Expression of a deep-sea bacterial laccase from Halomonas alkaliantarctica and its application in dyes decolorization

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Research Article

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Abstract

Introduction Laccase is a copper-containing polyphenolic oxidase widely found in bacteria, archaea, fungi, animals, and plants. As a green biocatalyst with considerable potential for numerous environmental and industrial applications, the enzyme production efficiency of laccase in nature is low, and the cost is high.

Purpose To examine the characterization and potential applications of laccase in this study, a novel laccase from *Halomonas alkaliantarctica* (LacHa) was cloned and heterologously expressed in *Escherichia coli*.

Results To achieve heterologous and efficient laccase expression, a bacterial laccase gene designed as LacHa from *Halomonas alkaliantarctica* of deep sea was cloned and expressed in *E. coli*. The results showed that the optimum temperature and pH of the enzyme reaction were 45°C and 7.5. The 100 μM Cu^{2+} and Fe^{2+} ions had the strongest stimulatory effect on laccase activity, the surface-active agent SDS and organic solvent 5% ethanol had opposite effect. EDTA, and 5% DMSO have no effect on LacHa activity. The activity of LacHa was enhanced 1.5 fold by chloride at concentrations lower than 500 mM, and 57.6% of its initial activity remained in the reaction system containing 1000 mM NaCl. Furthermore, LacHa showed decolorization rates ranging from 90.28% to 100% for indigo carmine and two azo dyes without mediators, with wide pH (5.0-9.0) and temperature (25-65°C) ranges.

Conclusions In the present study, LacHa was expressed and showed unusual properties, suggesting its great application potential in the treatment of textile industries or environmental applications.

Introduction

Laccases (benzenediol: oxygen oxidoreductases, EC1.10.3.2) belong to a group of multicopper oxidases (Guan ZB et al.,2018). They contain four copper atoms per monomer. Laccases possess conserved amino acid motifs responsible for binding to copper atoms, which are mainly composed of His-Cys and His residues (Hoegger PJ et al., 2006). These copper centers in the catalytic active sites of laccases can mediate the transfer of single electrons from phenolic compounds to oxygen and then generate water molecules (Enguita FJ et al., 2004). Because of their excellent catalytic properties, laccases are considered green and environmentally friendly biological catalysts in industrial applications, such as environmental remediation, textile wastewater decolorization, and medicine modification (Liu SH et al.,2020;Behrens CJ et al.,2017).

In the past, it was generally believed that fungi and plants were the main sources of laccase, but in fact, laccase activity was found in bacteria such as *Bacillus sphaeroides*, *Escherichia coli*, *Bacillus subtilis*, and *Bacillus amyloliquefaciens* (Guan ZB et al.,2018). Bacterial laccases exhibit rather low redox potential as compared with fungal laccases (Patel SKS et al.,2021). However, the long production cycle, poor thermostability, and low tolerance for the alkaline condition hinder the practical application of fungal laccases (Agrawal K et al.,2018). Recently, bacterial laccases have been found to possess

advantageous characteristics, including good stability under high temperature and alkaline conditions (Guan ZB et al.,2018;Tonin F et al.,2016). Besides, with the help of a redox mediator, bacterial laccases could gain the ability to degrade the recalcitrant substrates with higher redox potential than that of fungal laccases (Cañas AI et al.,2010;Janusz G et al.,2020). Therefore, bacterial laccases could be promising alternatives to fungal laccases for some specific industrial applications.

Wastewater in the textile industry contains a large number of dyes, including indigo carmine used for dyeing denim and azo dyes used in more than 70% of the textile industry, which has a serious impact on environmental safety (Xu GF et al.,2019). Laccase, a natural and mild biocatalyst, is widely used for the decolorization of dyes in wastewater treatment. Textile wastewater is usually alkaline and contains a high concentration of materials, which can cause most fungal laccases to exhibit stable catalytic activity (Fang ZM et al.,2012). In contrast, bacterial laccases have natural and stable alkaline catalytic activity, which is more suitable for textile wastewater treatment (Akram F et al.,2022). However, most bacterial laccases need to add mediators when decolorizing oxidized textile dyes, which may increase costs and lead to the uptake of other toxic substances (Gu Y et al.,2022). The temperature of textile wastewater is usually above 50^{o}C, which require the bacterial laccases must have high temperature stability (Hossain L et al.,2018). As a result, most bacterial laccases can not meet the requirements of textile wastewater treatment.

The genus *Halomonas alkaliantarctica*, which was established in 1980, belongs to the Halomonadaceae family of the order Oceanospirillales within the class Gammaproteo bacteria (Wang L et al.,2021). They are a group of gram-negative, halophilic or halotolerant, aerobic or facultative anaerobic, and non-sporulated bacteria (Vreeland RH et al.,1980). It also has some physiological characteristics that other microorganisms do not have, such as degradation of aromatic compounds (García MT et al.,2004), denitrification (Guo Y et al.,2013), and producing exopolysaccharides (Amjres H et al.,2011) and polyhydroxyalkanoates (Schwibbert K et al.,2011). In this study, a novel deep-sea laccase possessing dye decolorization ability without mediators and high temperature tolerance was developed. A hypothetical protein from *Halomonas alkaliantarctica* containing histidine-rich copper-binding motifs was expressed in *Escherichia coli*. In addition, the physico-chemical properties of the recombinant protein (LacHa) and its ability to decolorize different dyes were investigated.

Materials And Methods

Strains, culture media, and chemicals

H. antarctica was obtained from the Marine Culture Collection of China (MCCC No. 1A07573). *Escherichia coli* DH5α (General Biology, Chuzhou, China) was used for gene cloning and plasmid propagation. *E. coli* BL21 (DE3) (General Biol, Chuzhou, China) was used for the heterologous production of the recombinant laccase LacHa. The pET22b expression plasmid was obtained from Novagen. Standard M2 medium (per litre seawater containing 5 g sodium acetate, 0.5 g tryptone, 0.5 g yeast extract, 0.5 g glucose, 0.5 g sucrose, 0.05 g sodium citrate, 0.05 g DL-malic acid, 1 g ammonium nitrate, 0.2 g ammonium chloride,

and 0.5 g potassium dihydrogen phosphate, pH 7.6) was used for *H. antarctica* culture. Luria-Bertani (LB) medium (per litre containing 5 g yeast extract, 10 g tryptone, 10 g sodium chloride, and ampicillin were added to a final concentration of 100 μg/mL when necessary) was used for the *E. coli* culture and *H. antarctica* expression. Syringaldazine (SGZ), IPTG, ampicillin, and all dyes were purchased from Sangon Biotech (Shanghai, China). Syringaldazine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Bacterial Genomic DNA Extraction Kit Ver.3.0, PrimeSTAR® GXL DNA Polymerase, restriction endonuclease and T4-ligase were purchased from TaKaRa (Dalian, China). The GenRec Assembly Master Mix Kit was purchased from General Biology (Chuzhou, China). All other chemicals were of standard reagent grade.

Sequence Analysis And Three-dimensional Modeling Of Laccase

The open reading frame (ORF) of *LacHa* was predicted using DNAMAN 8. 0. The conserved domain was detected using the BlastP program of NCBI. Multiple sequence alignments were performed using ClustalW. A BLAST search was implemented in the Swiss Model server (http://swissmodel.expasy.org/), and *Saccharomyces cerevisiae* laccase (PDB ID: P) wasidentified as a structural template for three-D modelling of LacHa (sharing the sequence identity of 46.67%). The automatic sequence alignment obtained from this process was used for homology modeling with modelling using SWISS-MODEL (Benkert P et al.,2011). The acquired theoretical model of LacHa was displayed and analysed using the PyMOL molecular visualization system.

Plasmid And Expression Strain Construction

The *LacHa* gene (NCBI accession no. NZ_AYOZ01000034.1) form *H. antarctica* was amplified with genomic DNA as the template using the primer pair of *LacHa*-F(5' -ATGAACCCCTGGGGCCGCAGC-3') and *LacHa*-R(5'-AGAAACCTGAACAACGCGGA-3'). Plasmid pET22b pD was constructed using the pET-22b vector as the backbone followed the previous methods (Chang F et al.,2017). Briefly, the amplified *LacHa* gene was inserted into the *Nde* I- and *Not* I-linearized pET22b-pD vector by the seamless cloning method and then transformed into *E. coli* DH5α competent cells. A His_6-tag was fused to the C-terminal to facilitate further purification. The accuracy of the resulting plasmid, pET22b-pD-LacHa, was confirmed by sequencing (General Biol, Chuzhou, China) and then transformed into the competent cells of *E. coli* BL21 (DE3) to engineered *E. coli* BL21(DE3)/pET22b-pD-*LacHa*, which was used for protein expression. For the control of expression sample, pET22b-pD without the *LacHa* gene was also transformed into *E. coli* BL21(DE3).

Expression And Purification Of Recombinant Lacha

Escherichia coli BL21(DE3) cells harboring pET22b-pD-LacHa were grown in LB medium containing 1% ampicillin at 37°C with a shaking speed of 100 g. After culturing for 3–4 hours, the culture temperature

was lowered to 16°C, and IPTG was added to the culture at a final concentration of 0.2 mM to induce enzyme expression.

After an additional 16 h of incubation, the cells were centrifuged and collected (3000×g at 4°C for 10 min) and resuspended in 20 mL The accuracy of the resulting plasmid, pET22b-pD-LacHa, was confirmed by sequencing lysis buffer (containing 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 5 mM imidazole). The cell suspension was disrupted via sonication. The lysate was centrifuged at 12,000×g for 30 min at 4°C to remove the cell debris. The crude LacHa enzyme was loaded into a Ni-NTA column (4 mL, Sangon Biotech, Shanghai, China) for the binding of LacHa onto the column. Subsequently, the column was washed successively with wash buffer (containing 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 20 mM imidazole) and elution buffer (containing 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 200 mM imidazole). Dialysis was employed to remove NaCl and imidazole from the eluted fraction with 50 mM Na_2HPO_4-KH_2PO_4 buffer. The protein concentration of each fraction was determined using the Bradford method. Meanwhile, 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion chromatography (SEC) were used to determine the expression and purification of the recombinant protein.

Enzyme Activity Assay

LacHa activity was assayed according to the method described by Fang (Fang Z et al.,2011). One milliliter of enzymatic reaction mixture was composed of 20 μL of appropriately diluted enzyme stock and 980 μL of 50 mM Na_2HPO_4–KH_2PO_4 buffer (pH 7.5) supplemented with 100 μM syringaldazine and 100 μM $CuSO_4$. The Na_2HPO_4–KH_2PO_4 buffer containing $CuSO_4$ was pre-incubated at the assay temperature for 3 min before adding the enzyme and substrate to start the reaction. Then, the crude enzyme solution and pure enzyme solution were reacted for five minutes at 45°C and pH 7.5. Three groups of OD_{525} values were measured and averaged. One activity unit (U) was defined as the amount of LacHa required to oxidize 1 μmol of substrate per min. Boiling-treated LacHa cells were used as negative controls. The following formula was used to calculate U/ml = OD_{525}/(6.5×105×V/106). All assays were performed in triplicate.

Characterization Of Recombinant Lacha

A series of Na_2HPO_4–KH_2PO_4 buffers with different pH values of 5.5-8.0 were prepared, and the optimal pH value of laccase was evaluated at 45°C. The pH stability of the enzyme was determined by measuring the residual activity of LacHa after 1 h of incubation at 4°C in the aforementioned buffers. The effect of temperature on enzyme activity was measured by incubating LacHa at pH 7.5 and a temperature range of 25–55°C (gradient of 5). Thermal stability was determined by incubating LacHa at various temperatures (25–65°C) at pH 7.5 for 15 min. Values and standard deviations were calculated from three independent experiments.

The copper ion concentration gradient was set to 0 μM, 10 μM, 25 μM, 50 μM, 75μM, 100 μM, 150 μM, 200 μM, and each gradient was used as a control to explore the enzyme activity of Cu^{2+} ion pair impact. Reaction system: 50 μL of enzyme solution (pure enzyme solution in the experimental group, inactivated enzyme solution in the control group), 10 μL of SGZ, 930 μL of phosphate buffer, and Cu^{2+}10 μL of each concentration gradient. After each gradient sample reacted for 24 h, the OD value was measured using a spectrophotometer.

The effects of 1 mM Mg^{2+}, Ca^{2+}, Zn^{2+}, K^{+}, Fe^{2+}, EDTA, SDS, 5% ethanol, and 5% DMSO on LacHa activity were investigated by incubating LacHa cells with each effector for 15 min. Reaction system: 50 μL of enzyme solution (pure enzyme solution in the experimental group, inactivated enzyme solution in the control group), 10 μL of SGZ, 10 μL of $CuSO_4$, 10 μL of metal ions, protein inhibitors or organic solvents, and 920 μL of phosphate buffer. After the reaction of each sample, the optical density (OD) value was measured at the appropriate wavelength using a spectrophotometer. Kinetic parameters for LacHa were determined using different substrate concentrations. Origin software (version 8.0; Northampton, MA, USA) was used to fit the data to the Michaelis-Menten equation to calculate the estimates of the values.

NaCl effect was determined at concentrations ranging from 1 to 1,000 mM.

Decolorization Of Dyes

Indigo carmine (λmax = 610 nm), two azo dyes Congo red (λmax = 480 nm), and Eriochrome black T (λmax = 540 nm) were used in laccase decolorization experiments. The decolorization reaction system contained Cu^{2+} solution (10 mM, 10 μL), dye solution (10 mM, 10 μL), enzyme (50 U/L), and phosphate buffer (50 mM, pH 7.5). The decolorization experiments under pH (5–9) and temperature (25–65°C) were tested one by one according to single factor optimization. All tests were performed in triplicate. The absorbance of the reaction solution was measured at the wavelength of each dye after incubation. The decolorization rate of the dyes was calculated using the formula:

$$\text{Decolorization rate } (\%)=[(A_0 - A) \div A] \times 100\%$$

A_0 represents the initial absorbance of the dyes, and A_1 represents the final absorbance.

Results And Discussion

Sequence analysis of LacHa

The conserved sequence of *H. antarctica* was obtained from NCBI, and the ORF of *LacHa* contains 1854 bp that encodes 617 amino acids with a theoretical molecular weight of 68,487 Da. Its amino acid sequence was previously deposited in GenBank with accession number WP_133729725.1 previously. A putative conserved domain was detected according to the Pfam database (Pfam PF07732). From the alignment results, the putative conserved domain prediction results showed that *LacHa* belongs to the

Cu-oxidase-3 superfamily. As determined by the alignment analysis, the protein sequence has a high identity to laccases from other species, including laccase-like oxidase CopA (WP_054090693), *Paecilomyces variotii* laccase (XP_028481812), *Mycobacterium tuberculosis* laccase (WP_034169521), *Yersinia pseudotuberculosis* laccase (WP_050116815) and a multicopper oxidase of *Byssochlamys spectabilis* (RWQ92167). Module analysis revealed that *LacHa* possesses three conserved copper oxidase domains (Fig. 1), which are lacquer enzyme characteristics. The predicted 3-D structure of *LacHa* was built by homologous modelling using the structure of *Saccharomyces cerevisiae* laccase as a template (W) (Fig. 2). Laccases generally contain four copper atoms and have highly conserved catalytic centers, which are named T1, T2, and T3 copper centers (Beloqui A et al.,2006; Solomon EI et al.,1996). Similarly, three Cu-oxidase domains were also present in the active site of *LacHa*, T1 copper center (H551, C600, H497, and H605), T2 copper center (H154, H556 and H599), and T3 copper center (H1110, H112, H150, H152, H554 and H601).

Heterologous expression and purification of LacHa in E. coli

As mentioned above, we suggest that LacHa is a functional laccase. To verify this, LacHa was cloned, expressed, and purified by Ni-NTA chromatography. LacHa was purified by Ni-affinity chromatography, and a single band of approximately 68.5 kDa was detected by SDS—PAGE (Fig. 3). An activity assay showed that LacHa could oxidize syringaldazine, a typical laccase substrate. Under the corresponding optimum condition, the specificity of LacAn was 4.6 U mg-1. Additionally, the K_m and V_{max} of LacHa for SGZ were 10.6 μM and 12.7 μmol/min mg, respectively.

Effects Of Ph And Temperature On The Activity And Stability Of Lacha

When SGZ was used as the substrate, LacHa activity continued to increase with an increase in the pH between 5.5 and 7.5, and the activity reached a maximum at pH 7.5. The enzyme activity in Fig. 4B was above 85% at pH 6.5, 7.5 and 8.5 for 180 min. In general, bacterial laccases are functional in an alkaline environment and are active at pH 7.0–8.5, whereas fungal laccases are partially active in acidic environments (Claus H, 2003; Brander S et al.,2014). The alkaline activity of LacHa was similar to that of other bacterial laccases reported in previous studies (Table S1). However, some bacterial laccases exhibit acid-dependent activities. For example, for RL5, a bacterial laccase, the optimal pH for syringaldazine is 4.0–5.0, which was derived from a bovine rumen microflora metagenomic library (Beloqui A et al.,2006). In contrast, most laccases from fungi are unstable under alkaline conditions (Yang X et al.,2011). The laccase from *Ganoderma leucocontextum* Glacc110 was characterized using different parameters. It maintained > 90% activity after incubating in an acidic medium (pH 4.0) at 60°C for 16 min (Table S1) (Umar A et al.,2022).

In addition, at temperatures between 30°C and 45°C, the activity of LacHa increases with an increase in temperature. LacHa activity reached its maximum at 45°C. The enzyme activity at 25°C and 45°C was still more than 60% at 180 min, and the half-life reached 155 min at 65°C (Fig. 4C, 4D). LacHa is different from many other discovered bacterial laccases, which show great thermostability even at temperatures

below 45°C (Umar A et al., 2022; Li T et al., 2022) (Table S1). In addition, environmental factors also affect thermostability. *H. alkaliantarctica* originated from the deep ocean, which has a lower temperature environment, explaining the thermostability of LacHa. Similarly, marine microbes and *Marinomonas profundimaris* from the deep sea express laccases with similar properties to LacHa (Chang F et al.,2022; Fang ZM et al.,2011).However, the optimum temperature for syringaldazine of LacAn was 75°C, which is mainly because *Anoxybacillus ayderensis* SK3-4 originates from hot springs in a high-temperature environment (Wang J et al.,2020) (Table S1).

Effects Of Metal Ions And Organic Solvents On The Activity Of Lacha

Cu^{2+} was important for LacHa activity, as no activity was detected in the purified protein. The effect of Cu^{2+} on laccase activity was determined using the average value of three replicates in a concentration gradient system containing different Cu^{2+} concentrations. When the concentration of Cu^{2+} was lower than 100 μM, the LacHa activity of laccase increased with increasing Cu^{2+} concentration. When the concentration of Cu^{2+} was 100 μM, the activity of laccase LacHa was the highest (Fig. 5A). The stimulation of laccase activity by Cu^{2+} observed in this study was probably due to the filling of type I or II copper binding sites with copper ions, highlighting the importance of Cu^{2+} ions in laccase function (Nagai M et al.,2002.; Sonica S et al.,2014).

Metal ions bind to laccases and alter their stabilities. Fe^{2+} increased the enzyme activity up to 1.5-fold, but in the presence of Mg^{2+}, Ca^{2+}, Zn^{2+}, K^{+}, and other metal ions, the activity of laccase was inhibited (Fig. 5B). In the presence of K^{+}, laccase activity was the lowest, retaining 43.21% of its activity. Metal ions have different effects on different types of laccases, such as *Kabatiella bupleuri* G3 IBMi (Wiśniewska KM et al.,2021) and *A. ayderensis* SK3-4 (Fang ZM et al.,2011). KbLcc1 remained active at 1 mM Ni^{2+}, Cu^{2+}, Mn^{2+} and Zn^{2+} and 2 mM Co^{2+}, Ca^{2+} and Mg^{2}, but Fe^{2+} greatly inhibited laccase activity (Wiśniewska KM et al.,2021). The inhibitory effect of Fe^{2+} may be due to its interaction with the laccase electron transport system. Blockage of access to the substrate or transfer of electrons at the T1 site results in the inhibition of laccase activity (Murugesan K et al.,2009). However, the activation of LacHa in the presence of Fe^{2+} was in accordance with the results from previously characterized fungal laccases, such as *Coriolopsis gallica* NCULAC F1 (Cen Q et al.,2022) Fe^{3+} and Mn^{2+} strongly stimulate CGLac activity by 162.56% and 226.05%, respectively (Cen Q et al.,2022). Thus, the effect of metal ions on laccase activity had no obvious relationship with the species source and expression system. The stability of LacHa in the presence of some metal ions makes it suitable for applications where metal ions are present in high concentrations, such as in the pulp and paper industry and in wastewater containing heavy metals (Shraddha SR et al.,2011).

Chloride affects fungal laccase activities dramatically. In a high salinity solution with over 100 mM NaCl, most fungal laccases lose their activities (Jimenez-Juarez N et al.,2005). The possible reason for the occurrence of this inhibition effect on the laccase activity could be that the high concentration of chloride

disrupts the transfer of electrons from substrate to T1 copper or from T1 copper to T3 copper, which eventually influences the oxidation-reduction reaction mediated by the laccase. However, LacHa showed great tolerance against high concentration of NaCl and KCl, and the activity was enhanced to about 150% by NaCl at concentrations from 100 to 500 mM. Furthermor, it retained 57.6% of its residual activity in the solution with 1000mM NaCl (Fig. 6). Similar results have also been reported for other bacterial laccases, such as laccase Lbh1, found in *Bacillus halodurans* C-125, was reported to be stimulated by NaCl at concentrations of 100–450 mM (Ruijssenaars HJ et al. 2004). The laccase PPO1 from *Marinomonas mediterranea* was also tolerant to NaCl at pH 5 with an 150 value of 547 mM (Li T et al.,2020). Interestingly, laccase rLac of *Bacillus velezensis* from soil also be found chloride tolerance. It showed great tolerance against high concentration of NaCl, but as the NaCl concentration further increased, LacHa activity decreased slowly (Wang et al.,2019). Obviously, LacHa showed excellent chloride tolerant ability compared to other bacterial laccases. Therefore, laccases from different sources have remarkably different tolerance against NaCl (Rodrigues et al.,2009). Hence, the high-salinity tolerance of LacHa would be much more advantageous in bio- bleaching of paper pulp and dyestuffs processing, where most fungal laccases are unsuitable (Robles A et al.,2002).

In addition, 0.5 mM SDS significantly inhibited the enzymatic activity of laccase LacHa, reducing laccase activity to 16.22% (Fig. 5B). This result may be due to the binding of the ionic surfactant [below the critical micelle concentration (CMC)] to the enzyme, which may cause alterations in its enzymatic and physical characteristics (Sonica S et al.,2014; Robles et al.,2009; Zhang C et al.,2013) EDTA and 5% DMSO had no significant effect on laccase activity (Fig. 5B), like it in *Bacillus amyloliquefaciens* (Baldrian P et al.,2022) and *A. ayderensis* SK3-4 (Wang J et al.,2020). In the presence of 5% ethanol, the laccase activity was inhibited, and the enzyme activity decreased to 77.35%. Conversely, 1% ethanol had no effect on KbLcc1, although acetone and ethyl acetate reduced laccase activity (Wiśniewska KM et al.,2021).

Decolorization Of Dyes By Lacha

LacHa decolorized the three dyes without mediators. The decolorization rates of indigo carmine, chrome black T, and Congo red increased with increasing temperature (Fig. 7A). When it exceeded a certain temperature, the decolorization rate showed a downwards trend. The decolorization rate of indigo carmine reached 99.56% at 45–55°C and decreased to 94.62% after the temperature continued to rise to 65°C. Congo red showed the highest decolorization rate, reaching 94.59% at 50°C, which decreased when the temperature was above 55°C. With increasing temperature, the decolorization rate of chrome black T continuously increased. The decolorization rate reached a maximum value of 89.59% at 50°C and decreased to 77.16% at 65°C.

Indigo carmine showed the highest decolorization rate of 100% at pH 7.5, whereas the decolorization rate showed a downwards trend when the pH was below 7.0 or above 8.0 (Fig. 7B). The decolorization rate of chrome black T increased with increasing pH, reached a maximum value of 85.12% at pH 7.5, and

decreased to 57.72% at pH 9.0. The decolorization rate of Congo red also increased with increasing pH, reaching a maximum for 100% at pH 7.5 and decreasing significantly for 27.26% at pH 9.0 (Fig. 7B). The decolorization of LacHa for these dyes can reach a high level within a certain range, and it decreases when it exceeds a certain value.

The decolorization rate increased with increasing reaction time (Fig. 7C). The decolorization rate of indigo carmine reached 100% when the decolorization progressed to 12 h. The rate of Congo red decolorization also increased with time, reaching 100% at 24 h. The decolorization rate of chrome black T reached 90.28% in 12 h after the reaction and was 84.35% in 24 h, which remained stable.

From the results of the above experiments, the decolorization rates of indigo carmine and Congo red can reach 100%. Chrome black T can reach up to 90.28% and has a high decolorization rate. From the above data, it can be concluded that this laccase species has a high affinity for the azo and indigo dyes used in the experiment.

Indigo carmine and azo dyes are commonly used in the textile industry. As a result, they have become the main components of wastewater in these industries. Both fungal and bacterial laccases have been used in textile wastewater treatment because of their ability to catalyze dye degradation. The dye composition, temperature, and pH in textile wastewater are the main factors affecting laccase treatment. In this study, LacHa, a bacterial laccase that oxidizes oxidation substrates without mediators, was used to test the potential for dye decolorization. Interestingly, LacHa showed a high decolorization rate for indigo carmine and the two azo dyes without mediators.

The decolorization effect of LacHa at 25–65°C showed a trend of first increasing and then decreasing, but the decolorization rate generally remained in a high range. Even at 65°C, the LacHa decolorization rate for the three dyes exceeded 80%. The marine bacterial laccase Lac21 was used to decolorize azo dye-reactive deep blue M-2GE without a mediator, and the decolorization rate reached at 20°C but decreased with an increase in temperature (Fang ZM et al.,2011). Temperature can affect laccase activity by altering its structure. High temperatures made some minor adjustments to certain loops in laccase rLAC from *Bacillus pumilus.* It forms a more open channel and promotes binding of the substrate at the active site, thus shortening the distance between catalytic residues with elevated binding energy. Therefore, rLAC shows high activity at 80°C (Singh G et al.,2019). However, if the temperature is too high, this structural adjustment leads to a decline in laccase activity, and the decolorization of the dyes also decreases.

Most bacterial laccases cannot directly oxidize substrates with a high potential because of their low redox potential. Small-molecular-weight compounds are called redox mediators that act as electron transmitters between the enzyme and substrate to form stable free radicals and complete the oxidation reaction. Some nonspecific laccase substrates are oxidized in the presence of mediators (Singh G et al.,2019). Decolorization depends on the nature of the enzyme, biological source, chemical nature, and structure of the dyes. A novel laccase from *Geothermobacter hydrogeniphilus* was cloned and expressed to oxidize the malachite green dye MG, with a decolorization rate of only 10% without any mediators.

When 0.1 mM ABTS was added as a redox mediator, the decolorization rate increased to 90% (Mao G et al.,2021). When β-(10-phenothiazyl)-propionic acid was added as the mediator, a laccase from *Bacillus subtilis* could degrade cyan and malachite green above 80% (Coria-Oriundo LL et al.,2021). In comparison, LacHa can oxidize some azo dyes without mediators and has high decolorization, which indicates that LacHa may have a high redox potential.

pH usually affects laccase oxidation activity by changing the dissociation and charging state of the side chain groups, the structure of the active center, and the binding force between the enzyme and the substrate (Coria-Oriundo LL et al.,2021; Miranda-Blancas R et al.,2021). After optimizing the reaction conditions, LacHa oxidized indigo carmine and azo dyes in the pH range of 5–9 which improved the application potential of LacHa in dye decolorization.

In conclusion, LacHa cDNA from Halomonas alkaliantarctica was cloned and expressed in *E. coli* BL21(DE3). It showed potential advantages of activity and stability at high temperatures and alkaline pH conditions, and great chloride tolerance. LacHa could efficiently oxidize representative indigo carmine and azo dyes used in textile industries without any mediators, and the only product in the reaction process was water. Therefore, these results indicate that LacHa has potential applications in the treatment of wastewater from the textile industry.

Declarations

Acknowledgements

Jingjing Wang and Ran Zhang have the same contribution to this paper.

Author contributions

Conceptualization, Jingjing Wang and Ran Zhang; methodology, Jingjing Wang, Qiang Yin and Ran Zhang; validation, Jingjing Wang, Yan Zhang, Guilan Zhu, Lingling Wang and Ran Zhang; writing-review and editing, Jingjing Wang, Ran Zhang, Qiang Yin, Yan Zhang, Han Bai, Yong Qian and Xiazhen Zhou; supervision, Jingjing Wang, Ran Zhang, Qiang Yin, Yan Zhang; project administration, Yan Zhang; funding acquisition, Yan Zhang. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The raw sequencing data could be downloaded at the NCBI Sequence Read Archive (SRA) with the study accession number WP_133729725.1, the protein sequence has a high identity to laccases from other species, including laccase-like oxidase CopA (WP_054090693), *Paecilomyces variotii* laccase (XP_028481812), *Mycobacterium tuberculosis* laccase (WP_034169521), *Yersinia pseudotuberculosis* laccase (WP_050116815) and a multicopper oxidase of *Byssochlamys spectabilis* (RWQ92167).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflicts of interest

The authors declare that there is no conflict of interest.

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Figures

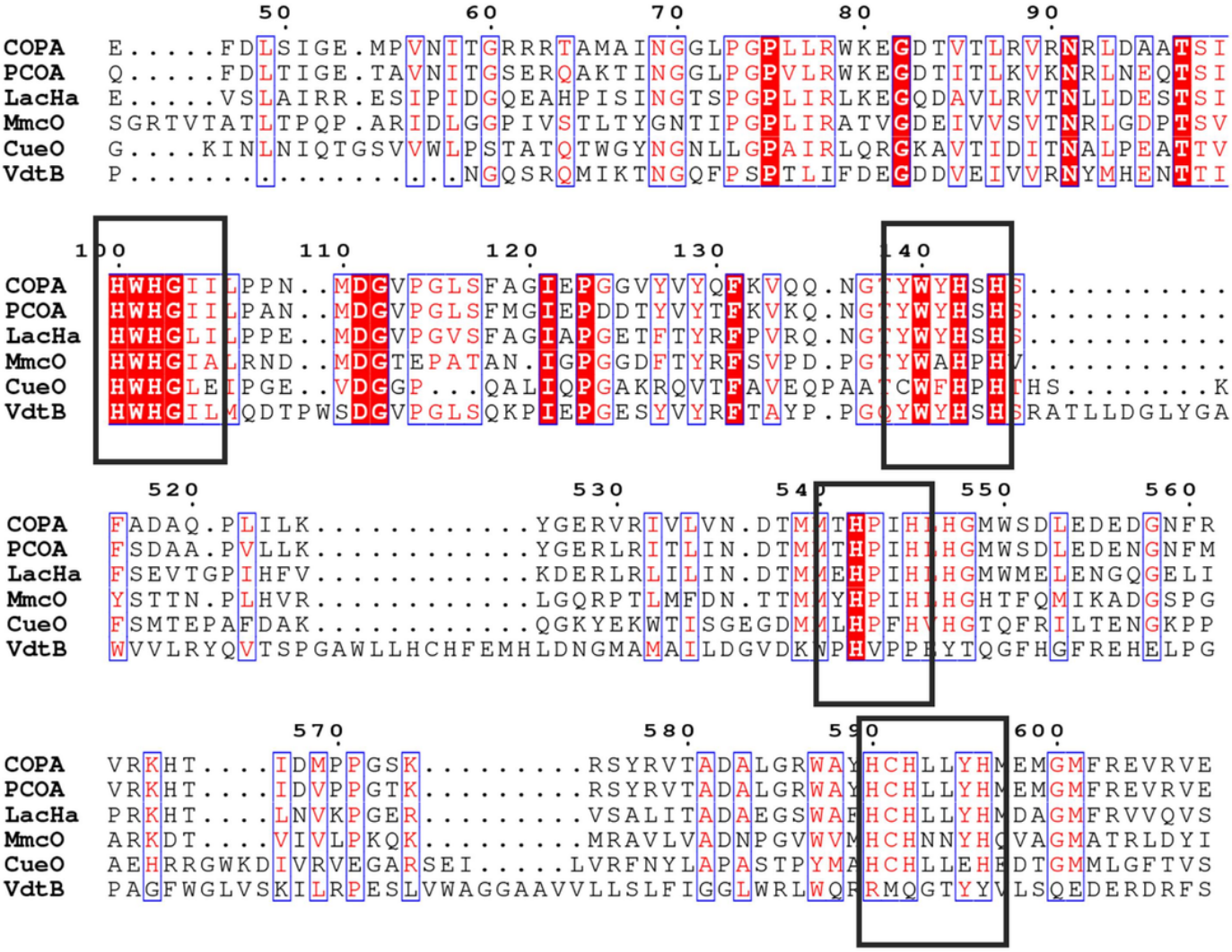

Figure 1

Multiple sequence alignment of LacHa with some other related proteins, and the amino acid sequence was retrieved from NCBI database. LacHa, a copper oxidase in this experiment (WP133729725); an uncultured bacterial laccase (ADM87301); a polyphenol oxidase of Marinomonas mediterranean MMB-1 (AAF75831); an anaerobic Bacillus copper oxidase (EPZ38526); an unclassified Halomonas IS1380 family transposase (WP159178014). Sequence alignment was performed using Clustal X 2.0 and GENEDOC. Full-length vertical boxes indicate four histidine-rich copper binding domain.

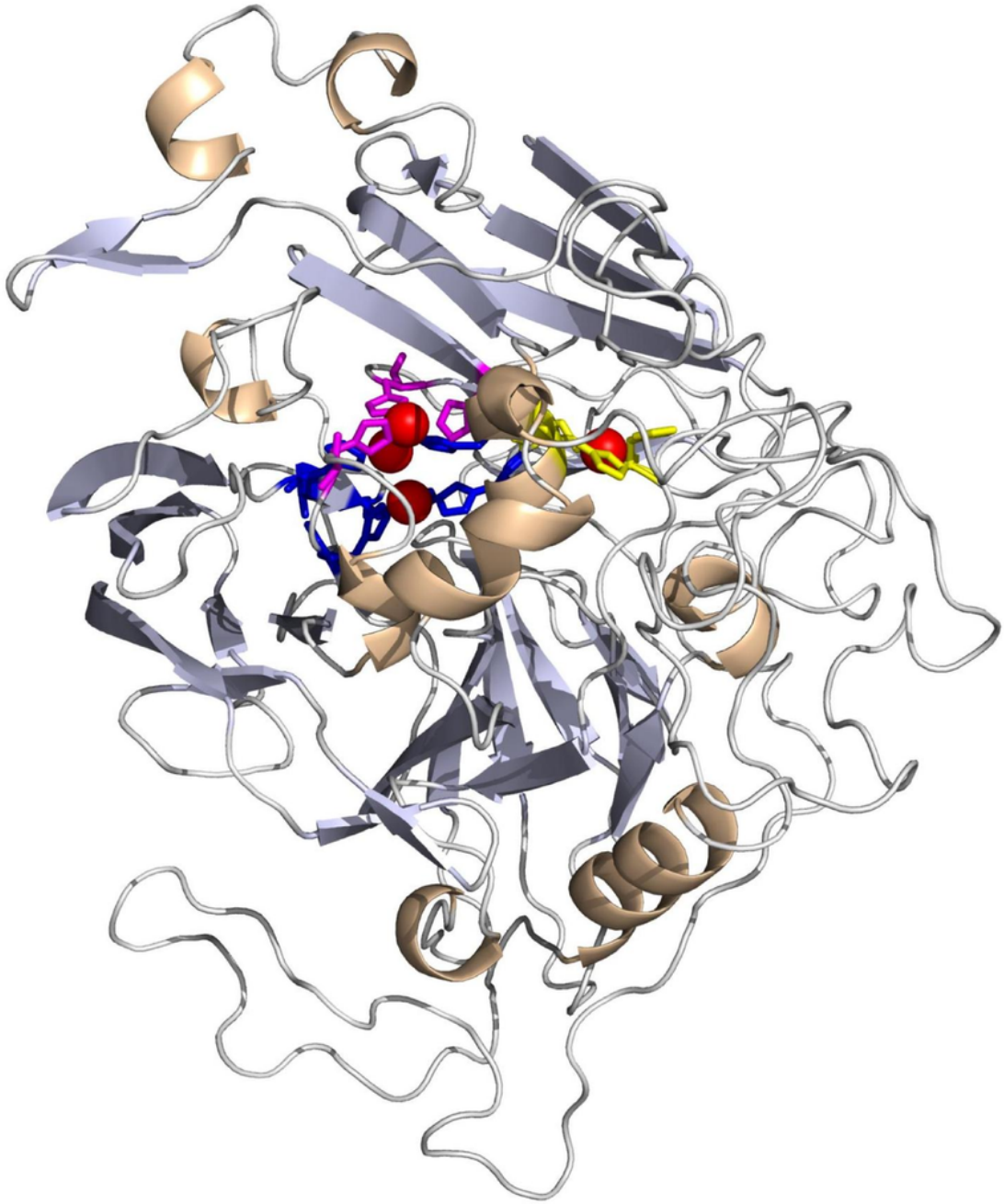

Figure 2

Predicted 3-D structure of LacHa. The model was built with the PyMOL molecular graphics system according to its sequence. Three Cu-bounding domains were predicted in the 3-D structure of LacHa: T1 copper center contained H551, C600 and H605 are highlighted in yellow; T2 copper center contained H154, H556 and H599 are highlighted in magentas; T3 copper center contained H110, H112, H150, H152, H554 and H601 are highlighted in blue.

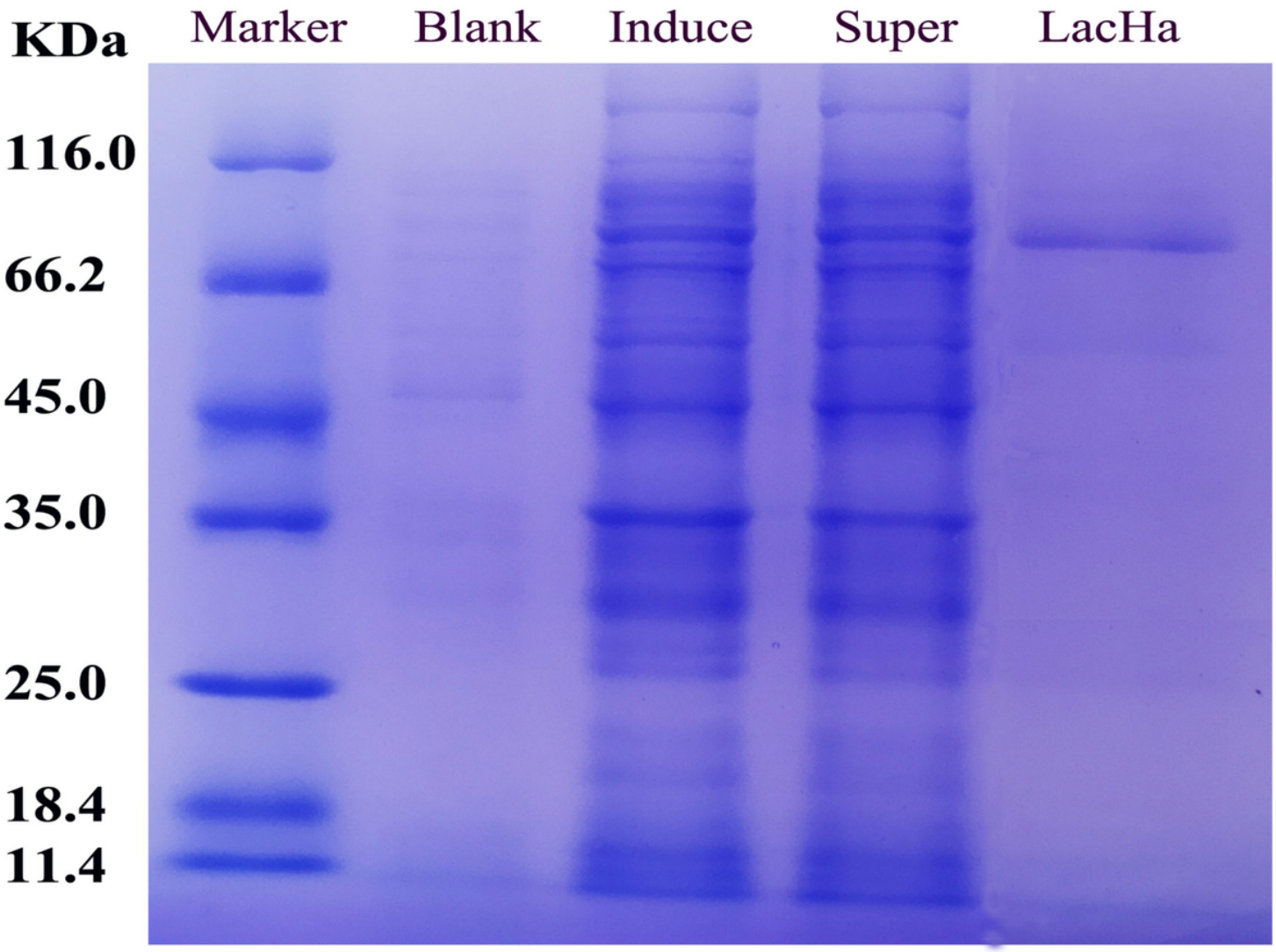

Figure 3

SDS-PAGE of LacHa expression using Ni^{2+}-NTA purified expression system in *E coli*. Marker is molecular marker, blank represents BL21 (DE3) without lacHa insertion, induce represents *E. coli* BL21(DE3)/pET22b-lacHa super represents supernatant after ultrasonic fragmentation of cells *(E. coli* BL21(DE3)/pET22b-lacHa), LacHa represents purified protein using Ni-NTA column chromatography. The band of LacHa is marked with black arrow.

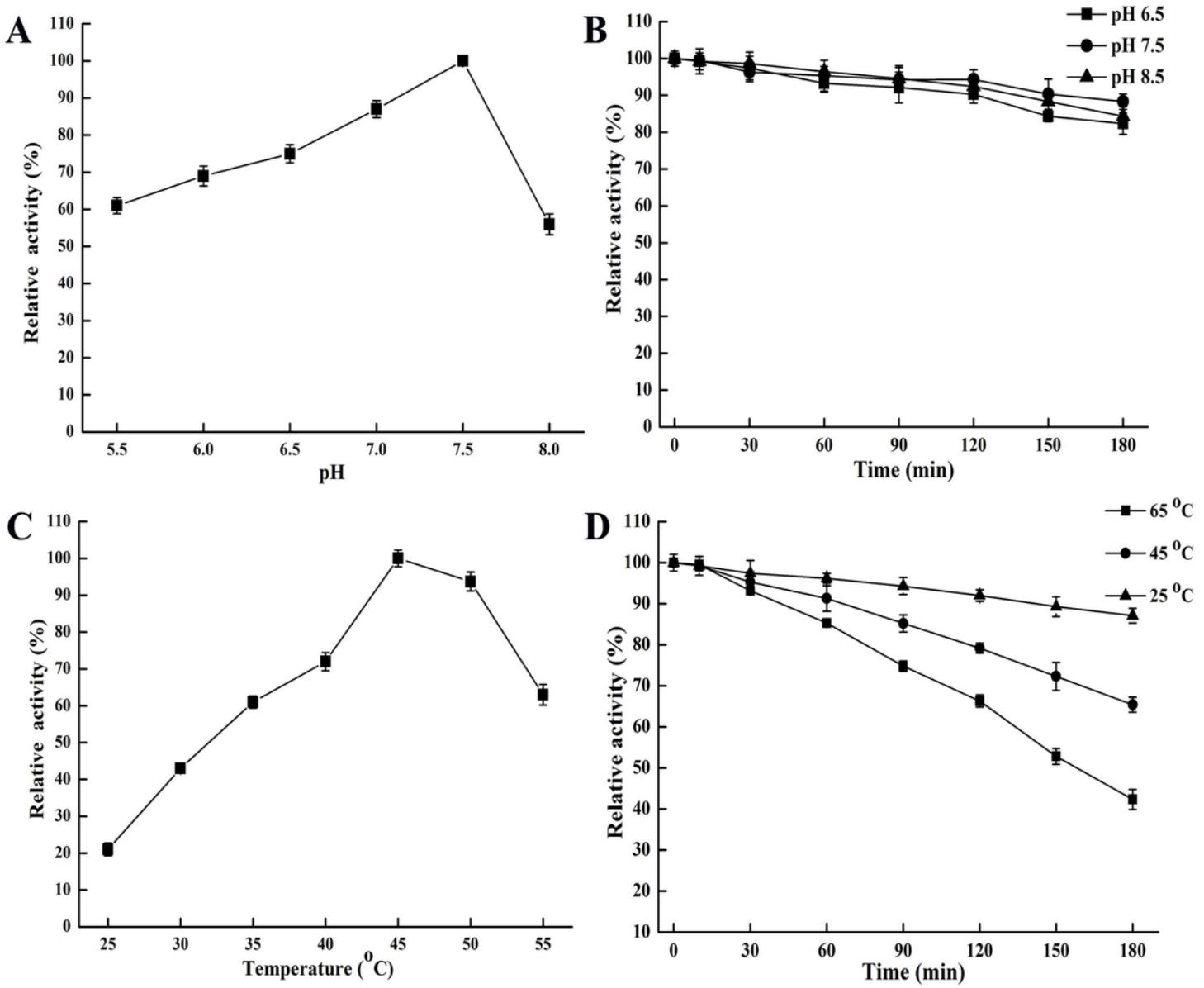

Figure 4

Effects of pH (A and B) and temperature (C and D) on the activity and stability of LacHa. The values and standard deviations are calculated based on three independent experiments.

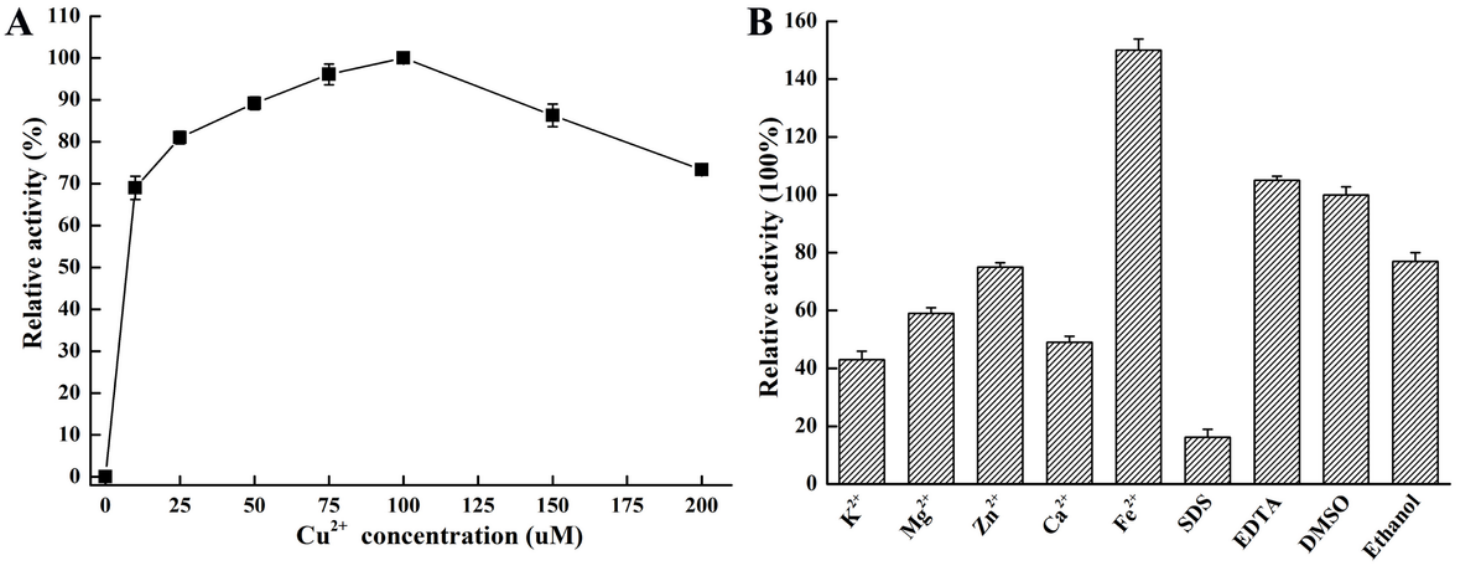

Figure 5

Effects of copper ion (A), metal ions and organic solvents (B) on the activity of LacHa. The values and standard deviations are calculated based on three independent experiments.

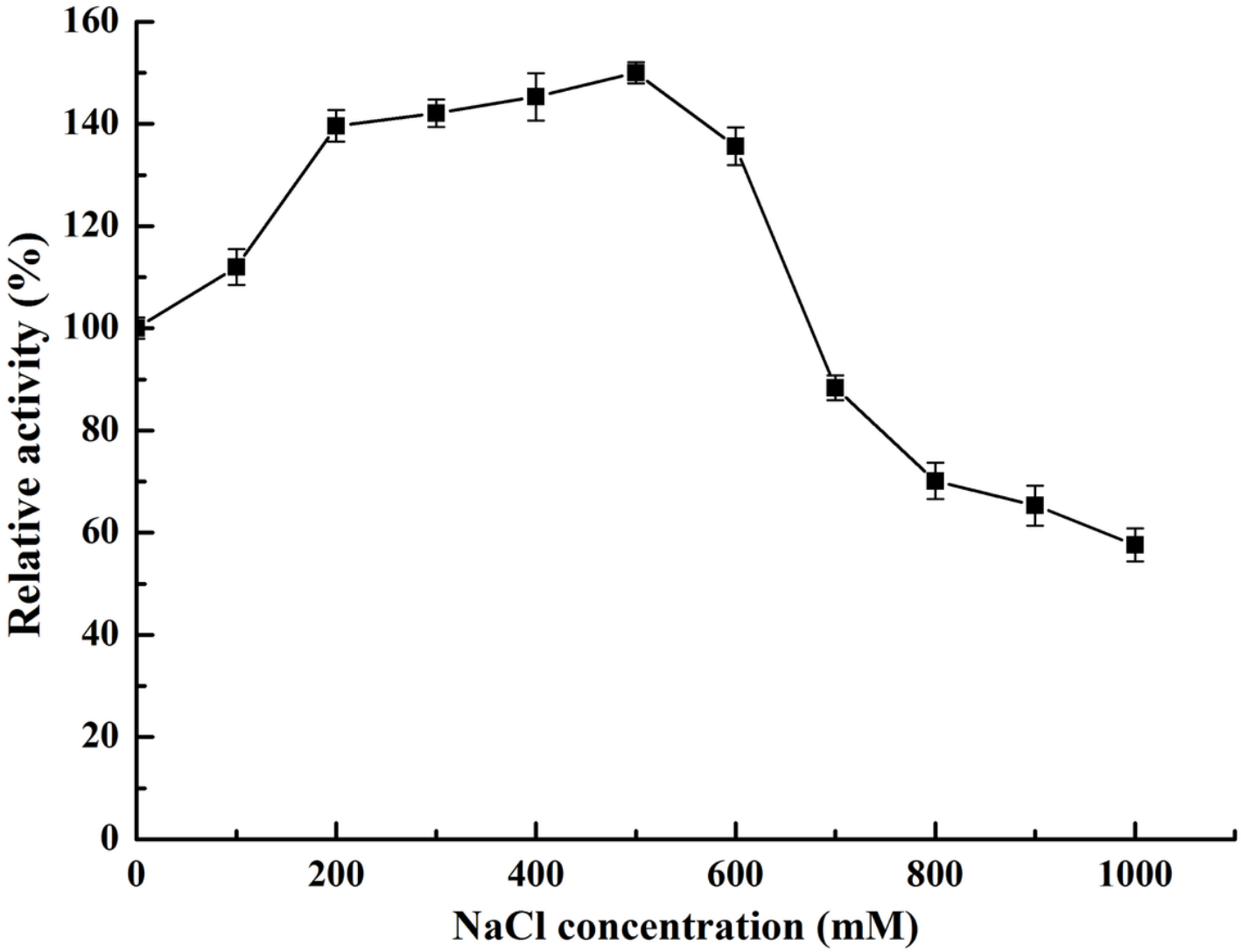

Figure 6

Effect of NaCl on the activity of LacHa. The activity was measured in 50 mM Na_2HPO_4–KH_2PO_4 buffer at pH 7.5, supplemented with 100 μM $CuSO_4$, at 45°C with 100 μM syringaldazine as substrate.

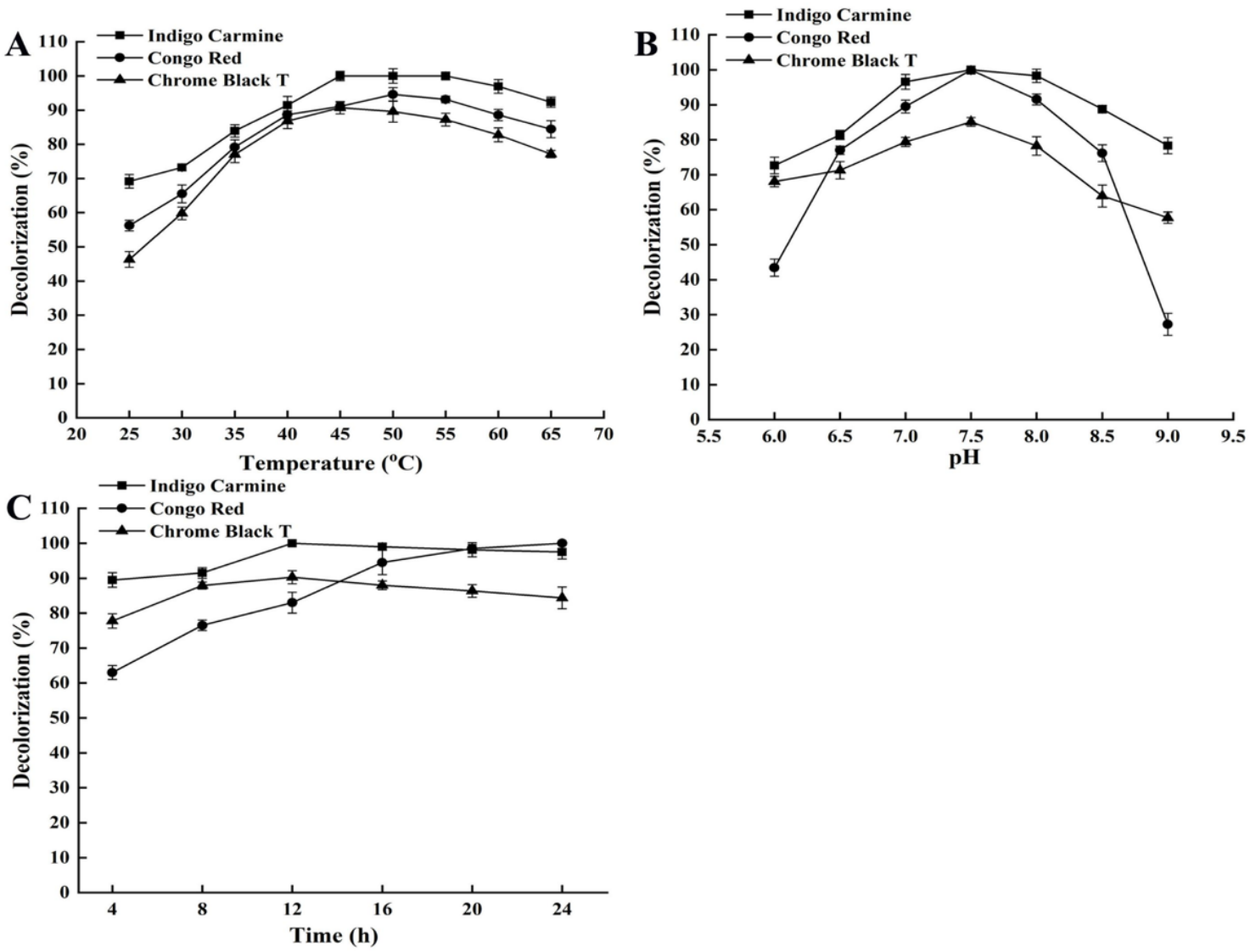

Figure 7

Effects of temperature (A), pH (B), time (C) on dye decolorization of LacHa. The values and standard deviations are calculated based on three independent experiments.

Supplementary Files

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- TableS1.pdf