recent years, many research articles appeared in literature on the use of laccases for color removal of dye effluents (Çifçi et al., 2019; Pereira et al., 2020).

Proteins and enzymes, the most versatile macromolecules in living systems, serve crucial functions in essential biological processes (Dobson, 2003). The structure of a protein describes the folding of a polypeptide chain into a specific, stable, functional, and three-dimensional construction in which the free energy is minimized (Khan et al., 2017). It is important to stabilize the functional state of proteins and diminish the types of degradation that occur during stress conditions such as the addition of chemical substances, changes in temperature or pH of the environment, ionic strength, co-solvents, agitation, presence of denaturants, e.g. urea and guanidine hydrochloride, etc. (Farhadian et al., 2016). The changes in a protein or enzyme structure may cause a malfunction or loss of activity (Dobson, 2003). Considering the enzymes' ability to catalyze industrial processes, tolerance is required against extreme temperatures, pHs, salts, alkalis, and surfactants (Day and Kempson, 2016; Pannu and Kapoor, 2014). Some strategies have been devoted to improving enzymes' stability for enhancing their utilization as industrial biocatalysts (Manning et al., 2010). Uses of osmolytes, organic solvents, ionic liquids, and enzyme immobilization on suitable carriers have revealed improvements in proteins' and enzymes' activity as well as stability (Pannu and Kapoor, 2014; Mancini et al., 2012).

Inspired by living organisms, the accumulation of molecularly small cosolutes, known as osmolytes, is a method of combating environmental stresses (Mancini et al., 2012; Wei et al., 2013). Small natural compounds, generally neutral non-electrolytes or zwitterions, also known as protective osmolytes, are classified in chemical groups such as amino acids (e.g., proline and glycine), methylamines (e.g., sarcosine, trimethylamine N-oxide, and betaine), and polyols (e.g., glycerol, sucrose, trehalose, and other certain sugars) (Day and Kempson, 2016; Wei et al., 2013). The osmolytes maintain a protein conformation to the native state without unfavorable interactions or perturbing their structure and function (Mancini et al., 2012). The mechanism of stabilization involves exclusion of protective osmolytes from the surface of proteins causing enhanced hydration of proteins (Mancini et al., 2012). As a consequence, solvent quality is decreased by the presence of protecting osmolytes, favoring intramolecular hydrogen bonding within the polypeptide and, therefore, the folded state of the protein (Canepa et al., 2020). Thus, in the presence of an osmolyte, proteins fold into an integrally compact tertiary structure with a reduced surface area (Mancini et al., 2012).

Betaine; N, N, N-trimethylglycine, is of importance for its role in osmoregulation in biological systems. In nature, many plants and animals endure complete environmental stresses such as low water content, high salinity, or extreme temperature by accumulating large amounts of betaine (Day and Kempson, 2016; Pannu and Kapoor, 2014). Betaine favors the compact protein conformation and decreases the diversity of conformations resulting in alterations in the folding thermodynamics (Day and Kempson, 2016). Osmolytes are becoming more useful for improving the conformational stability of enzymes (Lentzen and Schwarz, 2006).

The present study described the role of betaine in the structure, function, and stability of laccase. It aimed to investigate whether betaine could stabilize the enzyme in aqueous solutions when exposed to different thermal conditions. The decolorization and microtoxification ability of laccase was also studied on some recalcitrant synthetic dyes in the presence and absence of betaine under different thermal conditions.

2. Materials and methods

2.1. Chemicals

Laccase from *Trametes versicolor* (EC 1.10.3.2) with specific activity $\geq$ 0.5 U mg^{-1} and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were obtained from Sigma Aldrich (St. Louis, MO, USA). Betaine was attained from Fluka (Neu-Ulm, Germany). The organic dyes used in this study (Table S2) were purchased from Alvan Sabet Co. (Tehran, Iran). Tween 80, Tween 20, Triton X-100, and organic solvents were provided from Merck (Darmstadt, Germany). All other chemical substances were of the highest analytical grades commercially available.

2.2. Laccase activity assay

Laccase activity was assessed by monitoring the oxidation of ABTS at 420 nm. Briefly, the enzyme activity assay mixture contained a final volume of 1 mL of 0.05 mM ABTS dissolved in citrate buffer (100 mM, pH 4.5) plus 20 μL of appropriately diluted solution of laccase (1 U mL^{-1}). The aliquot was incubated in a glass tube in a 40 °C water bath for 15 min. The change in absorbance at 420 nm was recorded and the molar extinction coefficient of ABTS was used to determine

the catalytic activity ($_{420}$ = 36000 M^{-1} cm^{-1}). Laccase activity in the presence of betaine was also determined at the conditions described above for all experiments in which normal and thermal stresses were imposed (Fig. S1). The assays were performed under controlled pH and temperature with each experiment being repeated for three times (Lentzen and Schwarz, 2006).

2.3. Thermal stability studies

The enzyme stability study was carried out in glass tubes containing 1 U of laccase in a total volume of 1 mL prepared in citrate buffer (100 mM, pH 4.5) with and without betaine at a concentration of 1 M (Supplementary material). The residual enzyme activity was determined after a 12 h-incubation at 25 °C and 60 °C under the standard assay conditions as described above. The results were expressed as the enzyme residual activity (%) associated with the laccase activity measured at pH 4.5 at the beginning of the study.

2.4. Determination of kinetic constants

The influence of betaine on the kinetic behavior of laccase was investigated to discover if betaine could improve, or at least would not deteriorate, the stability of the enzyme. The kinetic study was performed in citrate buffer (100 mM, pH 4.5) containing various concentrations of ABTS (0–5 mM). Experimental data were analyzed via regression analysis using the Michaelis–Menten equation where the K_m and V_{max} constants were determined.

2.5. The enzyme structure assessment using analytical techniques

The potential of betaine for conformational changes of laccase was assessed by some instrumental analyses. Far-ultraviolet circular dichroism (far-UV-CD) and Fourier transform infrared (FTIR) spectroscopies generally evaluate the secondary structure content of a protein. The CD spectrum in the near UV region (320–260 nm) displays the environments of aromatic amino acid side chains, thus providing information on the tertiary (or 3D) structure of a protein (Yu et al., 2013). The unfolding state of a protein may be monitored by any changes in the CD signal. There are seven tryptophan residues found in laccase with each playing a certain role in CD signal measurement. Intrinsic fluorescence spectra of laccase may be recorded to understand the microenvironment around the tryptophan residues (Yu et al., 2013).

2.6. Differential scanning calorimetry (DSC)

Conformational stability of laccase was evaluated using an ultra-sensitive DSC. DSC thermograms of enzyme solutions were obtained via Mettler DSC 823e (Calorimetry Sciences Corporation, USA) equipped with platinum capillary cells (0.3 mL). The DSC spectra were obtained from 10–100 °C at a scan rate of 1 °C min^{-1}. Prior to DSC, all solutions were degassed. Then, the buffer background was removed from each sample scan with each thermogram representing the average of three scans. The temperature at the midpoint of the transition (T_m) was determined using Mettler STARe system software.

2.7. Dye decolorization

To carry out decolorization experiments, laccase (1 U mL^{-1}) was added to each dye solution (10 mg L^{-1}) with and without betaine 1 M in citrate buffer (100 mM, pH 4.5). The reaction mixture was then incubated at 25 °C and 60 °C and 150 rpm for 12 h. To determine the decolorization percentage, the absorbance was monitored by a UV–visible spectrophotometer (every 2 h until 12 h) at the highest absorbance level of each dye (Table S2). To measure the percentage of decolorization, the following equation was employed: decolorization (%) = $(A_i–A_t)/A_i$; where A_i represents the initial absorbance of the reaction mixture and A_t stands for the absorbance following incubation. In addition, a heat-inactivated enzyme was individually added to each dye solution and incubated as mentioned above which revealed a negative control. All experiments were repeated three times with the mean scores of decolorization being reported.

2.8. Dye toxicity

A susceptibility toxicity assay was performed in order to estimate the lethal effects of both the untreated- and treated-dye solutions (combination of thermal and enzymatic decolorization) based on the inhibitory growth of two gram-negative bacterial strains (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 9027) and two gram-positive bacterial strains (*Staphylococcus aureus* ATCC 6538 and *Streptococcus mutans* ATCC 35668). Each tested bacterial strain was firstly cultivated in nutrient broth to reach OD_{600} (optical density in 600 nm) of 0.1. Thereafter, the samples obtained from enzymatic treatment of each dye and the untreated dye solution (final concentration of 10 mg L^{-1}) were separately added to the prepared bacterial broth and incubated at 37 °C. Changes in OD_{600} of each bacterial strain were then recorded every 2 h for 10 h. A negative control (cultivated bacterial strain in the absence of dye) was also designed for each experiment. The percentage of growth inhibition (GI%) was defined as $[(1\text{-}D_{600S}/OD_{600C})\times 100]$; where OD_{600S} is the OD_{600} of sample and OD_{600C} is the OD_{600} of control. All experiments were performed in triplicate.

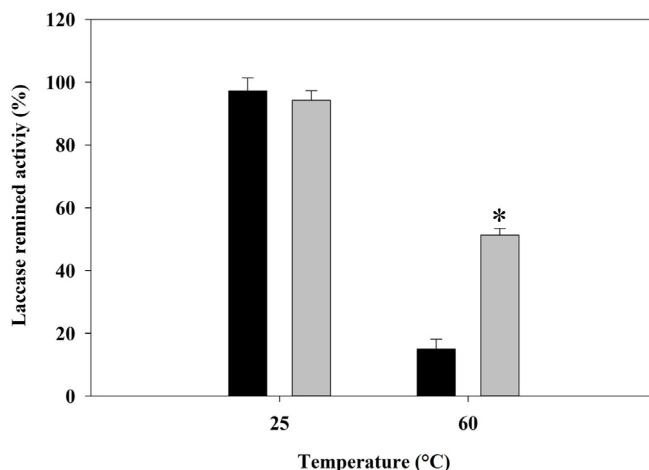

Fig. 1. Remained laccase activity in the absence (back bar) and presence (gray bar) of betaine at 25 °C and 60 °C in a 12 h-incubation. * is the level of significance ($p < 0.05$).

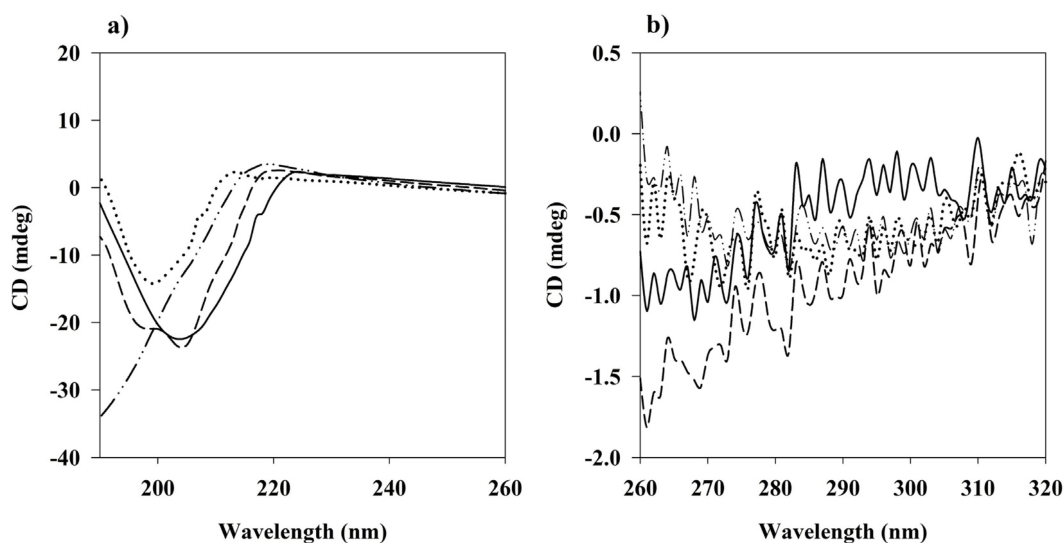

Fig. 2. Time-dependent far-UV-CD spectra (a) and time-dependent near-UV-CD spectra (b) of laccase in citrate buffer (100 mM, pH 4.5) in the absence (dotted line) or presence (solid line) of betaine 1 M at 25 °C; and in the absence (dash-dotted line) or presence (dashed line) of betaine 1 M at 60 °C after a 12 h-incubation.

2.9. Statistical analyses

The data obtained from each set of three experiments were reported as mean ± standard deviation. Statistical comparisons were conducted by one-way analysis of variance (ANOVA) through Tukey post hoc test. Probability values <0.05 were considered significant. The statistical analyses were provided using SigmaStat 12.3 software.

3. Results and discussion

3.1. Thermal stability of laccase

The stability of laccase was determined at 25 °C and 60 °C for 12 h in order to estimate the stabilizing effect of betaine under normal and thermal stressful conditions, respectively (Fig. S2). It was found that the ability of laccase for oxidizing the substrate ABTS diminished dramatically following extreme thermal conditions (60 °C). According to Fig. 1, the original activity of laccase was maintained at about 20% at 60 °C while the enzyme retained 60% of its initial activity ($p < 0.05$) in the presence of betaine at the same temperature. There was no significant distinction observed between the control and

the treated enzyme at 25 °C ($p > 0.05$). The extinction coefficient of the product was the same in the absence or presence of betaine. There are some reports suggesting that an osmolyte acts as a protein stabilizer in thermal stress conditions. It is well known that all types of osmolytes do not directly interact with many proteins, but they exhibit stabilizing effects on the structure of proteins and even enhance their activity (Wei et al., 2013). Arakawa et al. (2007) reported that sugar osmolytes (e.g. lactose, glucose, and sucrose) provided improved conformational stability to ribonuclease A, lysozyme, chymotrypsinogen A, β-lactoglobulin, bovine serum albumin, and ovalbumin by raising the free energy of the unfolded state relative to the native state. Plaza del Pino and Sanchez-Ruiz (1995) proved that the addition of the osmolyte sarcosine in a solution containing ribonuclease decreased the protein conformation diversity. As a result, reducing heterogeneity of the distribution of ribonuclease would cause changes in the dynamics of the protein. It was suggested that an osmolyte exerted its effect on the transition or intermediate states between the native and denatured protein conformations. If osmolytes compressed the structure of an unfolded protein, it could lead to protein aggregation and improper folding. Avanti et al. (2014) investigated various osmolytes (betaine, hydroxyectoine, trehalose, ectoine, and firoin) for the stability of lysozyme in aqueous solutions under different thermal conditions. They concluded that betaine could stabilize the protein under certain harsh conditions, but destabilized the same protein in an accelerated thermal condition.

3.2. Kinetic measurements

The kinetic parameters (K_m, V_{max}, and k_{cat}) of the enzyme on ABTS oxidation were determined (Table 1) in the absence or presence of betaine 1 M via nonlinear regression of the Michaelis–Menten equation using SigmaPlot version 12.3. The kinetic data obtained at 25 °C indicated that betaine did not significantly change the affinity of the enzyme to ABTS (although K_m was not exactly the same, it was very close) ($p > 0.05$). As shown in Table 1, the V_{max} for laccase in the absence of betaine was reduced from 22.09 ± 0.90 to 13.13 ±0.81 μM min^{-1} with temperature elevation. The reduction of k_{cat} may occur due to inactivation of the enzyme at 60 °C (Mancini et al., 2012). Since betaine was excluded from the vicinity of the enzyme, no direct interaction was observed between the osmolyte and no significant effect was found on K_m and k_{cat}.

3.3. FTIR and the enzyme secondary structure

The betaine–laccase interaction was characterized by FTIR and its derivative methods (Supplementary material). In the case of protein amide-I, no major spectral shifting was observed in band of 1635 cm^{-1} (mainly C=O stretch) upon betaine 1 M interaction at 25 °C and 60 °C (Fig. S3). The secondary structure of laccase was identified using the infrared self-deconvolution along with second derivative resolution enhancement and curve-fitting procedures (Nafisi et al., 2012). The structural data of laccase deposited in Protein Data Bank (http://www.rcsb.org/pdb/; accessed November 26, 2019) were used as a template. The enzyme secondary structure contains 6% α-helix (1658 cm^{-1}), 37% β-sheet (1621 cm^{-1}), 32% random coil (1636 cm^{-1}), 8% β-antiparallel, and 17% turn structure (1681 cm^{-1}). A secondary structure of the protein quantitative analysis was carried out on laccase samples (Fig. S4). FTIR results indicated that betaine 1 M did not directly interact with laccase molecule. In the presence of betaine, augmented intensity of the amide-I band was discovered due to stabilization of the protein structure. The increase in α-helix was indicative of protein stabilization upon interaction between betaine and laccase. Although the enzyme and betaine got into contact with each other, the established interaction did not lead to a complex formation. Crowe et al. (2010) investigated phosphofructokinase structuring by some osmolytes such as sugar, glycerol, proline, and trimethylamine N-oxide. They suggested that hydrogen bonding of the additives to polar and charged groups protected the secondary structure of the protein. This can be explained based on the exclusion mechanism of betaine from the enzyme surface, which consequently adds to the structured water around this area.

3.4. CD analysis of the secondary and tertiary structures of laccase

The far-UV-CD spectra of laccase were recorded and analyzed to understand the effect of betaine on the structure of laccase incubated at 25 °C and 60 °C (Fig. 2(a)). The secondary structure compositions (i.e., fractions of α-helix and β-strand) were assigned using Circular Dichroism analysis through Neural Networks (CDNN) (Table 2). By increasing the temperature from 25 °C to 60 °C, a major change was observed in the intensity of laccase spectra. In the presence of 1 M betaine, the intensity of the CD peaks at 25 °C increased to a minor extent relative to that of the protein in buffer; however, the overall α-helical and β-sheet characteristics of the protein were maintained constant. The enzyme treated with 1 M betaine at 60 °C retained considerably more α-helix and formed less β-strand compared to the betaine-free sample. Further, the betaine-treated sample showed no significant changes in β-sheet and α-helix at 25 °C and 60 °C compared to those of betaine-free laccase solutions ($p > 0.05$). The obtained result is in agreement with the hypothesis that osmolytes act as structure-inducing agents by their ability to induce helical structure (Mojtabavi et al., 2019). Near UV region (320–260 nm) of the CD spectrum was prepared to discover the status of aromatic amino acid side chains and hence to learn about the tertiary structure of the protein. A secondary structure of protein quantitative analysis was performed on laccase samples close to UV region of the CD spectrum. Fig. 2(b) illustrates that the tertiary structure of laccase was altered because of the lengthy interaction between laccase and betaine. It is notable that each molecule of

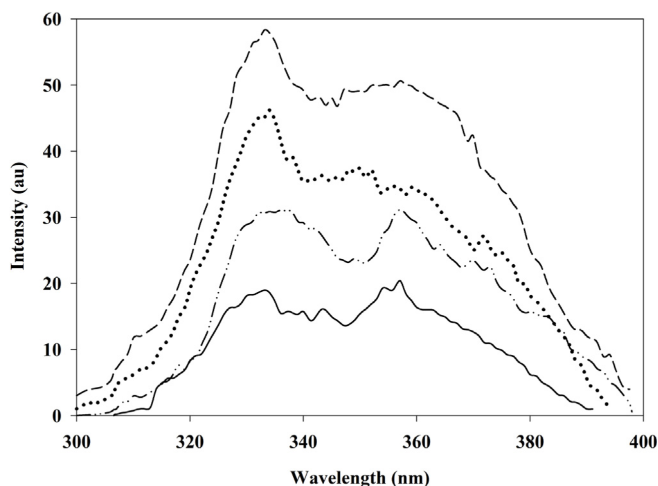

Fig. 3. Intrinsic fluorescence spectra of laccase in citrate buffer (100 mM, pH 4.5) in the absence (dotted line) or presence (solid line) of betaine 1 M at 25 °C; and in the absence (dash-dotted line) or presence (dashed line) of betaine 1 M at 60 °C after a 12 h-incubation.

Table 1
Kinetic parameters of ABTS oxidation by laccase (1 U mL^{-1}) with and without betaine in citrate buffer (100 mM, pH 4.5).

Betaine (M)	Temperature (°C)	K_m (μM)	V_{max} (μM min^{-1})	k_{cat} (S^{-1})
–	25	497.07 ± 1.21	22.09 ± 0.90	90.20 ± 0.21
1	25	499.81 ± 0.81	20.10 ± 0.53	89.32 ± 0.10
–	60	341.54 ± 1.20	13.13 ± 0.81	55.90 ± 0.39
1	60	436.12 ± 2.01	18.80 ± 0.33	83.34 ± 0.50

laccase is featured with seven tryptophan residues with each exerting a specific effect on the measured CD signal. Still, the different intensities of the spectra imply that betaine causes different effects on the laccase structure. Nash et al. (1982) showed that betaine might alter the physicochemical characteristics of some enzymes (mitochondrial enzymes) directly or indirectly. This suggests that the accumulation of betaine might be of adaptive significance under stress conditions. In addition, Belluzo et al. (2011) found that betaine affected the entire protein structure of catalase and induced minor stabilization within the secondary structure of the enzyme.

3.5. Fluorescence data analysis

The fluorescence spectra of laccase were obtained in the absence or presence of betaine incubated at 25 °C and 60 °C to gain a deep insight into its influence on the tertiary structure of the protein. The fluorophore tryptophan is highly sensitive to its surrounding environment; therefore, the conformational changes of the enzyme caused by the addition of betaine could be monitored using fluorescence spectroscopy. The maximum intensity of fluorescence (I_{max}) and the maximum emission wavelength (λ_{max}) reflect the changes the conformation of laccase in the absence or presence of betaine (Manning et al., 2010). No shift was observed in the maximum excitation wavelength (λ_{max}) of the laccase fluorescence spectra. As shown in Fig. 3, fluorescence intensity of the native laccase was slightly upper than that of the enzyme in the presence of betaine treated for 12 h at 25 °C and higher than that of the enzyme with 12 h of thermal treatment at 60 °C. The intrinsic fluorescence of laccase was studied by adding betaine, as a quencher, in concentrations from 0 to 2.5 M at 25 °C (Fig. S5). Furthermore, considerably higher fluorescence intensity was detected in the stressed laccase solution containing betaine in comparison to of the case in the absence of the osmolyte (Fig. 3). This indicated that betaine was able to inhibit considerable denaturation of laccase due to the more compactness of the enzyme structure (Manning et al., 2010). Belluzo et al. (2011) showed that betaine could quench with the amino acid tryptophan of catalase because of its quaternary amine. Based on the fluorescence spectrum (Fig. 3), it seems that similar interactions also occur between betaine and tryptophan residues of laccase at 25 °C. The emission band intensity of laccase increases by the high temperature, while the peak position remains unchanged because of more exposed to the tryptophan residues due to the cosolute–protein interaction (Belluzo et al., 2011). A probable mechanism of quenching is that betaine traps oxygen via a boosting foam. The oxygen molecule is one of the best-known collisional quenchers that quench almost all known fluorophores (Khodaverdian et al., 2018). To investigate this hypothesis, samples were degassed to remove dissolved oxygen in the solution for obtaining reliable fluorophore. Once the mentioned process occurred, the obtained results were the same as the data before degassing (data not shown). This, based on the results, this hypothesis is not approved.

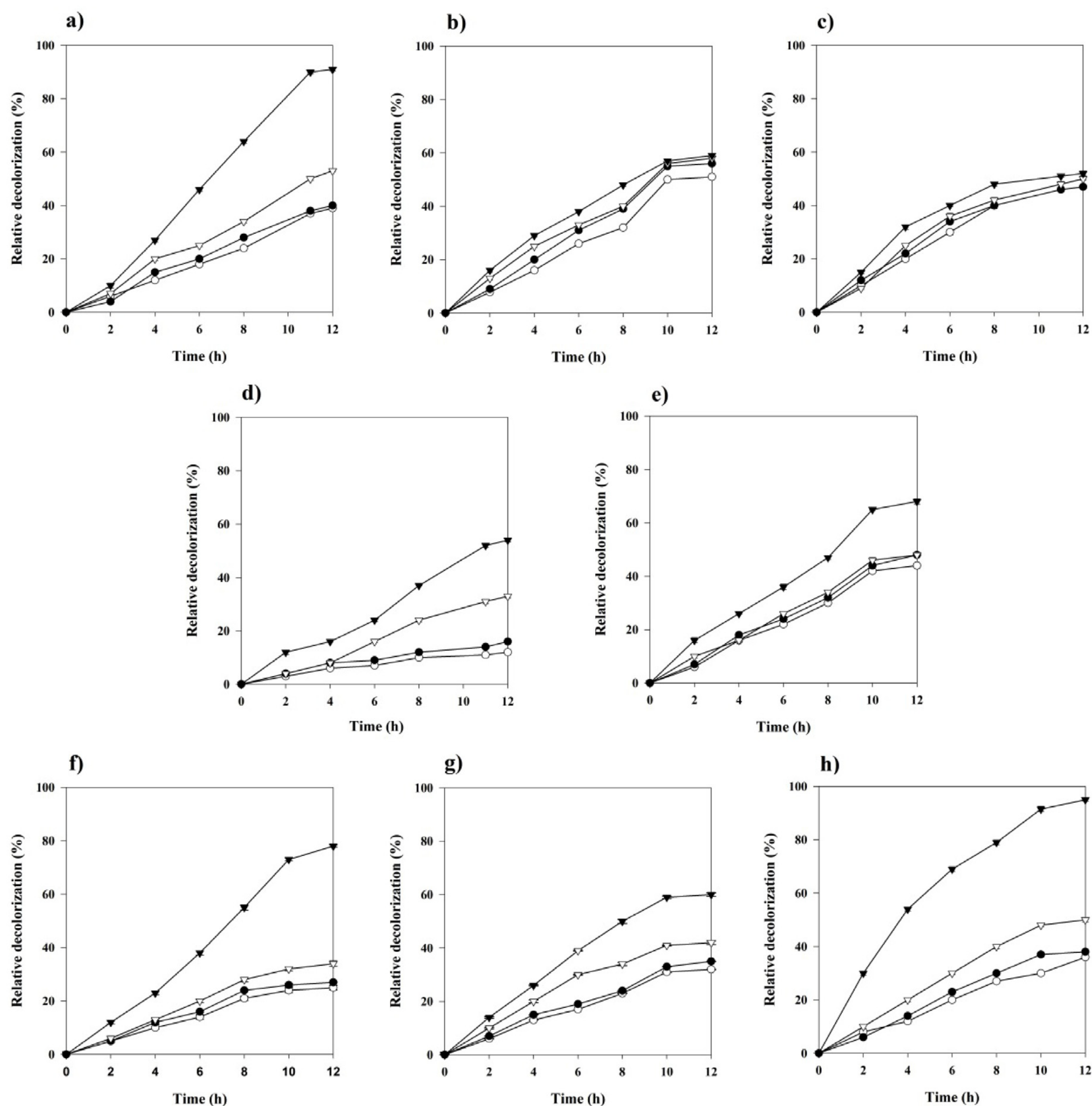

Fig. 4. Relative decolorization of some recalcitrant synthetic dyes by a combination of the enzymatic removal in the presence (black symbols) or absence (hollow symbols) of betaine 1 M and thermal treatment at 25 °C (circle) and 60 °C (triangle) on (a) Acid blue 25, (b) Acid orange 7, (c) Acid yellow 36, (d) Amido black, (e) Congo red, (f) Direct black 166, (g) Eosin, and (h) Reactive blue 29 for 12 h. Data are expressed as mean ± SD (n=3).

Table 2
The percentages of secondary structures of laccase (1 U mL^{-1}) with and without betaine in citrate buffer (100 mM, pH 4.5) determined by CDNN Deconvolution Software.

Betaine (M)	Temperature (°C)	α-Helix (%)	Antiparallel (%)	Parallel (%)	β-Turn (%)	Random Coil (%)
–	25	6.10 ± 0.23	7.90 ± 0.12	33.80 ± 0.11	20.10 ± 0.14	32.10 ± 0.16
1	25	7.81 ± 1.09	5.98 ± 0.23	30.11 ± 0.23	34.90 ± 0.12	21.20 ± 0.37
–	60	2.02 ± 0.07	3.98 ± 0.12	39.71 ± 0.14	10.18 ± 0.15	44.11 ± 0.23
1	60	15.98 ± 0.23	5.02 ± 0.11	18.97 ± 0.21	36.50 ± 0.16	23.53 ± 0.17

Table 3
Thermodynamic parameters (T_m and ΔH_{cal}) obtained from DSC of laccase (1 U mL^{-1}) with and without betaine in citrate buffer (100 mM, pH 4.5).

Betaine (M)	Temperature (°C)	T_m (°C)	ΔH_{cal} (J g^{-1})
–	25	70.20 ± 0.11	39.87 ± 0.67
1	25	89.07 ± 0.02	53.61 ± 0.99
–	60	28.71 ± 0.48	30.02 ± 0.42
1	60	96.70 ± 0.93	89.07 ± 0.87

Table 4
Removal percentage of some synthetic dyes by laccase in the presence or absence of betaine (1 M) after 12 h incubation at 25 and 60 °C in citrate buffer (100 mM, pH 4.5).

Dye	Removal at 25 °C (%)				Removal at 60 °C (%)			
	No betaine	Control[a]	Betaine	Control[b]	No betaine	Control[a]	Betaine	Control[b]
Acid blue 25	39.09 ± 0.71	1.09 ± 0.1	40.08 ± 0.40	2.09 ± 0.11	53.34 ± 0.31	13.10 ± 0.21	91.32 ± 0.10	14.06 ± 0.23
Acid orange 7	51.12 ± 0.20	0.00 ± 0.00	56.09 ± 0.41	1.00 ± 0.03	58.32 ± 0.52	10.09 ± 0.42	59.11 ± 0.70	10.42 ± 0.22
Acid Yellow 36	47.21 ± 0.41	1.09 ± 0.10	47.08 ± 0.51	1.08 ± 0.01	50.09 ± 0.43	5.06 ± 0.71	52.23 ± 0.61	4.09 ± 0.92
Amido black	12.09 ± 0.31	2.08 ± 0.01	16.03 ± 0.91	0.02 ± 0.01	33.09 ± 0.61	11.09 ± 0.61	55.32 ± 0.22	10.09 ± 0.23
Congo red	44.09 ± 1.30	1.11 ± 0.02	48.09 ± 0.51	1.11 ± 0.21	47.11 ± 0.31	21.12 ± 0.10	68.22 ± 0.31	22.67 ± 0.13
Direct black 166	25.08 ± 0.61	0.12 ± 0.02	24.05 ± 0.41	0.03 ± 0.01	34.82 ± 0.72	11.12 ± 0.60	78.09±0.44	11.65 ± 0.64
Eosin	32.17 ± 0.50	2.09 ± 0.01	36.07 ± 0.34	1.01 ± 0.01	42.23 ± 1.01	16.09 ± 1.01	60.06 ± 0.71	15.76 ± 1.04
Reactive Blue 29	36.08 ± 0.21	0.01 ± 0.01	38.22 ± 0.52	0.12 ± 0.30	48.23 ± 0.20	23.98 ± 0.11	95.08 ± 0.24	21.09 ± 0.41

[a]Samples containing heat-inactivated enzyme (in boiling water for 1 min) without betaine in dye solutions.
[b]Samples containing heat-inactivated enzyme (in boiling water for 1 min) with betaine in dye solutions

3.6. Effect of betaine on the protein unfolding temperature (T_m)

T_m (which indicates the level of protein stability) was used to evaluate the structural integrity of laccase. The DSC thermal scan of laccase exhibited a single sharp endothermic transition peak, showing a T_m of 70.20 ±0.11°C and ΔH_{cal} of 39.87 ± 0.67 J g^{-1}, respectively. Thermal denaturation profile of laccase in the presence of betaine at 25 °C and 60 °C revealed a transition point with the T_m value elevation to 89.07 ±0.02°C and 96.70 ±0.93°C, respectively. The scans of free-betaine samples incubated at 60 °C showed descending thermal transition peaks (T_m = 28.71 ±0.48°C) (Table 3). Measurement of T_m is useful to predict the stabilizing effect of betaine. There was a direct correlation between T_m and the presence of betaine in the enzyme solutions. The ΔH_{cal} values of laccase solution decreased upon temperature rise of incubation, which can be in line with a partially denatured conformation (Sanchez-Ruiz et al., 1988). In addition, Belluzo et al. (2011) demonstrated that some osmolytes such as betaine, sucrose, and mannitol affected the conformational stability and dynamics of catalase by improving the rigidity of the overall enzyme structure, at least in certain surface areas. It could be concluded that increased protein stability can be explained by the theory of preferential hydration of the protein. However, some studies (Decca et al., 2019) revealed that osmolytes acted in an opposite way on completely different proteins; in the case of lysozyme, sarcosine induced an increase in T_m, while complete unfolding took place even in the presence of the osmolyte and the lysozyme irreversibly aggregated. This ambiguous behavior could be associated with the exposure of polar and apolar parts of the protein to solvents or osmolytes (Macchi et al., 2012). The study by Söderlund et al. (2002) indicated that betaine caused more compact folding of lipase from *Thermomyces lanuginosa*. Hence, the rotation of Trp in lipase was attenuated and their average microenvironment became more hydrophobic, and the surface tension of water increased. The increased surface tension of water caused by betaine thermodynamically favored the native state of lipase.

3.7. Dye decolorization by laccase in the presence of betaine

Dyes are known as one of the most noticeable environmental contaminants even at very low concentrations. The removal of colors from effluents is therefore a major concern in industries, especially with the increase in stringent legislation. Nowadays, the use of enzyme is a promising means for wastewater treatment. However, some synthetic dyes are very stable and resistant to enzymatic removal and it is difficult to remove them from effluents only via conventional biological procedures. Combining some removal processes may be useful to ensure proper treatment of the dyes with these features. In this research, enzymatic decolorization together with thermal treatment was selected as one of the most versatile and economical methods. Enzymatic decolorization of organic dyes by laccase is presented in Table 4. The results indicated that there was no significant change ($p > 0.05$) in dye decolorization percentages with and without betaine after a 12 h-incubation by laccase at 25 °C for all applied dyes. No color removal was detected either in the negative control. The changes in decolorization percentage for Acid yellow 36 and Acid orange 7 were negligible after 12 h of thermal treatment at 60 °C. The addition of betaine could not help in oxidizing these dyes by laccase at 60 °C. The relative decolorization activity of laccase in the presence or absence of betaine 1 M after a 12 h-incubation at 25 °C and

Table 5
Growth inhibition percentage (GI%) of untreated and treated dyes with laccase in the presence of betaine at 60 °C against four bacterial strains. Values are averages of three replicates ± standard deviation.

Dye names	Bacterial strains							
	S. aureus		*S. mutants*		*P. aeruginosa*		*E. coli*	
	Un-treated	Treated	Un-treated	Treated	Un-treated	Treated	Un-treated	Treated
Acid blue 25	28.01 ± 0.01	9.21 ± 0.12	35.17 ± 0.27	6.13 ± 0.21*	26.14 ± 0.91	6.18 ± 0.08	30.16 ± 0.37	10.02 ± 0.81
Amido black	31.02 ± 0.11	8.18 ± 0.17*	36.82 ± 0.61	9.45 ± 0.04*	33.31 ± 0.43	12.25 ± 0.37	45.32 ± 0.99	17.15 ± 0.08
Congo red	33.10 ± 0.02	22.16 ± 0.01	33.32 ± 0.51	2.43 ± 0.12*	42.04 ± 0.57	15.24 ± 0.16	45.18 ± 0.13	23.23 ± 0.39
Direct black 166	39.12 ± 0.06	12.23 ± 0.34	39.24 ± 0.10	10.13 ± 0.35	49.01 ± 0.61	23.15 ± 0.47	51.04 ± 0.18	19.81 ± 0.37
Eosin	35.14 ± 0.08	10.91 ± 0.01	32.05 ± 0.41	1.02 ± 0.01*	29.14 ± 0.81	8.16 ± 0.85	30.01 ± 0.59	9.87 ± 0.19
Reactive Blue 29	27.13 ± 0.21	10.62 ± 0.91	22.32 ± 0.32	6.05 ± 0.98*	18.15 ± 0.81	6.18 ± 0.72	20.14 ± 0.94	8.01 ± 0.61

*Significancy was determined using independent sample t-test (p value < 0.05).

60 °C on all applied dye solutions is displayed in Fig. 4. The results indicated that except for Acid yellow 36 and Acid orange 7, enzymatic decolorization of the rest of the organic dyes increased after the thermal treatment at 60 °C. The study also showed that the enzymatic decolorization percentages in the presence of betaine for Reactive blue 29, Direct black 166, Acid blue 25, Amido black 166, Eosin, and Congo red were considerably better than in the case with no betaine at 60 °C. The dye removal values indicated that Reactive blue 29 (95.08 ± 0.24%) and Acid blue 25 (91.32 ± 0.10%) showed a higher degree of decolorization followed by Direct black 166 (78.09 ± 0.44%), Congo red (68.22 ± 0.31%), Eosin (60.06 ± 0.71%), and Amido black 166 (55.32 ± 0.22%) after 12 h of enzymatic incubation with betaine at 60 °C. Several studies have shown the ability of laccases to oxidize a wide range of chemical organic dyes (Ashrafi et al., 2013; Gholami-Borujeni et al., 2011). However, some organic dyes (e.g. Amido black, Congo red, Acid black, etc.) are recalcitrant to enzymatic removal at ambient temperature (Aghaie-Khouzani et al., 2012; Gholami-Borujeni et al., 2011). In a previous research, Amido black, Congo red, and Acid black were found to be the most recalcitrant dyes against enzymatic decolorization (Forootanfar et al., 2011). This study showed that the thermal treatment partly removed recalcitrant organic dyes. When laccase was added to the solutions, dyes exhibited less resistance to decolorization as compared to the case with no laccase. The enzymatic removal of these dyes considerably improved in the presence of betaine at a high temperature. Apparently, betaine functions as a stabilizer and induces changes in the laccase structure, which facilitates hydration of the enzyme. This demonstrates greater accessibility of dyes to the enzyme at 60 °C.

3.8. Microtoxicity study

Several studies have been conducted on the capability of physicochemical or enzymatic procedures to achieve safe metabolites after treatment of hazardous pollutants. It was tried to examine the toxic effects of formed metabolites after remediation (Ashrafi et al., 2013). For this purpose, a practical method is to evaluate growth inhibition of standard microbial strains (e.g. *E. coli* and *S. aureus*) that are mostly used for the assessment of toxicity of synthetic dyes. The outcomes of the present study indicated that the growth rate (GI%) was notably declined after adding dye solutions to cultivation media of gram-negative and positive bacterial strains. The percent of growth inhibition (GI%) with all treated dyes demonstrated a notable decline in comparison to the dye solutions. The azo dye Direct black 166 was the most toxic dye, inhibiting *S. aureus, S. mutants*, *P. aeruginosa,* and *E. coli* by 39.12%, 39.24%, 49.01%, and 51.04%, respectively, after a 10-h exposure to each bacterial strain. However, growth of the mentioned bacterial strains was remarkably improved in the presence of treated Direct black 166 with laccase stabilized at 60 °C (Table 5). The anthraquinonoid dye Reactive blue 29 showed the lowest inhibition against tested bacterial species (Table 5). After a treatment of Eosin, only 1.02% toxicity toward *S. mutants* remained. Ashrafi et al. (2013) studied decolorization of 13 organic dyes using laccase from *T. versicolor*. They reported that toxicity (based on GI% in *S. typhi*, *B. subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa*) notably decreased following the treatment of dye solution using enzyme. In addition, the growth of *E. coli* and *B. megaterium* was increased from 11% to 94% and from 2% to 99%, respectively, following the incubation of malachite green exposed to the purified laccase obtained from *S. thermophilum* (Ashrafi et al., 2013). Decolorization of 18 synthetic dyes was also examined by Mendes et al. (2011) using a recombinant CotA-laccase from *B. subtilis*. The results showed that except for Reactive Black 5, Acid Black 210, Direct Red 80, Acid Black 194, Reactive Yellow 145, Direct Black 38, and acid Red 266, toxicity (based on GI% in *S. cerevisiae*) significantly declined following treatment of dyes using purified laccase (Mendes et al., 2011).

4. Conclusion

The addition of betaine to laccase-contained samples enhanced the stability of laccase and maintained the catalytic efficiency of the enzyme under high temperature conditions. These changes in the enzyme stability were well associated with the variations in the laccase structure conformation under high temperature as evidenced by FTIR, CD, and fluorescence spectra. The connection between tryptophan and betaine at van der Waals radii turns laccase structure to the native state. Overall, betaine-stabilized laccase could be applied for combined thermal and enzymatic decolorization and detoxification of recalcitrant synthetic dyes in aqueous solutions and wastewater.

CRediT authorship contribution statement

Somayeh Mojtabavi: Involved in the sections of "laccase structure assessment using analytical techniques", "Thermal stability studies", "Dye decolorization", Statistical analysis, Did the section "microtoxicity study, Writing - original draft. **Mohammad Reza Khoshayand:** Statistical analysis. **Mohammad Reza Fazeli:** Did the section "microtoxicity study". **Nasrin Samadi:** Involved in purchasing of required materials and instruments, Designing of the experiments, Analyzing of data, Reviewing of the manuscript. **Mohammad Ali Faramarzi:** Involved in purchasing of required materials and instruments, Designing of the experiments, Analyzing of data, Reviewing of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

All authors read and approved the final manuscript. This work was financially supported by the grant No. 97-03-33-40087 from Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. This research was also partially supported by Biotechnology Research Center, Tehran University of Medical Sciences, Tehran, Iran. The study is a part of the Ph.D. thesis of Dr. Somayeh Mojtabavi.

Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2020.101046.

References

Aghaie-Khouzani, M., Forootanfar, H., Moshfegh, M., Khoshayand, M.R., Faramarzi, M.A., 2012. Decolorization of some synthetic dyes using optimized culture broth of laccase producing ascomycete *Paraconiothyrium variabile*. Biochem. Eng. J 60, 9–15. http://dx.doi.org/10.1016/j.bej.2011.09.002.

Ahn, C., Zeng, X., Li, L., Obendorf, S.K., 2014. Thermal degradation of natural dyes and their analysis using HPLC-DAD-MS. Fash. Text. 1 (22), http://dx.doi.org/10.1186/s40691-014-0022-5.

Arakawa, T., Kita, Y., Timasheff, S.N., 2007. Protein precipitation and denaturation by dimethyl sulfoxide. Biophys. Chem. 131, 62–70. http://dx.doi.org/10.1016/j.bpc.2007.09.004.

Ashrafi, S.D., Rezaei, S., Forootanfar, H., Mahvi, A.H., Faramarzi, M.A., 2013. The enzymatic decolorization and detoxification of synthetic dyes by the laccase from a soil-isolated ascomycete, Paraconiothyrium variabile. Int. Biodeterior. Biodegrad. 85, 173–181. http://dx.doi.org/10.1016/j.ibiod.2013.07.006.

Avanti, C., Saluja, V., Van Streun, E.L., Frijlink, H.W., Hinrichs, W.L., 2014. Stability of lysozyme in aqueous extremolyte solutions during heat shock and accelerated thermal conditions. PLoS One 9, e86244. http://dx.doi.org/10.1371/journal.pone.0086244.

Belluzo, S., Boeris, V., Farruggia, B., Picó, G., 2011. Influence of stabilizers cosolutes on catalase conformation. Biol. Macromol. 49, 936–941. http://dx.doi.org/10.1016/j.ijbiomac.2011.08.012.

Canepa, J., Torgerson, J., Kim, D.K., Lindahl, E., Takahashi, R., Whitelock, K., Heying, M., Wilkinson, S.P., 2020. Characterizing osmolyte chemical class hierarchies and functional group requirements for thermal stabilization of proteins. Biophys. Chem. 12, 106410. http://dx.doi.org/10.1016/j.bpc.2020.106410.

Çifçi, D.İ., Atav, R., Güneş, Y., Güneş, E., 2019. Determination of the color removal efficiency of laccase enzyme depending on dye class and chromophore. Water Sci. Technol. 80, 134–143. http://dx.doi.org/10.2166/wst.2019.255.

Crowe, J.H., Crowe, L.M., Carpenter, J.E., Petrelski, S., Hoekstra, F.A., Araujo, P.D., Panek, A.D., 2010. Anhydrobiosis: cellular adaptation to extreme dehydration. Compr. Surv. 1445–1477. http://dx.doi.org/10.1002/cphy.cp130220.

Dai, L., Zhu, W., He, L., Tan, F., Zhu, N., Zhou, Q., Hu, G., 2018. Calcium -rich biochar from crab shell: An unexpected super adsorbent for dye removal. Bioresour. Technol. 267, 510–516. http://dx.doi.org/10.1016/j.biortech.2018.07.090.

Day, C.R., Kempson, S.A., 2016. Betaine chemistry, roles and potential use in liver disease. Gen. Subj. 1860, 1098–1106. http://dx.doi.org/10.1016/j.bbagen.2016.02.001.

Decca, M.B., Borioli, G.A., Durand, E.S., Moreschi, A., Hallak, M.E., Montich, G.G., 2019. Thermal unfolding of calreticulin, structural and thermodynamic characterization of the transition. Biochim. Biophys. Acta 1867, 175–183. http://dx.doi.org/10.1016/j.bbapap.2018.12.002.

Dobson, C.M., 2003. Protein folding and misfolding. Nature 426, 884–890. http://dx.doi.org/10.1038/nature02261.

Farhadian, S., Shareghi, B., Saboury, A.A., Momeni, L., 2016. Counteraction of lactose on the thermal stability and activity of α-chymotrypsin: thermodynamic, kinetic, and docking studies. RSC Adv 6, 72201–72212. http://dx.doi.org/10.1039/C6RA11833J.

Forootanfar, H., Faramarzi, M.A., Shahverdi, A.R., Yazdi M.T, 2011. Purification and biochemical characterization of extracellular laccase from the ascomycete *Paraconiothyrium variabile*. Bioresour. Technol. 102, 1808–1814. http://dx.doi.org/10.1016/j.biortech.2010.09.043.

Gholami-Borujeni, F., Mahvi, A.H., Naseri, S., Faramarzi, M.A., Nabizadeh, R., Alimohammadi, M., 2011. Application of immobilized horseradish peroxidase for removal and detoxification of azo dye from aqueous solution. Res. J. Chem. Environ. 15, 217–222.

Khan, S., Farooq, U., Kurnikova, M., 2017. Protein stability and dynamics influenced by ligands in extremophilic complexes -a molecular dynamics investigation. Mol. BioSyst. 13, 1874–1887. http://dx.doi.org/10.1039/c7mb00210f.

Khodaverdian, S., Dabirmanesh, B., Heydari, A., Dashtban-Moghadam, E., Khajeh, K., Ghazi, F., 2018. Activity, stability and structure of laccase in betaine based natural deep eutectic solvents. Int. J. Biol. Macromol. 107, 2574–2579. http://dx.doi.org/10.1016/j.ijbiomac.2017.10.144.

Kumar, P., Prasad, B., Mishra, I.M., Chand, S., 2007. Catalytic thermal treatment of desizing wastewaters. J. Hazard. Mater. 149, 26–34. http://dx.doi.org/10.1016/j.jhazmat.2007.03.051.

Lentzen, G., Schwarz, T., 2006. Extremolytes: natural compounds from extremophiles for versatile applications. Appl. Microbiol. BioTechnol. 72, 623–634. http://dx.doi.org/10.1007/s00253-006-0553-9.

Macchi, F., Eisenkolb, M., Kiefer, H., Otzen, D.E., 2012. The effect of osmolytes on protein fibrillation. Int. J. Mol. Sci. 13, 3801–3819. http://dx.doi.org/10.3390/ijms13033801.

Mancini, R.J., Lee, J., Maynard, H.D., 2012. Trehalose glycopolymers for stabilization of protein conjugates to environmental stressors. J. Am. Chem. Soc. 134, 8474–8479. http://dx.doi.org/10.1021/ja2120234.

Manning, M.C., Chou, D.K., Murphy, B.M., Payne, R.W., Katayama, D.S., 2010. Stability of protein pharmaceuticals: an update. Pharm. Res. 27, 544–575. http://dx.doi.org/10.1007/s11095-009-0045-6.

Mendes, S., Farinha, A., Ramos, C.G., Leitão, J.H., Viegas, C.A., Martins, L.O., 2011. Synergistic action of azoreductase and laccase leads to maximal decolourization and detoxification of model dye-containing wastewaters. Bioresour. Technol. 21, 9852–9859. http://dx.doi.org/10.1016/j.biortech.2011.07.108.

Mojtabavi, S., Samadi, N., Faramarzi, M.A., 2019. Osmolyte-induced folding and stability of proteins: concepts and characterization. in: IJPR. http://dx.doi.org/10.22037/ijpr.2020.112621.13857.

Nafisi, S., Panahyab, A., Sadeghi, G.B., 2012. Interactions between β-carboline alkaloids and bovine serum albumin: investigation by spectroscopic approach. J. Lumin. 132, 2361–2366. http://dx.doi.org/10.1016/j.jlumin.2012.03.075.

Nash, D., Paleg, L.G., Wiskich, J.J., 1982. Effect of proline, betaine, and some other solutes on the heat stability of mitochondrial enzymes. Plant Biol. 9, 47–57. http://dx.doi.org/10.1071/PP9820047.

Nejad, Z.G., Borghei, S.M., Yaghmaei, S., 2019. Biodegradation of synthetic dye using partially purified and characterized laccase and its proposed mechanism. Int. J. Environ. Sci. Technol. 16, 7805–7816. http://dx.doi.org/10.1007/s13762-019-02226-5.

Pannu, J.S., Kapoor, R.K., 2014. Microbial laccases: a mini-review on their production, purification, and applications. Int. J. Pharm. Arch. 3, 528–536. http://dx.doi.org/10.1071/PP9820047.

Pereira, C.S., Kelbert, M., Daronch, N.A., Michels, C., de Oliveira, D., Soares, H.M., 2020. Potential of enzymatic process as an innovative technology to remove anticancer drugs in wastewater. Appl. Microbiol. BioTechnol. 104, 23–31. http://dx.doi.org/10.1016/J.SCITOTENV.2018.09.373.

Plaza del Pino, I.M., Sanchez-Ruiz, J.M., 1995. An osmolyte effect on the heat capacity change for protein folding. Biochemistry 34, 8621–8630. http://dx.doi.org/10.1021/bi00027a011.

Sanchez-Ruiz, J.M., Lopez-Lacomba, J.L., Cortijo, M., Mateo, P.L., 1988. Differential scanning calorimetry of the irreversible thermal denaturation of thermolysin. Biochemistry 27, 1648–1652. http://dx.doi.org/10.1021/bi00405a039.

Seshadri, S., Bishop, P.L., Agha, A.M., 1994. Anaerobic/aerobic treatment of selected azo dyes in wastewater. Waste Manage 14, 127–137. http://dx.doi.org/10.1016/0956-053X(94)90005-1.

Söderlund, T., Zhu, K., Jutila, A., Kinnunen, P.K., 2002. Effects of betaine on the structural dynamics of *Thermomyces* (*Humicola*) *lanuginosa* lipase. Colloids Surf. B 26, 75–83. http://dx.doi.org/10.1016/S0927-7765(02)00032-2.

Wei, Y., Thyparambil, A.A., Latour, R.A., 2013. Quantification of the influence of protein-protein interactions on adsorbed protein structure and bioactivity. Colloids Surf. B 110, 363–371. http://dx.doi.org/10.1016/j.colsurfb.2013.04.018.

Younes, S.B., Bouallagui, Z., Sayadi, S., 2012. Catalytic behavior and detoxifying ability of an atypical homotrimeric laccase from the thermophilic strain *scytalidium thermophilum* on selected azo and triarylmethane dyes. J. Mol. Catal. B: Enzym 79, 41–48. http://dx.doi.org/10.1016/j.molcatb.2012.03.017.

Yu, X., Zou, F., Li, Y., Lu, L., Huang, X., Qu, Y., 2013. Effect of three trifluoromethanesulfonate ionic liquids on the activity, stability, and conformation of laccase. Int. J. Biol. Macromol. 56, 62–68. http://dx.doi.org/10.1016/j.molcatb.2012.03.017.