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Thermostable Bacterial Laccase: Catalytic Properties and Its Application in Biotransformation of Emerging Pollutants

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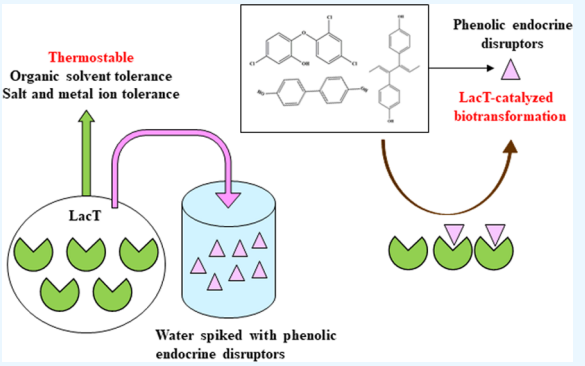
ABSTRACT: Laccases have been predominantly reported in fungi, and primarily, fungal laccases are currently exploited in industrial applications. However, extremophilic bacterial laccases possess immense potential, as they can withstand extreme temperatures, pH, and salt concentrations. In addition, unlike fungal laccases, the production of bacterial laccases is cost-effective. Therefore, bacterial laccases are gaining significant attention for their large-scale applications. Previously, we reported a novel thermostable laccase (LacT) from *Brevibacillus agri*. Herein, we have confirmed that LacT shares a high sequence similarity with CotA laccase from *Bacillus amyloliquefaciens*. Peptide mass fingerprinting of LacT was conducted via matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF/MS-MS). Inductively coupled plasma-optical emission spectroscopic (ICP-OES) analysis revealed the presence of ∼3.95 copper ions per protein molecule. Moreover, the secondary and tertiary structure of LacT was studied using circular dichroism (CD) and fluorescence spectroscopy. The absence of notable shifts in CD and fluorescence spectra with an increase in temperature established that LacT remains intact even at elevated temperatures. Analysis of the thermal denaturation profile of LacT by thermogravimetric analysis (TGA) also confirmed its temperature stability. Thereafter, we exploited LacT in its application for the bioremediation of phenolic endocrine disruptors, namely, triclosan, 4,4′-dihydroxybiphenyl, and dienestrol. LacT oxidizes 4,4′-dihydroxybiphenyl and triclosan but no LacT activity was detected with dienestrol. The rate of biotransformation of 4,4′-dihydroxybiphenyl and triclosan increased in the presence of $CuSO_4$ and a redox mediator, ABTS. Transformation of dienestrol was observed only with LacT in the presence of ABTS. This study establishes the application of LacT for the bioremediation of phenolic compounds.



1. INTRODUCTION

Laccases (*p*-diphenol: dioxygen oxidoreductases EC 1.10.3.2) are multicopper polyphenol oxidases. They can catalyze the oxidation of various types of aromatic and nonaromatic compounds. They are involved in prominent mechanisms, for example, cross-linking of monomers, ring cleavage of aromatic complexes, and degradation of polymers. By virtue of this, laccases are useful in numerous biotechnological and industrial applications such as the synthesis of organic compounds, biopulping agents in the paper industry, biobleaching of denim, biofuel cells, biosensors, and bioremediation. Laccases are widely distributed in major classes of fungi, actinomycetes, bacteria, and higher plants. Especially, white-rot fungi are predominant laccase producers. Some laccases are also reported in lichens, sponges, oysters, and arthropods.[1,2]

Fungal laccases, by virtue of their high redox potential, have been extensively researched. However, fungal laccases have many shortcomings such as low enzyme yield, nonfunctionality at extreme pHs, temperature, high concentration of salts/metals/organic solvents, etc., which makes them nonfeasible for industrial deployment. The slow growth rate of fungi and the high maintenance cost for culturing further hinder the practical application of fungal laccases. In addition, the accumulation of biomass during the large-scale production of fungal laccases creates a disposal problem.[3] On the contrary, bacterial laccases are stable at high temperatures and extreme pH ranges. They exhibit a good tolerance to salts, metals, organic solvents, and inhibitory agents. Moreover, the production of bacterial laccases is fast, easy, and economical.[4] Unlike that of fungi, the management of bacterial cellular debris after laccase production is convenient. On account of the above-mentioned advantages of bacterial laccases, their demand in the industrial sector is increasing at a faster pace.[3,5]

One of the emerging fields of laccase application is the bioremediation of contaminants of emerging concern (CECs).

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CECs are a wide range of deleterious chemicals that are not regulated by law.[6] Endocrine disruptors are a type of CEC that can have potentially harmful effects on both human beings and wildlife as they can mimic the body's endogenous hormones, such as estrogen, or affect their production, release, metabolic breakdown, and removal.[7] Due to this, they have been implicated in reproductive disorders and various types of malignancies.[8] Triclosan (TCS), dienestrol (DS), and 4,4′-dihydroxybiphenyl (DHBP) are examples of such endocrine-disrupting chemicals. Due to broad-spectrum antimicrobial activity of TCS, it is used as a preservative in different consumer goods such as cosmetics, personal care products, plastics, paints, etc.[9] DS is a nonsteroidal synthetic estrogen. Due to its ability to stimulate estrogen receptors, it has been used to treat atrophic vaginitis, a condition that can be induced by low estrogen in the body.[10] It is also used in animal husbandry as an additive in animal feeds to promote growth in animals to attain more muscle meat.[11] DHBP is a significant industrial chemical. For instance, vulcanized rubber is susceptible to oxidative damage from heat, light, and natural aging. DHBP is used as an antioxidant to retard such damage in rubbers and plastics.[12] Furthermore, hydroxylated biphenyls are one of the most used active ingredients in pesticides.[13] The estrogenic effects of these compounds have been extensively studied.[14−16]

At present, conventional wastewater treatment plants are not equipped to completely remove such compounds. Due to the growing menace of such pollutants, researchers have developed specialized chemical and physical methods such as reverse osmosis and advanced oxidation processes (AOPs) to ultimately obliterate such synthetic chemicals from water. Unfortunately, the aforementioned methods suffer from various limitations such as high costs, excessive energy consumption, inadequate infrastructure, the requirement of technical expertise, and the use of toxic chemicals.[17] Therefore, it is a pressing priority to develop green and cost-effective technologies to eliminate such contaminants. In this direction, the application of enzymes especially laccases for pollutant remediation is gaining enormous attention. The application of enzymes in bioremediation can be impeded by their inability to withstand high temperatures, salts, organic solvents, etc. Previously, we reported a novel thermostable laccase (LacT) from *Brevibacillus agri* that exhibited thermostability with high salt, organic solvent, and metal tolerance.[18] Such a thermostable laccase can prove to be highly beneficial in the bioremediation of contaminated water than mesophilic enzymes.

The use of synthetic chemicals and their subsequent discharge into the environment have grown significantly over the years, raising concerns about their detrimental effects on health. Industrial expansion, agrochemical use, and human consumption have escalated the release of these chemicals into the environment.[17] Triclosan, dienestrol, and 4,4′-dihydroxybiphenyl are endocrine disruptors that can have detrimental effects. For instance, Stoker et al.[14] studied the effect of triclosan on the pubertal development of female rats. The researchers observed that oral exposure to triclosan (150 mg/kg) increased uterine weight, which is indicative of estrogenic action. These observations suggest that triclosan is capable of interfering with estrogen-dependent functions. Similarly, Schreiber et al.[15] confirmed that exposure of male rats to dienestrol during gestation and lactation is toxic to their reproductive systems. The authors observed decreased sperm mobility and viability with an irregular morphology upon maternal exposure to dienestrol. Paris et al.[16] studied the estrogenicity of phenyl derivatives, including DHBP, and reported that DHBP activates both estrogen receptor α (ERα) and β (ERβ). They ascribed very high estrogenicity to DHBP on par with bisphenol A, a well-known documented endocrine disruptor.

In the present study, we performed the biophysical characterization of previously isolated LacT from *B. agri*[18] by using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF/MS-MS), inductively coupled plasma-optical emission spectroscopic (ICP-OES) analysis, thermogravimetric analysis, and temperature-dependent fluorescence and circular dichroism spectroscopies. Thereafter, we assessed LacT in the biotransformation of three endocrine disruptors, namely, triclosan, dienestrol, and 4,4′-dihydroxybiphenyl.

2. EXPERIMENTAL METHODOLOGY

2.1. Materials and Chemicals. 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS, >98%) and trypsin were purchased from Sigma-Aldrich. Triclosan (TCS, >98%), 4,4′-dihydroxybiphenyl (DHBP, >98%), and dienestrol (DS, >96%) were obtained from Tokyo Chemical Industry Co., Ltd. (Japan). Basic chemicals, namely copper sulfate, salts, solvents, and buffers, were procured from Himedia Pvt. Ltd. (India). Quartz cuvettes used in spectroscopy were from Hellma Analytics (Germany). All other chemicals used were of either analytical grade or molecular grade as per the requirement.

2.2. Microorganism, LacT Production, and Purification. In our previous studies, we isolated *B. agri* (NCBI GenBank accession number SUB7371747 VP-1 MT422060) from hot sulfur spring water.[18] The culture of *B. agri* was maintained on malt-agar slants (stored at 4 °C) and glycerol stock (stored at −80 °C). A novel acidophilic and thermostable laccase (LacT) was identified and characterized from *B. agri*. Thereafter, the production and purification of LacT were optimized. LacT was purified by a series of chromatographic techniques, namely, anion exchange and size exclusion chromatography. The purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram analysis.[18,19] This study focuses further on the biophysical characterization of LacT and its potential application in bioremediation.

2.3. MALDI-TOF/MS-MS Analysis of LacT. Peptide mass fingerprinting of purified LacT was conducted by using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF/MS-MS) (Autoflex II TOF/TOF Bruker Daltonics). The protein band showing laccase activity in the zymogram was excised from its corresponding SDS-PAGE gel having Coomassie Brilliant Blue G-250 dye. Then, the excised band was digested with trypsin and peptides were extracted according to the method devised by Shevchenko et al.[20] The data obtained from mass spectrometry were analyzed by a BLAST search on the National Centre for Biotechnology Information (NCBI) database. The evolutionary comparison between the query sequence and reference sequences from the NCBI database was conducted in the molecular evolutionary genetics analytics (MEGA) version 11.[21]

2.4. Metal Content of Laccase: ICP-OES. We examined LacT for the presence of metal ions, namely, copper, iron, zinc, cobalt, manganese, and magnesium, by using ICP-OES

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Table 1. List of Peptide Fragments after Trypsin Digestion (MALDI-TOF/MS-MS)

s. no.	mass	range	peptide sequence
1	2313.965	1−20	-.MALEKFADELPIIETLKPQK.T
2	3304.889	147−175	R.GALLWYHDHAMAITRLNVYAGLAGMYIIR.E
3	1570.493	162−175	R.LNVYAGLAGMYIIR.E
4	2120.726	181−198	K.QLKLPAGEYDVPLMIMDR.T
5	1684.558	339−353	R.VTKPLKGEDTSRKPK.Y
6	1613.530	351−364	R.KPKYLSAMPDMTSK.R
7	2048.724	354−370	K.YLSAMPDMTSKRIHNIR.T
8	993.836	366−373	R.IHNIRTLK.L
9	1207.438	393−402	R.WHDPVTEAPR.L

(Agilent Technologies 5110). Protein samples (1 mg/mL) were added in 3.0−3.5 mL of ultrapure nitric acid (∼65−70%). Then, the mixture was digested at 200 °C for 10−15 min in a microwave reaction system (MARS, CEM corporation). The digested samples were diluted with 2% nitric acid at the time of the metal analysis. The concentrations of metal ions were measured from the standard calibration curve of each metal.

2.5. Fluorescence Spectroscopy. In the present study, we have examined the effect of temperature on the intrinsic fluorescence emission of LacT by using a fluorescence spectrometer (Cary 600 series, Agilent Technologies, Inc.). The excitation wavelength was fixed at 280 nm. The emission spectrum was recorded in the wavelength range of 305−405 nm with an interval of 1 nm. Both emission and excitation slits were set at a 5 nm bandwidth. The quartz cuvette having a path length of 1 cm was used. The samples contained 0.3 mg/mL LacT in 20 mM sodium acetate buffer (pH 5.0). Fluorescence emission spectra were studied by incubating LacT at various temperatures of 30, 50, 60, 70, and 80 °C. All spectra were recorded after subtracting the emission of 20 mM sodium acetate buffer (pH 5.0). The reported spectra were the averages of five independent scans.

2.6. Circular Dichroism Spectroscopy. In the present study, CD spectroscopy was performed to understand the changes in the secondary structure of LacT after exposure to high temperatures. CD spectra were recorded by using a CD spectropolarimeter (JASCO Corporation, Japan model no. J-815) fitted with a Peltier-type temperature-controlled cuvette holder (PTC-517) at a scan rate of 100 nm/min. The samples contained 3 μM LacT in 20 mM sodium acetate buffer (pH 5.0) in a quartz cuvette of a 0.1 cm path length (300 μL cell). Spectra were recorded at the far-ultraviolet (UV) region (195−240 nm) with an interval of 1 nm. The samples were incubated at various temperatures of 30, 50, 60, 70, and 80 °C. All spectra were background-corrected by subtracting the blank spectra (20 mM sodium acetate buffer, pH 5.0). The reported spectra were the averages of five independent scans.

2.7. Thermogravimetric Analysis (TGA). We performed a thermogravimetric analysis of LacT to understand its thermal denaturation profile. TGA (PerkinElmer, Inc.) was conducted in a nitrogen atmosphere with a flow rate of 20.0 mL/min. Lyophilized (freeze-dried) purified LacT samples in the range of 4−5 mg were subjected to analysis by TGA. A TGA experiment was conducted in the temperature range of 30−110 °C with a heating rate of 10 °C/min. The reported scans are the mean of three independent iterations.

2.8. Laccase-Mediated Biotransformation of Organic Contaminants. Three phenolic endocrine disruptors, viz., triclosan, 4,4′-dihydroxybiphenyl, and dienestrol, were subjected to *B. agri* LacT treatment. LacT (2U) was added to a reaction mixture containing 100 mM citrate-phosphate buffer (pH 3.0) spiked with 10 mg/L triclosan, 4,4′-dihydroxybiphenyl, or dienestrol. Due to the poor solubility of the compounds in water, the buffer was prepared with 20% ethanol. The laccase-mediated removal of these organic pollutants was assessed by monitoring the decrease in absorbance at 280 nm for triclosan, 263 nm for DHBP, and 228 nm for dienestrol, with a UV−visible (UV−vis) spectrophotometer (model no. V-730; JASCO, Japan) at 50 °C against a blank solution containing only LacT. The LacT-mediated removal of the organic pollutants was also monitored in the presence of 10 mM copper sulfate or 1.0 μM ABTS in the reaction solution. In these cases, buffer containing LacT and ABTS or $CuSO_4$ was used as the blank. The percentage removal efficiency was calculated according to the following formula

$$R\ (\%) = (A_0 - A_t)/A_0 \times 100$$

where R is the percentage removal efficiency and A_0 and A_t are the absorbance at the λ_{max} of the pollutants at time (t) = 0 and 60 min, respectively. Kinetic parameters of LacT with DHBP, TCS, and DS were calculated by measuring the initial velocity of the laccase-mediated biotransformation with different concentrations of the substrate (0−70 μM). Kinetic studies were carried out with LacT (2U) at 50 °C. The kinetic parameters were also determined in the presence of ABTS (1.0 μM) and Cu^{2+}(10 mM).

3. RESULTS AND DISCUSSION

It was found that the characteristics of biological molecules are influenced by various environmental factors such as temperature, pH, organic solvents, salts, metals, inhibitors, etc. Therefore, various spectroscopic techniques were developed to understand the properties of biomolecules under different environmental conditions.[22] Especially, temperature is an important physical factor that greatly affects the structure and stability of proteins. In the present work, we have studied the effect of temperature on the stability of LacT by using well-known techniques, namely, fluorescence and circular dichroism spectroscopy. Moreover, the behavior of LacT with a function of temperature was studied by using thermogravimetric analysis. Based on the TGA results, the thermal denaturation profile of LacT was constructed.

3.1. MALDI-TOF/MS-MS and Metal Content Analysis of LacT. In the previous study, we purified LacT by ion exchange and size exclusion chromatography. The purification of LacT resulted in an ∼38% yield and an ∼3.2 purification fold. The homogeneity of purified LacT (molecular weight of ∼65 kDa) was confirmed by SDS-PAGE and zymogram

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ACS Omega 2023, 8, 34710−34719

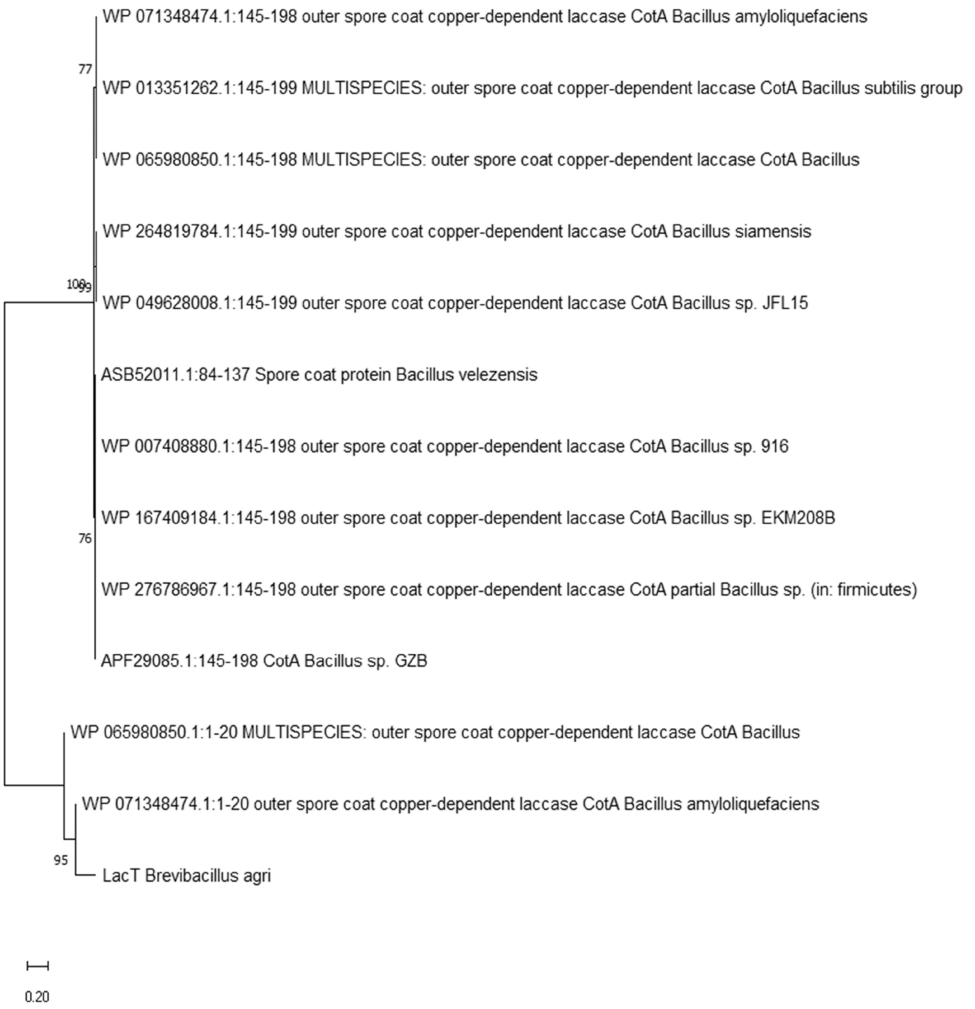

Figure 1. Phylogenetic tree showing the evolutionary relationships between LacT and other bacterial laccases.

analysis.[19] In this study, we performed peptide mass fingerprinting by the MALDI-TOF/MS-MS technique to further identify and characterize LacT in detail. The list of peptide fragments obtained after trypsin digestion of LacT is presented in Table 1.

Thereafter, a phylogenetic tree was constructed to examine the relationship of LacT to different bacterial laccases, as shown in Figure 1. The evolutionary history was determined using the minimum evolution method[23] using the stated scale. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method[24] and are in the units of the number of amino acid substitutions per site. The analysis involved 13 amino acid sequences. Evolutionary analyses were conducted in MEGA11.[21] The phylogenetic analysis of the peptide fragments of LacT stipulated that it shares the highest similarity with outer spore coat copper-dependent laccase CotA from *B. amyloliquefaciens* (WP 071348474.1).

Furthermore, we used ICP-OES to measure the metal content in the purified LacT samples. The results revealed an ∼3.95 molar copper/protein ratio in LacT. This implied that LacT is mainly a copper-loaded enzyme. These data also suggested that LacT belongs to the category of four-copper oxidase. On the other hand, the concentrations of zinc and cobalt metals in LacT were found to be very low. LacT showed a molar metal/protein ratio of ∼0.3 and ∼0.1 for zinc and cobalt, respectively. Iron, manganese, and magnesium metal ions were not detected in the protein samples. The concentration of various metal ions in purified LacT determined by ICP-OES is listed in Table 2.

Table 2. ICP-OES Analysis of Purified LacT

element	molar metal/protein ratio
copper	3.95 ± 0.04
iron	nd
zinc	0.3 ± 0.05
cobalt	0.1 ± 0.05
manganese	nd
magnesium	nd

ND: Not detectable.

The concentrations of copper and other metal ions vary among laccase enzymes. In the literature, laccase enzymes were reported to contain 1−6 copper atoms per protein molecule. Reiss et al.[25] reported a 3.9 ratio of molar Cu/protein in a novel thermostable CotA-type laccase from *Bacillus pumilus*. They concluded that this novel laccase was a fully Cu-loaded protein. In another study, Lcc1 from *Cyathus bulleri* (a ligninolytic fungus) showed a ratio of ∼3.76 copper ions/mol and ∼181 ppb zinc ions in the native enzyme.[26] Conversely, in

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ACS Omega 2023, 8, 34710−34719

other published studies, instead of only copper atoms, other types of metal ions such as iron and zinc were also found in the laccase enzyme. For instance, a purified laccase from *Pleurotus ostreatus* HP-1 contained three types of metal ions, namely, copper, iron, and zinc, in the stoichiometric ratio of 1:1:2, respectively.[27] A novel extracellular bacterial laccase was isolated from *Bacillus* sp. CF96 (symbiotic bacterium existing in the digestive system of termite) displayed a stoichiometric ratio of 4:1:1 for copper, iron, and zinc, respectively.[28] As the zinc and cobalt molar ratio in the current study was found to be much lower than 1, it indicated that LacT very unlikely utilizes Zn^{2+} or Co^{2+} for its catalytic activity.

3.2. Tertiary-Structure Analysis by Fluorescence Spectroscopy. Fluorescence spectroscopy is extensively used to investigate the conformational changes in the structure of proteins, degree of hydrophobicity, and local mobility around fluorophores.[29] In the fluorescence studies, the tertiary conformation of the protein is investigated by observing the environment around aromatic amino acids. Tryptophan and tyrosine are commonly used aromatic amino acids that are buried in the hydrophobic cores of protein. They are highly sensitive to their surroundings during protein folding and unfolding. Therefore, they are used as indicators for understanding the tertiary structures of protein.[26]

In this study, we examined the conformational property of LacT by recording its intrinsic fluorescence from 305 to 405 nm at different temperatures after excitation at 280 nm. The native LacT at 30 °C showed fluorescence intensity (in terms of counts per second) of ∼9174 at λ_{max}. It was observed that the fluorescence intensity slowly decreased with an increase in incubation temperature from 30 to 80 °C. It was probably due to the exposure of tryptophan residues to the polar environment. This suggested that the residues of tryptophan and tyrosine were buried inside native LacT in the hydrophobic environment.[29] However, there was no distinct heat-dependent shift in the maximum fluorescence wavelength of LacT. No major blue or red shift was observed in the fluorescence of LacT after heat treatment. These findings suggested that the LacT protein contains stable domains that remained intact even at higher temperatures (Figure 2).

In the literature, many thermostable laccases also exhibited similar results. For instance, Kumar and Srikumar observed an identical pattern in the laccase isoforms (OV137 and CP137) from xerophytic plants *Opuntia vulgaris* and *Cereus pterogonus*, respectively.[30] They also reported that the increase in temperature negligibly altered the fluorescence spectrum of OV137 and CP137. In another reported study, TthLAC, a thermostable recombinant laccase from *Thermus thermophilus* (strain HB 27), showed no change in the fluorescence maxima even after heating up to 90 °C.[31]

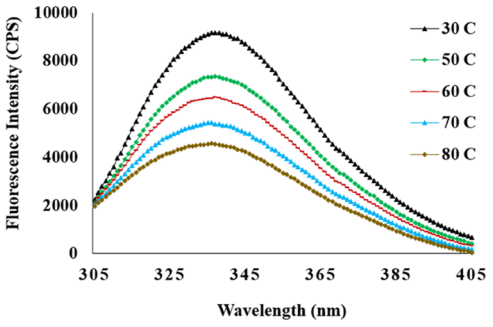

Figure 2. Intrinsic fluorescence emission spectra of LacT at temperatures ranging from 30 to 80 °C. Fluorescence intensity was measured in counts per second (CPS). Emission and excitation slits were fixed at 5 nm. Protein samples contained 0.3 mg/mL LacT in a 20 mM acetate buffer (pH 5.0). All fluorescence spectra were background-corrected with buffer. The final spectrum was the mean of five independent scans.

3.3. Secondary-Structure Analysis by Circular Dichroism Spectroscopy. The CD spectrum of proteins in the far-UV region is a commonly used technique to determine the secondary structure of proteins. It helps us to understand the conformation of the peptide backbone in proteins. This information is crucial for the folding and unfolding studies of proteins.[32,33] In a protein molecule, α-helices are mainly stabilized through backbone hydrogen bonding, while in the case of β-sheets, both backbone hydrogen bonding and hydrophobic intermolecular interactions are involved in the stabilization of β-sheets.[29] Especially, thermophilic proteins have some peculiar structural and sequential attributes that provide them elevated intrinsic thermal stability. The attributes responsible for the stability of thermophilic proteins are surface loop deletion, greater hydrophobicity, decreased occurrence of thermolabile residues, better salt bridge formation, fewer or smaller cavities, effective atom packing, enhanced hydrogen bonding, decreased looping, expanded polar surface area, substitution of residues in the secondary structures, and the presence of more proline residues in loops.[34]

In the present research, the thermostability of LacT was studied by using a far-UV spectrum of CD in the range of 195−240 nm. The CD spectrum of purified LacT revealed a distant negative peak at ∼218 nm (Figure 3). This indicated

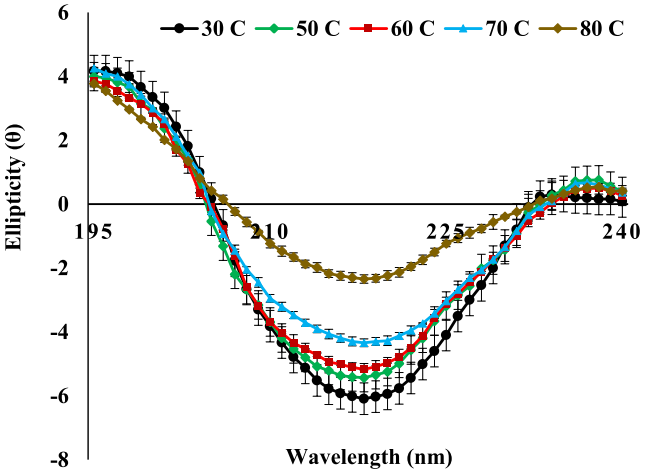

Figure 3. Far-UV circular dichroism spectra of LacT at temperatures ranging from 30 to 80 °C. CD spectra were recorded from 195 to 240 nm with an interval of 1 nm. The protein samples contained 3 μM LacT in 20 mM acetate buffer (pH 5.0). The scan rate was 100 nm/min. All spectra were background-corrected by the blank spectra (20 mM acetate buffer, pH 5.0). The final spectra were the mean of five independent scans.

the predominance of β-sheets in the secondary structure of LacT. This finding was in agreement with many other reported thermostable laccases, for instance, Shafiei et al.[35] found 66% β-sheet in thermostable laccase from *Cohnella* sp. A01. In another study, Tian et al.[36] reported 36.5% β-sheet in Lac-Q (thermostable fungal laccase from *Pycnoporus* sp. SYBC-L10).

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ACS Omega 2023, 8, 34710−34719

A thermostable laccase from *T. thermophilus* also found a clear negative peak at ∼218 nm.[31]

There was a gradual decline in ellipticity (θ) with an increase in the incubation temperature. However, there was no shift in the maximum peak (λ_{max} = 218 nm) even after incubation at 80 °C. The heating of LacT at 70 °C reduces its ellipticity by only ∼28% at λ_{max}. These results indicated that LacT was able to maintain its secondary structure after heat treatment and was resistant to complete unfolding. In previous studies, other thermostable laccases also showed similar trends. Kumari et al.[31] reported only 20% unfolding in the protein structure after heating from 20 to 90 °C in TthLAC, a thermostable laccase. Similarly, laccase from *Geobacillus thermopakistaniensis* did not show any notable change in CD spectra until 90 °C.[37]

3.4. Thermal Denaturation Profile by TGA. TGA is a highly effective technique used to measure the thermal stability of various substances. In this technique, the weight of material is monitored with respect to an increase in temperature. TGA can also evaluate the concentrations of volatile compounds and moisture contents in a given material. The apparatus of TGA consists of a microfurnace and a heating element comprised of platinum, which can withstand ∼1000 °C.[38]

In this study, the potential of LacT to withstand high temperatures was investigated using TGA. The purified LacT samples were lyophilized overnight. The solid powder of native LacT was subjected to heat treatment at the temperature range of 30−110 °C having a 10 °C/min heating rate. The loss in the weight of the enzyme during heating was calculated in terms of the weight (%). The weight of the original sample (before heating) was expressed as 100%.

At the end of heating from 30 to 110 °C, LacT showed an ∼21% loss of its original weight.

TGA data indirectly reveal the bond strength between water molecules and proteins. In nonthermostable proteins, the bond between molecules of water and proteins is very weak. Therefore, upon heating, the water molecules quickly escape from proteins. Whereas, in the case of thermostable proteins, water molecules are strongly held by protein molecules. So, the removal of water molecules from proteins is not easy, even after heating at high temperatures. In the case of LacT, during heat treatment, there was a gradual loss in the weight of protein samples, which indicated that water molecules are tightly held by protein. Therefore, water molecules were not easily removed from LacT with an increase in temperature. TGA analysis of LacT with respect to temperature is represented in Figure 4. This result was in agreement with those of other reported thermostable laccases. For instance, Kumar and Srikumar[30] reported a 10−18% loss in the weight of thermophilic laccase isoforms from xerophytes after heat treatment from 30 to 100 °C.

3.5. Biotransformation of Organic Contaminants by LacT. There are currently no sustainable and efficient ways to remove these micropollutants.[39] Laccases, therefore, show promise for the successful treatment of such compounds. However, wastewater from different industries may contain heavy metals, high salt concentrations, and organic solvents that may interfere with laccase's ability to act on its target compounds. In addition, enzymes are notoriously susceptible to temperature and pH changes. Therefore, in such cases, laccases like LacT (*B. agri*) that exhibit thermostability and thermotolerance as well as tolerance toward salts, metals, and solvents[18] can be a major step forward in developing sustainable technologies for bioremediation and translating

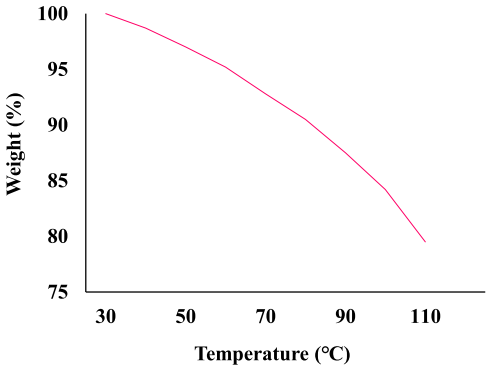

Figure 4. Characterization of LacT by thermogravimetric analysis. Lyophilized protein (∼5 mg) was taken. The experiment was carried out under a nitrogen atmosphere (flow rate of 20 mL/min). Protein samples were heated between temperatures of 30−110 °C. The heating rate was fixed at 10 °C/min. The final TGA plot was the mean of three independent readings.

such research to practical applications. Therefore, in this study, we analyzed the effect of LacT on the concentrations of DS, TCS, and DHBP in spiked water samples.

Treatment with LacT resulted in a decrease in the concentration of DHBP and TCS, indicated by the reduction in the absorbance at 262 and 280 nm, respectively (Figures 5a and 6a). The addition of LacT resulted in the removal of ∼8% of DHBP and ∼37% of TCS in 1 h. Previously, the bioremediation of TCS with the *Providencia rettgeri* MB-IIT strain exhibiting laccase and manganese peroxidase activity has been reported.[40] The isolated strain degraded ∼98% TCS in 24 h. The bioconversion of DS and DHBP with bacterial laccase has not been outlined in the literature. Moreover, the bioconversion rate of DHBP and DS with T. versicolor laccase (TvL) is higher than that observed with LacT, as percentage removal efficiency was ∼68% for DHBP and ∼11% for DS in 1 h.[41] However, T. versicolor laccase has suboptimal temperature and pH working ranges as compared to LacT, which makes LacT a better candidate for biocatalytic applications in extreme conditions. Moreover, the activity of TvL is negatively affected in the presence of salt and organic solvents.[41]

On the other hand, LacT did not have any effect on the concentration of DS (Figure 7a). In our previous study, we demonstrated the biotransformation of DS with TvL.[41] There is an array of factors that can influence the catalytic activity of laccases, such as the electron donation propensity of the substrate, the redox potential of the laccase, and substrate binding to name a few.[42] Bacterial laccases are recognized to possess a lower redox potential than their fungal counterparts.[5] It is possible that DS is endowed with a high redox potential or is encumbered to properly fit into the active site of LacT. Under such circumstances, redox mediators have proven to be useful.

Therefore, we treated DS spiked water samples with LacT in the presence of the redox mediator, ABTS, and observed a slight decrease in the absorbance at its λ_{max} value, indicating a decrease in the concentration of DS (Figure 7b). However, the observed hypochromic shift with DS was less significant when compared to the other two phenolic compounds. The mechanistic pathway of such a catalytic reaction involves the oxidation of ABTS by laccase and the oxidation of the substrate by the oxidized form of ABTS, allowing LacT to expand its substrate range that can be oxidized. The addition of

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ACS Omega 2023, 8, 34710−34719

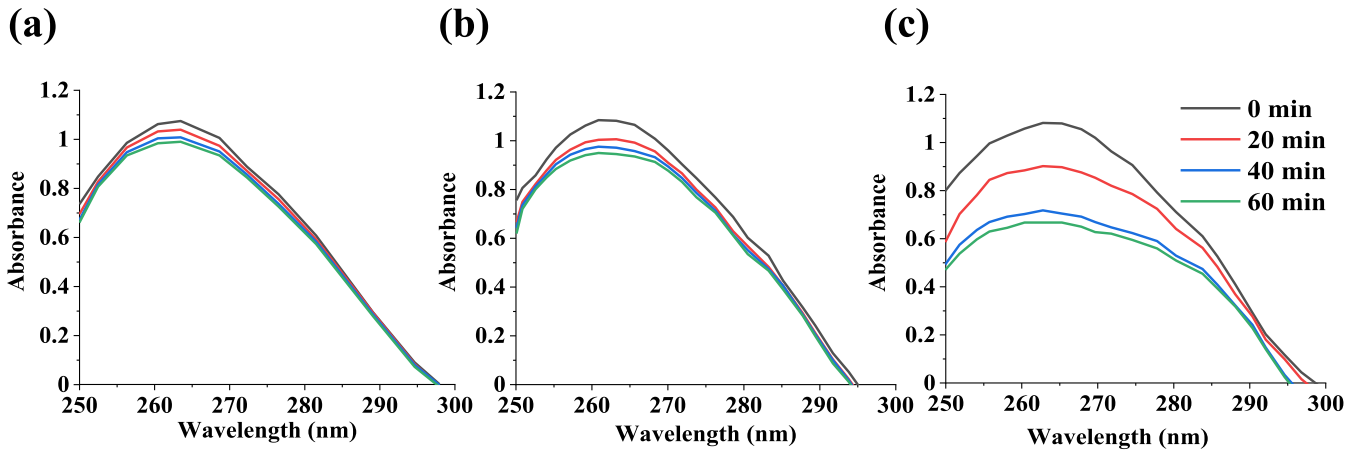

Figure 5. LacT-catalyzed transformation of DHBP (a) in the absence of both ABTS and $CuSO_4$, (b) in the presence of ABTS (1 μM), and (c) in the presence of $CuSO_4$ (10 mM). LacT (2U) was added to the spiked samples containing DHBP (10 mg/L) in 0.1 M citrate-phosphate buffer (pH 3.0) and incubated at 50 °C for 1 h. The laccase-mediated transformation was monitored by a decrease in the absorbance at the λ_{max} (263 nm) at an interval of 20 min.

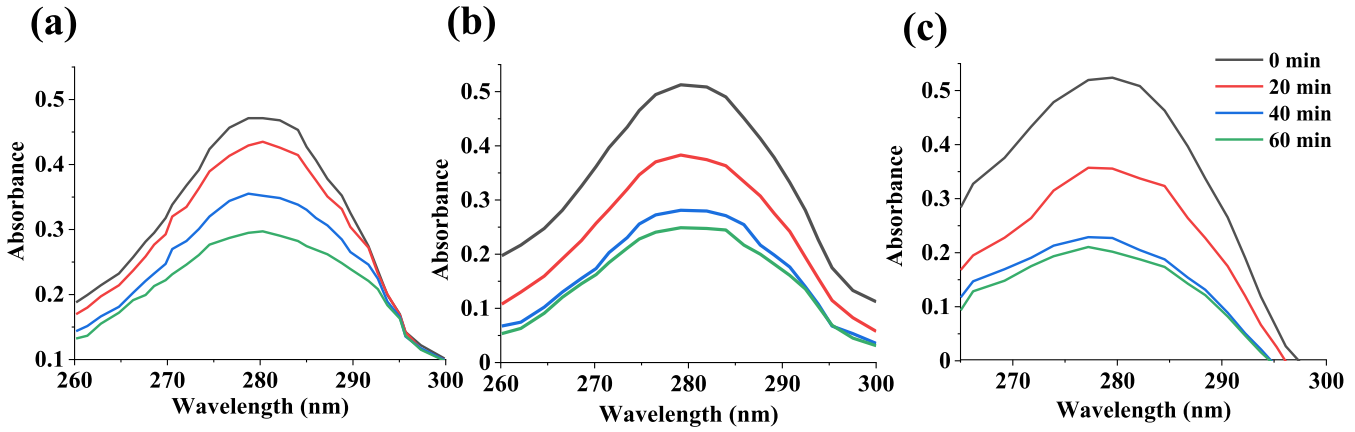

Figure 6. LacT-catalyzed transformation of triclosan (a) in the absence of both ABTS and $CuSO_4$, (b) in the presence of ABTS (1 μM), and (c) in the presence of $CuSO_4$ (10 mM). LacT (2U) was added to the spiked samples containing triclosan (10 mg/L) in 0.1 M citrate-phosphate buffer (pH 3.0) and incubated at 50 °C for 1 h. The laccase-mediated transformation was monitored by a decrease in the absorbance at the λ_{max} (281 nm) at an interval of 20 min.

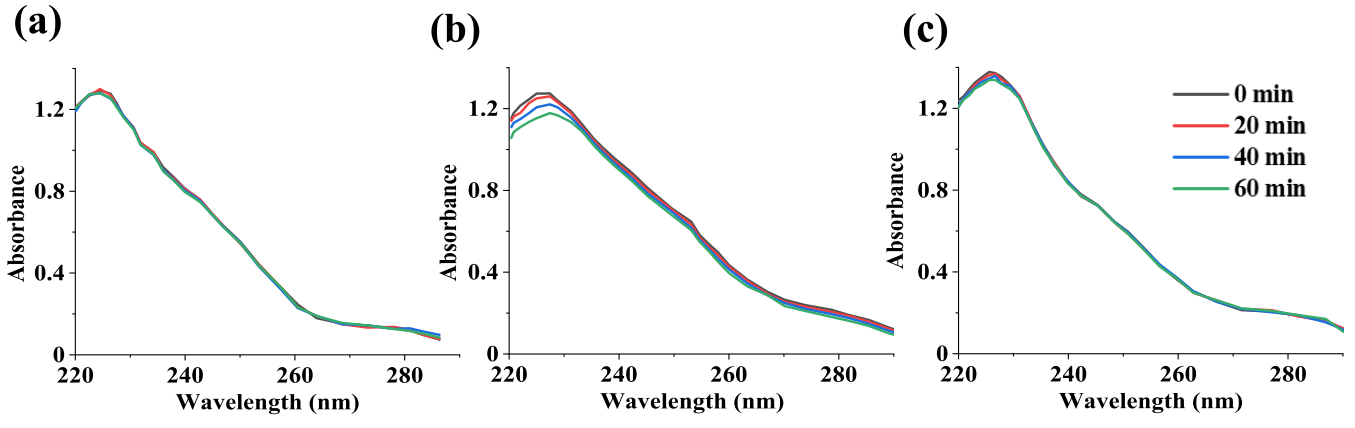

Figure 7. LacT-catalyzed transformation of dienestrol (a) in the absence of both ABTS and $CuSO_4$, (b) in the presence of ABTS (1 μM), and (c) in the presence of $CuSO_4$ (10 mM). LacT (2U) was added to the spiked samples containing dienestrol (10 mg/L) in 0.1 M citrate-phosphate buffer (pH 3.0) and incubated at 50 °C for 1 h. The laccase-mediated transformation was monitored by a decrease in the absorbance at the λ_{max} (229 nm) at an interval of 20 min.

ABTS also improved the removal of DHBP (Figure 5b) and TCS (Figure 6b) by ~1.6 and ~1.4 fold, respectively. The increase in biotransformation rates of laccase in the presence of ABTS has been reported previously.[43]

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ACS Omega 2023, 8, 34710−34719

Table 3. Summary of Percentage Removal Efficiency and Kinetic Parameters for the Biotransformation of DHBP, DS, and TCS by LacT under Different Conditions

condition	removal efficiency (%)	K_m (μM)	V_{max}(μM/min)
DHBP + LacT	7.86 ± 0.40	53.73 ± 1.35	3.45 ± 0.49
DHBP + LacT + ABTS	12.63 ± 0.69	36.49 ± 3.88	5.96 ± 0.68
DHBP + LacT + $CuSO_4$	38.3 ± 2.1	45.21 ± 4.92	16.91 ± 1.47
TCS + LacT	36.91 ± 2.0	42.35 ± 1.99	17.27 ± 1.61
TCS + LacT + ABTS	51.33 ± 2.7	37.26 ± 1.49	24.43 ± 3.26
TCS + LacT + $CuSO_4$	61.46 ± 3.5	40.84 ± 1.18	31.30 ± 1.18
DS + LacT			
DS + LacT + ABTS	5.81 ± 0.3	58.21 ± 4.53	2.56 ± 0.14
DS + LacT + $CuSO_4$			

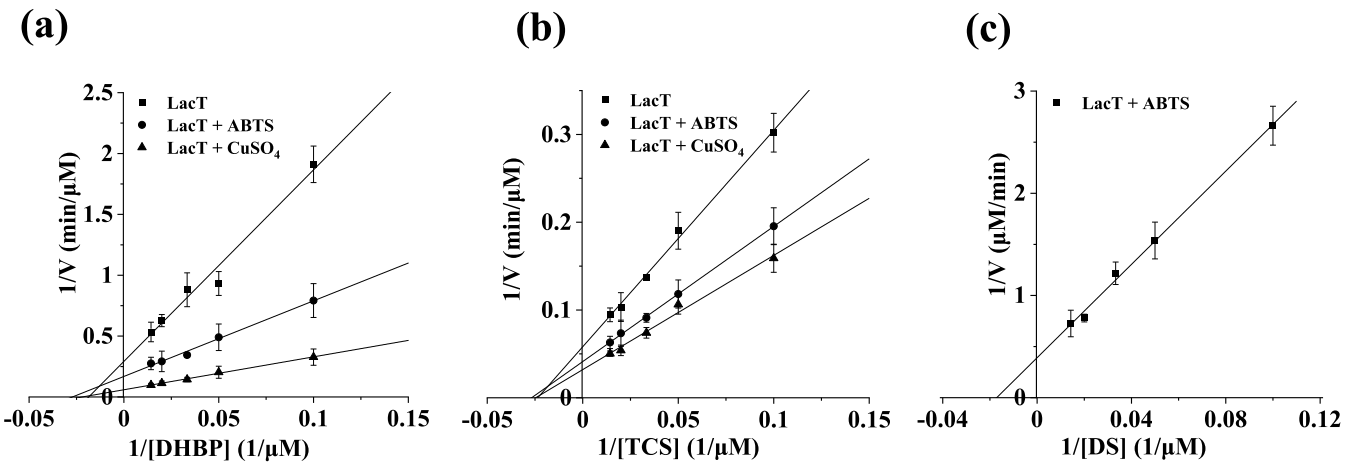

Figure 8. Lineweaver–Burk plot for the biotransformation of (a) DHBP, (b) TCS, and (c) DS by LacT in the absence and the presence of ABTS (1 μM) or $CuSO_4$ (10 mM). LacT (2U) was added to the spiked samples containing 10–70 μM DHBP, TCS, or DS in 0.2 M citrate-phosphate buffer (pH 3.0) and incubated at 50 °C for 1 h. The initial velocity of the biotransformation was monitored by a decrease in the absorbance at the respective λ_{max}.

Laccases belong to the group of multicopper oxidases, which implies that they contain copper atoms in their active site. The presence of copper in LacT is further confirmed by ICP-OES. The addition of copper to purified laccase was reported to activate and stabilize the enzyme, which led to an increase in catalytic activity.[44] The activation and stabilization of laccase by copper can prove to be useful in bioremediation applications; therefore, the laccase-mediated removal of the three micropollutants was also studied in the presence of $CuSO_4$. The addition of Cu^{2+} led to an improvement in the removal of DHBP and TCS by ∼4.8 and ∼1.7-fold, respectively (Figures 5c and 6c). However, no such improvement in the case of DS was observed (Figure 7c). A summary of the percentage removal efficiency of LacT under different conditions is shown in Table 3. In addition, the control reaction mixture, which consisted of the substrate and copper or ABTS without LacT, did not exhibit any decrease in the absorbance at the λ_{max} (Figures S1–S3). Therefore, our findings suggest that the observed improvement in the biotransformation of phenolic compounds is solely due to the positive effect of copper or ABTS on LacT's activity and negates the possibility of copper or ABTS directly acting on the substrate.

Laccase oxidizes phenolic compounds into the corresponding phenoxy radicals, which undergo nonenzymatic reactions such as the production of quinone or polymerization.[45,46] A decrease in the absorbance at the λ_{max} of the micropollutants indicates the deterioration of the parent phenolic compound by LacT. It is believed that the elimination of the parent substance can help diminish the toxicity associated with such pollutants.[47] The phenolic endocrine disruptors used in this study are known to exhibit estrogenic activity.[14,16,48] Paris et al.[16] demonstrated that the extent of hydroxylation as well as the position of the hydroxyl groups in phenylphenols can play a key role in determining the estrogenic activity of the compound. They observed that 4-OH phenylphenol (*para*) presented higher estrogenic activity than its *meta* and *ortho* counterparts. In addition, estrogenic activity increased with an increase in hydroxyl groups. For instance, 4,4′-biphenol displayed higher estrogenic activity than 4-OH phenylphenol. Therefore, oxidation of the *para*-OH groups in the tested substrates by LacT could aid in reducing the estrogenic activity of the compounds. On this basis, we can conclude that LacT can be an effective and sustainable tool in the removal of such emerging phenolic contaminants from domestic wastewater and industrial effluents.

3.6. Determination of Kinetic Parameters. As it has been demonstrated that ABTS and Cu^{2+} successfully improve the LacT's rate of biotransformation of the pollutants, we wanted to investigate the change in kinetic parameters of LacT brought upon by ABTS and Cu^{2+} ions (Figure 8). As is evident in Table 3, the addition of ABTS led to an increase in the maximal biotransformation rate (V_{max}) of LacT with DHBP and TCS as substrates. Moreover, LacT exhibited catalytic activity with DS only in the presence of ABTS. For DHBP and TCS, the lowest apparent K_m and, by extension, the highest

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ACS Omega 2023, 8, 34710−34719

substrate affinity were observed in the presence of ABTS. The addition of ABTS resulted in the decrease of K_m with DHBP and TCS from 53.73 ± 1.35 to 36.49 ± 3.88 μM and 42.35 ± 1.99 to 37.26 ± 1.49 μM, respectively. On the other hand, the maximal bioconversion rate (V_{max}) was achieved in the presence of Cu^{2+} as it improved from 3.45 ± 0.49 to 16.91 ± 1.47 μM/min with DHBP and from 17.27 ± 1.61 to 31.30 μM/min for TCS. The addition of Cu^{2+} also resulted in the decrease of the apparent K_m.

4. CONCLUSIONS

We conducted the biophysical characterization of a novel thermostable laccase from *B. agri*. BLAST search of the peptide sequences obtained from MALDI-TOF indicated that LacT had high sequence homology with CotA laccase from *B. amyloliquefaciens*. Temperature-dependent fluorescence and circular dichroism spectroscopy revealed that an increase in temperature did not cause any shifts in the spectra, which is suggestive of thermal stability. TGA also confirmed the high-temperature stability of LacT. Furthermore, we have investigated the potential of LacT for the biotransformation of three important phenolic endocrine disruptors, namely, triclosan, dienestrol, and 4,4′-dihydroxybiphenyl. LacT-mediated bioconversion was observed with 4,4′-dihydroxybiphenyl and triclosan as the substrates; however, the biotransformation of dienestrol was detected only when LacT was used in conjunction with a redox mediator, ABTS. The addition of ABTS and $CuSO_4$ enhanced the enzymatic activity and improved the substrate affinity (K_m) and V_{max} of the LacT-mediated biotransformation of TCS and DHBP. Thus, the present study demonstrates the feasibility of using LacT for the bioremediation of phenolic contaminants and reiterates the importance of $CuSO_4$ and the mediator (ABTS) to enhance laccase activity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c03627.

Ultraviolet spectra of 4,4′-dihydroxybiphenyl in the presence of copper sulfate and ABTS, ultraviolet spectra of dienestrol in the presence of ABTS, and ultraviolet spectra of triclosan in the presence of copper sulfate and ABTS (PDF)

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Author Contributions

‡V.P. and S.L. contributed equally to this work. T.D., S.L., and V.P. conceptualized and designed the experiments. S.L. and V.P. performed the experiments. S.L. curated and analyzed the original data. T.D., S.L., and V.P. wrote the paper. T.D. and S.L. reviewed and finalized the draft.

Notes

The authors declare no competing financial interest.

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