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Research Article 研究报告

灰盖鬼伞中过表达的碱性真菌漆酶在高盐染料废水中的应用潜力

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WANG Xianhua, ZHU Xueling, LIN Jiayi, GU Xianfu, PENG Qixia, XIAO Yazhong, Ursula Kües, FANG Zemin. An alkaline fungal laccase overexpressed in *Coprinopsis cinerea* shows application potential in treating high-salt dye wastewater[J]. Acta Microbiologica Sinica, 2024, 64(8): 3014-3029.

摘　要：【目的】本研究利用双孢菇(*Agaricus bisporus*) *gpdII* 启动子，实现了碱性真菌漆酶 PIE5 (*Cc*PIE5)在灰盖鬼伞(*Coprinopsis cinerea*) FA2222 中的同源过表达。【方法】在 mKjalke 液体培养基中 37 ℃培养 7 d，漆酶活力达到(24.2±1.1) U/mL。纯化的 *Cc*PIE5 在 pH 8.0 和 60 ℃下表现出最佳催化活性。【结果】与已经研究报道的真菌漆酶不同，*Cc*PIE5 能耐受高浓度 NaCl，当 NaCl 浓度从 0 升至 1.5 mol/L 时，*Cc*PIE5 的 k_{cat} 和 K_m 均呈下降趋势，表示 *Cc*PIE5 在纺织印染废水脱色中有潜在应用价值。进一步在染料废水脱色应用中，以丁香酸作为介体，*Cc*PIE5 在 pH 8.5 和 60 ℃条件下可高效降解(92.9±2.3)%的靛蓝胭脂红。通过 LC-MS 分析确认，Isatin 5-sulfonic acid (ISA)是靛蓝胭脂红降解的主要副产物。【结论】*Cc*PIE5 在高温、碱性和含盐条件下高效脱色染料，是处理环境和工业特定应用的理想选择。

关键词：真菌漆酶；过表达；灰盖鬼伞；高盐；靛蓝胭脂红

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An alkaline fungal laccase overexpressed in *Coprinopsis cinerea* shows application potential in treating high-salt dye wastewater

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Abstract: [Objective] To homologously overexpress an alkaline fungal laccase PIE5 (*Cc*PIE5) in *Coprinopsis cinerea* FA2222 under the control of the *Agaricus bisporus gpdII* promoter. **[Methods]** The laccase activity reached (24.2±1.1) U/mL in the supernatant after 7 days of cultivation at 37 ℃ in the mKjalke medium. The purified *Cc*PIE5 showcased the best performance at pH 8.0 and 60 ℃. **[Results]** Unlike other characterized fungal laccases, *Cc*PIE5 was tolerant to high concentrations of NaCl. Particularly, both k_{cat} and K_m decreased when the concentration of NaCl was increased from 0 to 1.5 mol/L, which indicated that *Cc*PIE5 demonstrated application potential in the dye decoloring of texile finishing. In dye decolorization, *Cc*PIE5 efficiently degraded (92.9±2.3)% indigo carmine at pH 8.5 and 60 ℃, with syringic acid as the mediator. Isatin 5-sulfonic acid (ISA) was identified by LC-MS as the primary byproduct of indigo carmine degradation. **[Conclusion]** *Cc*PIE5 is best-suited in decolorizing dyes under high temperatures and alkaline and salty conditions. It serves as a good candidate for specific applications in the environment and industry.

Keywords: fungal laccase; overexpression; *Coprinopsis cinerea*; high-salt; indigo carmine

Synthetic dyes are widely used in food, cosmetics, paper, and textile industries. It is reported that more than 700 000 tons of synthetic dyes are produced annually worldwide, with more than 15% of them emitted into the environment as effluents. The complexity of wastewater generated from modern textile industries' dyeing processes is steadily increasing[1]. For example, the dye wastewater resulting from contemporary textile finishing procedures exhibits characteristics such as high chroma (ranging from 10 to 250 mg/L), fluctuating alkaline pH levels (between 6.0 and 11.8), and elevated temperatures (ranging from 50 ℃ to 70 ℃). Additionally, high concentrations of salts (ranging from 40 g/L to 100 g/L) are commonly utilized in dye baths to ensure maximal dye fixation onto fibers (with the mass fraction of NaCl and Na_2SO_4 exceeding 6% and 5%, respectively)[2]. Typically, seawater salinity is characterized by the presence of 3.5% *W*/*W* salts[3]. Wastewater exhibiting salinity levels surpassing those found in oceans is classified as hypersaline (3.5%), while wastewater with salinity ranging from 1.0% to 3.5% *W*/*W* NaCl is termed highly saline[4]. Moreover, the presence of salinity in wastewater significantly hampers the efficacy of various wastewater treatment methodologies. The persistence of highly stable textile finishing

dyes has emerged as a leading contributor to water pollution, exerting detrimental effects on the environment and posing a grave threat to ecological and environmental integrity.

With society's increasing concerns for the ecological environment and health, the treatment of dye wastewater has become an essential part of social development. Many physical and chemical approaches have been developed to treat dye wastewater, including biosorbents, coagulants, electrochemical oxidation, ultrasonic chemical oxidation, ozonation, and irradiation[5]. However, they are costly and susceptible to secondary pollution, restricting their wide application. In contrast, biological processes have advantages of high decolorization rate, low cost, simple operation, low toxicity or non-toxicity of the end product, and have gradually become the primary way for dye wastewater treatment[6-7]. Specifically, the utilization of heme-containing oxidoreductases, including manganese, lignin, and versatile peroxidases, as well as oxidative laccases, is recognized as an effective catalysis for dye biodegradation[8]. Although peroxidases have a higher redox potential (up to 1.40 V *vs*. a standard electrode), they depend on hydrogen peroxide, leading to heme bleaching and enzyme deactivation even in moderate hydrogen peroxide concentrations[9-10]. By comparison, laccases are four-copper-containing O_2-dependent oxidoreductases that oxidize a wide variety of substrates by electron transfer across the copper atoms at the T1, T2, and T3-T4 sites, with water as the only other end product, making them more promising for the biodegradation of dyes[8].

Several fungal and bacterial laccases have been evaluated in dye wastewater treatment with or without a low-molecular-weight mediator (laccase mediator system, LMS)[11-13]. Usually, fungal laccases catalyze the dyes at acidic conditions, showing the characteristics of a high redox potential at the substrate-binding T1 copper, high specific activity and superior affinity for a broader range of substrates[14]. In comparison, most bacterial laccases show optimum activity at neutral and alkaline conditions and the advantages of thermostability and high chloride tolerance. However, bacterial laccases possess a relatively low redox potential (430−460 mV *vs*. >730 mV of fungi) and specific activity (<10 U/mg *vs*. >100 U/mg with ABTS (2,2′-azino-bis(3-ethylbenzothazoline-6-sulfonate)) as the substrate)[15]. Thus, it is still challenging to degrade modern-process-produced dye wastewater using any of the characterized natural fungal and bacterial laccases, due to their inherent technical deficiencies. More efforts remain to find specific fungal laccases that show high activity at alkaline pH and high chloride tolerance for wastewater treatment processes.

Because of their low natural production yields, fungal laccases secreted from native sources are typically unsuitable for large-scale industrial applications[16]. Thus, large-scale production of laccases at lower costs is another bottleneck for applying laccases in dye wastewater treatment. Various strategies, including optimizing culture conditions and adding inducers for overexpression, have been tried to scale up the production of laccases but have resulted in only limited improvements[17]. Expressing a laccase gene in heterologous organisms has sometimes been shown to be an effective strategy. For example, more than 30 fungal laccases have been heterologously expressed in *Pichia pastoris* with various productivities[16,18]. However, the laccase obtained often changed in biochemical properties because protein modification by glycosylation differed depending on the heterologous host cells[17]. To this end, the over-expression of laccase in the homologous host cells should enable the production of a well-glycosylated laccase at low cost[19].

Basidiomycete fungal laccases have been instrumental in integrating biotechnological processes across diverse sectors such as paper and pulp, food and feed, textile, and pharmaceutical

industries. The prevailing milieu for most basidiomycete laccases is within mildly acidic parameters (pH 4.0−6.0) and a temperature range of 30−50 ℃, which may preclude their utilization in contexts necessitating alkaline conditions and elevated temperatures. *Coprinopsis cinerea*, a prominent model basidiomycetous fungus, harbors 17 distinct laccase genes within its genome and boasts well-established protocols for genetic manipulation[20]. Various inquiries have underscored the promise of heterologous expression of laccases from *C. cinerea* in efficacious dye wastewater treatment. Notably, *Cc*Lcc2 and *Cc*Lcc6, when expressed heterologously in *P. pastoris*, exhibit potential in decolorizing dye-contaminated wastewater[21-22]. Additionally, a newly identified laccase, Lac1, from the basidiomycete *Coprinus comatus*, sharing 66% genetic similarity with the laccase gene of *C. cinerea*, has shown remarkable prowess in decolorizing three different azo dyes[23]. Recently, the basidiomycete laccase, *C. cinerea* Lcc9, has emerged as exceptionally thermophilic and alkaliphilic, demonstrating promise in decolorizing dye-contaminated wastewater[24-25]. Fungal laccase PIE5, a mutant derived from the native laccase Lcc9 of *C. cinerea*, represents the foremost thermo- and alkali-philic fungal laccase engineered with an optimal catalytic pH in alkaline realms (pH range of 8.0−8.5), particularly towards guaiacol, offering potential for the treatment of dye wastewater. Nevertheless, the expression level of PIE5 reached only 1.2 U/mL when expressed in *P. pastoris*, presenting challenges for its industrial-scale application. In this current investigation, PIE5 was successfully overexpressed in *C. cinerea*, yielding a final output of (24.2±1.1) U/mL. This alkali-philic enzyme demonstrated exceptional chloride tolerance, presenting a novel advantage for the biotreatment of high-salt dye wastewater, as illustrated in this study through the degradation of indigo carmine under highly saline alkaline conditions.

1 Materials and Methods

1.1 Strains, plasmids and chemicals

C. cinerea FA2222 (*A5*, *B6*, *acu1*, *trp1-1*, *1-6*), used as a *trp1*-auxotrophic monokaryon in transformation, is available at the DSMZ in Braunschweig. *C. cinerea* expression vector pYSK7 with laccase gene *lcc1* with its native terminator sequences subcloned behind the *Agaricus bisporus gpdII* promoter[19] was used in vector construction, marker-plasmid pBD5 with the *C. cinerea trp1*$^{+}$ gene[26] for selection for prototrphy in *C. cinerea* co-transformations. *P. pastoris* GS115 and the expression vector pPIC9K were from Invitrogen. Plasmid pPIC9K-*pie5* from a *lcc9* mutant library contains the developed allele *pie5*[27]. 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid ammonium salt (ABTS), guaiacol and indigo carmine were from Sigma-Aldrich. All other chemicals and reagents were of analytical grade.

1.2 Expression of PIE5 and wild-type Lcc9

The expression of PIE5 in *P. pastoris* GS115 using pPIC9K-*pie5* was carried out under the control of the inducible AOX1 promoter, along with the α-factor signal coding sequences from the pPIC9K vector for secretion. The laccase expressed in *P. pastoris* was subsequently purified from the culture supernatant, as detailed by Xu et al.[25], and hereafter referred to as *Pp*PIE5. The wild-type Lcc9, originating from the coculture of *C. cinerea* and *Gongronella* sp. w5, was synthesized and purified following the procedures outlined by Pan et al.[24].

1.3 Construction of a PIE5 overexpression *C. cinerea* FA2222 strain

Plasmid pPIC9K-*pie5* was used as the template to amplify PCR of the *lcc9* mutant allele *pie5*[27]. A primer pair was used with homologous overlaps to the DNA regions of the *gpdII* promotor and the *lcc1*-terminator present in plasmid pYSK7[19,28], pYSK7-*pie5*F 5′-AATCCACCATCTCCGTTTTCTCCCATCTACACACAACAAGCTTATCGCCATGCAAATCCTCGGCCCC-3′ and pYSK7-*pie5*R: 5′-TGACTATAGCAGCCTCC

TACCACTGGCCCTCTGGTCAACTATAATATTAT TTAAGGGGTAGGGACGATTTGG-3′ (underlined fragments are homologous overlaps sites). PCR was conducted using the PrimeSTAR® Max DNA Polymerase Kit (TaKaRa). Plasmid pYSK7-*lcc1* was linearized using *Hpa* I and *Bam*H I (TaKaRa) to double-digest. *Saccharomyces cerevisiae* strain Y1H (TaKaRa) was transformed with the *pie5* PCR amplicon and the linearized pYSK7 to generate the final plasmid pYSK7-*pie5* based on *in vivo* homologous recombination[19]. The *Saccharomyces cerevisiae* plasmid was transformed into *Escherichia coli* JM109 to increase its copy number.

C. cinerea FA2222 was cultivated on solid YMG/T medium (per liter 4 g yeast extract, 10 g malt extract, 4 g glucose, 100 mg tryptophan, and 1% agar) at 37 ℃. Protoplast transformation was performed using oidia collected from fully grown plates and harvested in sterile water, filtered through sterile glass wool, washed, protoplasted, and transformed as described before by Dörnte and Kües et al.[28]. For each co-transformation, 1 μg plasmid pYSK7-*pie5* and 1 μg pBD5 harboring the *trp1*$^+$ gene were used. Transformed protoplasts were plated onto regeneration agar according to Dörnte et al.[28]. When required to test laccase activities of obtained transformants in further growth, 0.5 mmol/L ABTS (1st round of selection) and 1 mmol/L guaiacol (2nd round of selection) were added for further testing into regeneration agar, respectively. Finally, prototrophic transformants were collected onto minimal medium[26] and cultivated for further use at 37 ℃.

To further screen transformants with high laccase activity, clones positive in the two rounds of screening on regeneration agar were then cultivated at 37 ℃ and 120 r/min in 100 mL mKjalke liquid medium (per liter: 10 g yeast extract, 20 g glucose, 2 g KH_2PO_4, 0.5 g $CaCl_2·2H_2O$, 50 mg $MgSO_4·7H_2O$) supplemented with 0.1 mmol/L $CuSO_4$ in 500 mL Erlenmeyer flasks[17]. Laccase activity in supernatants was tested every 24 h.

1.4 Purification of PIE5 expressed in *C. cinerea* FA2222

To purify overexpressed PIE5 from *C. cinerea* FA2222, the *C. cinerea* FA2222-*pie5* transformant with highest laccase activity (clone 14) was cultured in 0.1 mmol/L Cu-supplemented mKjalke medium at 37 ℃, as described above. The culture broth was collected on the 6th day of incubation, centrifuged at 4 ℃ and 8 000×*g* for 10 min, and further concentrated using an ultrafiltration device (Millipore) to get the crude enzyme solution. After dialysis using citrate-phosphate buffer (20 mmol/L, pH 6.5), the crude enzyme solution was applied onto a DEAE-Sepharose FF column (10 mm×200 mm, Amersham Pharmacia, Uppsala) and eluted according to protocols reported previously[25]. The purified protein was designated as *Cc*PIE5.

The homogeneity of the purified protein was determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) with a 12% polyacrylamide gel. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. The protein concentration was assayed using the Bradford method at 595 nm, with bovine serum albumin as the standard.

1.5 Laccase assays

Laccase activity was determined at 30 ℃ for 3 min in 100 mmol/L sodium tartrate buffer at pH 4.0 with 1 mmol/L ABTS as the substrate (ε_{420}= 36 000 L/(mmol·cm)). The reaction was initiated by adding the enzyme into the solution. The absorbance was measured at 420 nm[29]. Alternative substrates for measuring laccase activity were 1 mmol/L guaiacol (ε_{465}=12 000 L/(mol·cm)), 0.5 mmol/L 2,6-dimethylphenol (2,6-DMP, ε_{468}= 49 600 L/(mol·cm)), 100 μmol/L syringaldazine (SGZ) (ε_{525}=65 000 L/(mol·cm)). One unit of activity was defined as the amount of enzyme needed to oxidize 1 μmol substrate per minute. All measurements were performed in triplicates from three biological repeats of culture.

1.6 Additional biochemical characterization of laccase

The effect of pH on laccase activity was

assayed in 50 mmol/L citrate-phosphate buffer (4.0−8.0) and 50 mmol/L Tris-HCl buffer (7.5−9.5) at 25 ℃ using 1 mmol/L guaiacol as the substrate. The effect of temperature was determined by incubating protein in a temperature range of 35−75 ℃ at optimum pH. The enzyme stabilities against pH and temperature were determined by incubating proteins at various temperatures and pH 6.5 and 8.5. Then, the residual activities were determined with 1 mmol/L guaiacol as mentioned in section 2.4. The effect of ions (NaCl, NaBr, Na_2SO_4, KCl, NH_4Cl, and $CaCl_2$) on PIE5 activity was tested at 60 ℃. The salt concentration was always 1.0 mol/L but for NaCl that ranged from 0.0 mmol/L to 2.0 mol/L. All experiments were performed at least three independent times from three different purified protein batches, with at least three technical repeats of measurement per batch.

1.7 Kinetic properties

The kinetic parameters of the enzyme were tested under optimum conditions, and K_m and V_{max} were calculated by nonlinear regression of the Michaelis-Menten equation (Eq. 1) with GraphPad Prism 8.0 (GraphPad Software).

$$v_0 = \frac{V_{max}[S]}{K_m + [S]} \quad \text{(Eq. 1)}$$

1.8 The dye removal properties of *Cc*PIE5

*Cc*PIE5 was used to decolorize indigo carmine in the presence of 1.0 mol/L NaCl. The initial testing system contained Tris-HCl buffer (50 mmol/L, pH 8.5), purified enzyme (0.2 U/mL), and indigo carmine (100 mg/L), and mediators (vanillin, citronellal, syringic acid (SA), ferulic acid, vanillic acid, and SGZ) when required (final concentration, 200 μmol/L) in a total volume of 1 mL. The reaction mixtures were incubated at 60 ℃ for 2 h.

The effects of concentrations of enzymes, mediators and their concentrations, pH, temperature, and incubation time on dye decolorization were evaluated one by one, based on a single-factor optimization strategy. The decolorization rate of indigo carmine by laccases was monitored according to the decrease in absorbance at 610 nm. All experiments were performed at least three independent times with at least three technical repeats of measurement. The heat-treated enzyme was used as the negative control.

The decolorization ratio was calculated using the following equation (Eq. 2).

$$\text{Decolorization ratio (\%)} = (A - A_0)/A_0 \times 100 \quad \text{(Eq. 2)}$$

A_0 and A represent the initial and final absorbance of indigo carmine, respectively.

1.9 End product determination

The reaction mixture was separated using centrifugal filters (3 kDa cut-off value) to discard *Cc*PIE5 to analyze the degradation products. Electrospray ionization mass spectrometry (ESI-MS) analysis was performed with a mass spectrometer (ThermoFisher Scientific) operating in the negative ion mode. The conditions of the MS were: auxiliary gas flow rate of 15 bar; sheath gas flow rate of 70 bar; capillary voltage of 15 V; capillary voltage of 15 V; and capillary temperature of 350 ℃[30]. The expected product structures were drawn with the aid of the mass fragmentation tool of KingDraw and these were then verified with the *m*/*z* values obtained from the ESI-MS study[31].

2 Results and Discussion

2.1 Overexpression of PIE5 in *C. cinerea* FA2222 and purification

A total of 56 positive transformants were obtained after the two rounds of regeneration medium plate screening, using ABTS and guaiacol as the substrates (Figure 1A). The same positive clone is shown in reaction with ABTS (middle) and guaiacol (right). Reactions with guaiacol in the regeneration agar were, however, less intense, with visually smaller orange halos generated around the orange-brown stained colonies. Randomly selected transformants were then cultured in mKjalke liquid medium (Figure 1B). Because a target gene is randomly inserted at ectopic sites into the *C. cinerea* genome with different copy numbers during transformation[32],

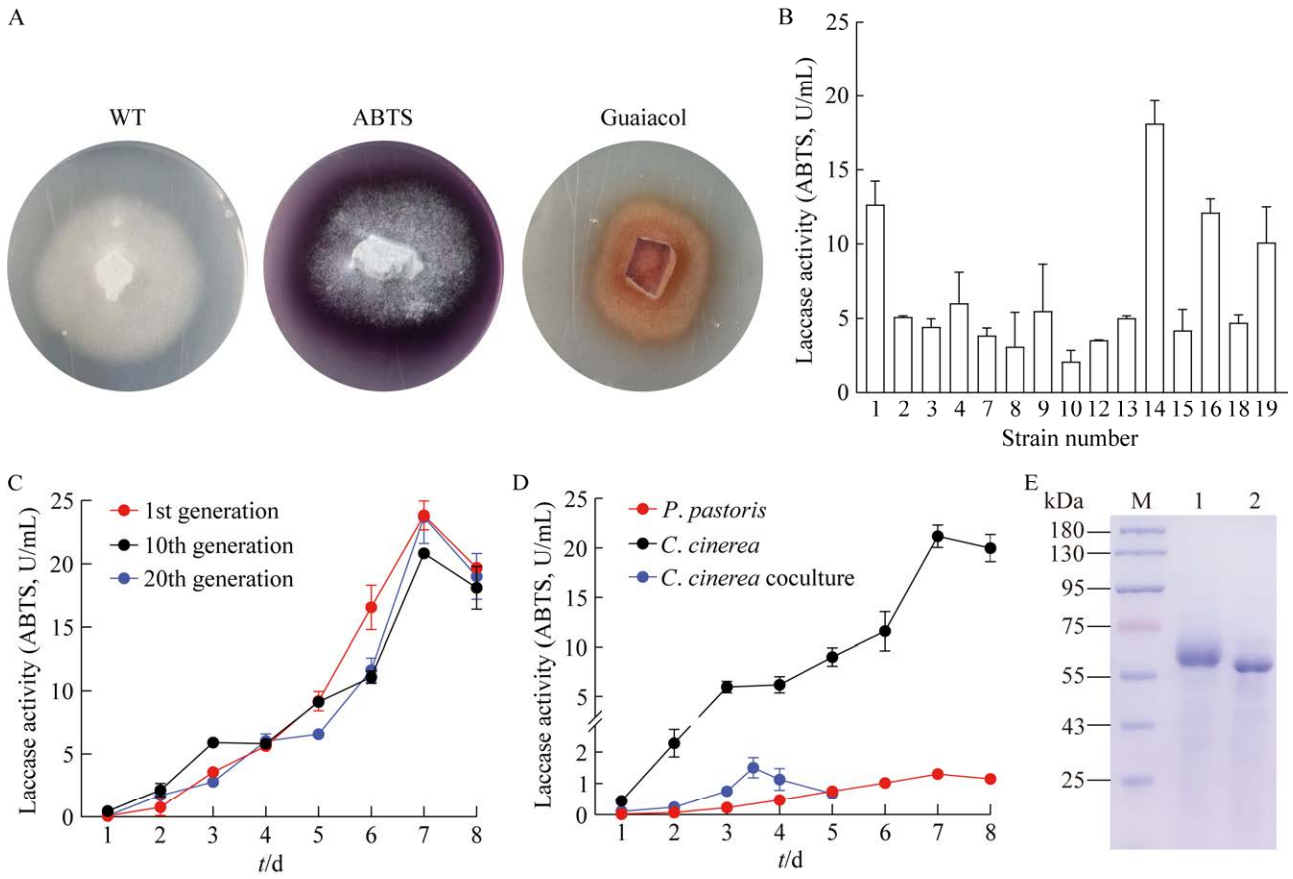

Figure 1 Overexpression of *Cc*PIE5 in *Coprinopsis cinerea* FA2222 and its purification. A: Positive colony screening with ABTS or guaiacol as the substrates on regeneration agar plates. B: Laccase activity of 19 randomly selected positive transformants. C: Laccase activity of the positive transformant FA2222 *Cc*PIE5-14 after the 1st, 10th, and 20th-generation fermentation. D: Comparison of laccase activities of PIE5 from *C. cinerea*, *Pichia pastoris*, and wild-type Lcc9. E: SDS-PAGE of protein marker (M), purified *Pp*PIE5 (lane 1) and *Cc*PIE5 (lane 2).

transformants showed different laccase activities of (2.1±0.5) U/mL to (18.1±1.1) U/mL in liquid cultures, measured with ABTS (Figure 1B), which in its large variability is similar to previous observations from culture supernatants (YMG/T plus 0.1 mmol/L $CuSO_4$) of transformants carrying *lcc1* under the control of different basidiomycetous promoters[19]. The transformant with the highest laccase activity of about 18.0 U/mL (clone 14, Figure 1B, from now on called *Cc*PIE5-14) was chosen for further research in this work.

Laccase activity in the culture supernatant of clone 14 reached (24.2±1.1) U/mL when using ABTS as the testing substrate after 7 days of cultivation in shaking flasks (Figure 1C). The laccase production was stable even after the overexpression transformant *Cc*PIE5-14 was subcultured for up to 20 generations (Figure 1C). The enzymatic activity of *Cc*PIE5 recombinantly expressed by *C. cinerea* FA2222 *Cc*PIE5-14 was 20-fold higher than that of *Pp*PIE5 expressed in *P. pastoris* GS115[27] and was about 13-fold higher than that expressed by *C. cinerea* monokaryon Okayama 7 from the endogenous *lcc9* gene during fungal coculture in competition with *Gongronella* sp. w5 in our former studies[33] (Figure 1D). Furthermore, the yield of recombinant laccase activity in this study was 2.6-fold higher

than in the best case observed so far. Previously, five laccases (Lcc1, Lcc2, Lcc6, Lcc8, and Lcc9) from *C. cinerea* have been heterologously or homologously recombinantly expressed[17,21-22,25,34], where the laccase yield of the *lcc1* transformant pYSK7-26 reached 9.2 U/mL in Cu-supplemented mKjalke medium at 25 ℃ and represented the highest recombinant laccase activity reported before in *C. cinerea*[17,24,35-36].

*Cc*PIE5 was purified 12.71-fold from the culture supernatant (Figure 1E), with a final yield of 40.41% (Table 1). It showed a specific activity of 640.12 U/mg. By comparison, when expressed in *P. pastoris*, *Pp*PIE5 showed a specific activity of 318.00 U/mg[27]. Varied glycosylation may cause the changed specific activity of PIE5. As shown on a SDS-PAGE gel, although PIE5 had the predicted molecular weight of 55.6 kDa, *Pp*PIE5 and *Cc*PIE5 showed heavier molecular weights than 55.6 kDa, indicating that they were glycosylated proteins[37]. Furthermore, *Cc*PIE5 shifted faster in gels than *Pp*PIE5, suggesting it had lighter glycosylation than the latter. Similar observations with distinct degrees in glycosylation were reported for the wild-type Lcc9 when expressed in the native host and in *P. pastoris*. Glycosylation were shown to contribute to protein stability. N313 and N454 were determined in mutagenesis approaches to be the specific amino acid that are glycosylated in Lcc9[25]. Generally, the effect of glycosylation on laccase activity is still understudied[38]. However, the studies on a fungal laccase in seven different *Steccherinum ochraceum* strains suggested a substantial role of *N*-linked glycosylation in the moderation of enzymatic properties of laccases[39].

2.2 Biochemical characterization of *Cc*PIE5

The effects of pH and temperature on purified *Cc*PIE5 activity were analyzed using the phenolic compound guaiacol as the substrate. *Cc*PIE5 showed the optimum pH and temperature at 7.5−8.0 and 55−60 ℃, respectively, with more than 60% of activity retained at pH of 7.0 and 8.5 and temperatures of 50 ℃ and 65 ℃ (Figure 2A, 2B). In addition, *Cc*PIE5 showed a half-life time of 45 min at pH 6.5 and 8.5 at 60 ℃ (Figure 2C). A comparison of the biochemical properties between *Cc*PIE5 and *Pp*PIE5 showed that they shared nearly identical properties, including optimum pH, temperature, and thermo- and pH-stabilities (Figure 2A−2C), despite their apparent difference in glycosylation.

Dye wastewater usually contains high salt concentrations of more than 1.0 mol/L NaCl or Na_2SO_4[2]. Thus, the biochemical properties of the two PIE5 versions were tested at 1.0 mol/L NaCl. The optimum pH and temperature changed to 8.0−8.5 and 65 ℃ (Figure 2D−2F). The half-life time of the two PIE5 versions under these conditions at 65 ℃ was 40 min. These results suggested that PIE5 is a thermostable fungal laccase showing high activity at alkaline conditions in the presence of high concentrations of salt. Beyond high salt concentration, wastewater from denim processing is in addition characterized by high pH (>6.0) and high temperature (above 50 ℃)[40]. Thus, the ability of laccases to operate at higher temperatures is particularly important because elevated temperatures often favor high reaction rates in many processes, which means higher decolorization velocity. Furthermore, utilization of thermostable laccases with properties such as the developed PIE5 here avoids the

Table 1 Summary of the purification steps of extracellular *Cc*PIE5 from *Coprinopsis cinerea*

Purification steps	Total volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yields (%)	Purification (fold)
Crude enzyme	1 800	40 300	756.00	53.31	100.00	1.00
Ultrafiltration	150	32 200	135.18	238.21	90.65	4.73
Dialysis	110	30 442	87.91	339.81	75.54	6.75
DEAE-sepharose FF	160	16 285	25.44	640.12	40.41	12.71

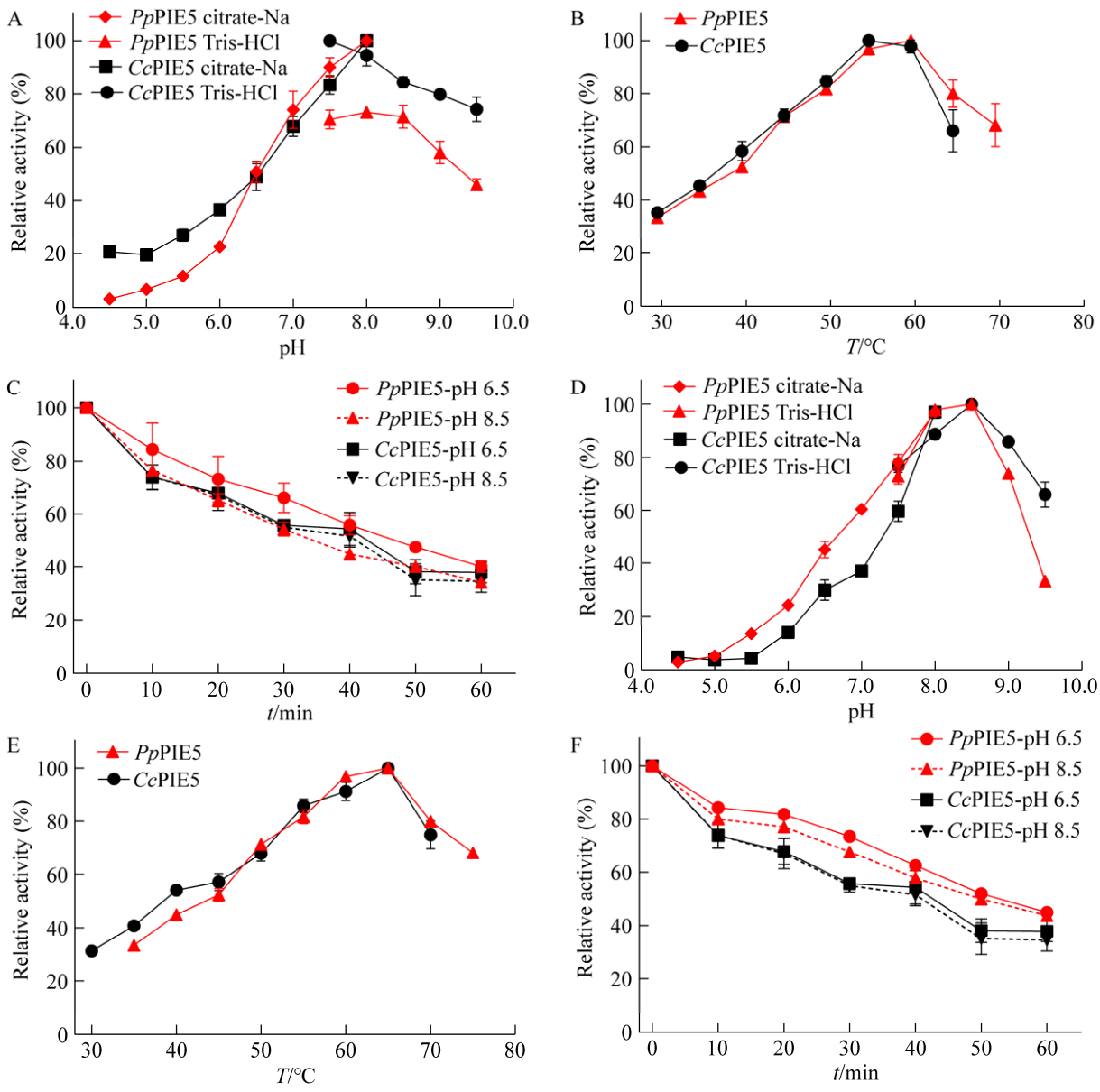

Figure 2 Enzymatic characterization of purified PpPIE5 and CcPIE5 enzymes. A−C: Definition of optimal pH, temperature and thermostability at pH 6.5 and 8.5 without NaCl. D−F: Definition of optimal pH, temperature and thermostability at pH 6.5 and 8.5 in the presence of 1.0 mol/L NaCl.

additional cooling process, reduces the operation cost, and fully uses the high temperature of dyeing wastewater to achieve the maximum decolorization rate in a short time[15].

2.3 Chloride tolerance of PIE5

The salt tolerance ability of *Pp*PIE5 and *Cc*PIE5 was further evaluated by employing NaCl and Na_2SO_4 as salts that are commonly used in dyeing processes[40]. These salts did not negatively affect the PIE5 laccase activity. Laccase activity was even stimulated by 1.0 mol/L Cl^- when tested, using guaiacol as the substrate (Figure 3A). Further experiments showed that halide ions, including Cl^- and Br^-, stimulated the laccase activity, by up to 300% (Figure 3A). Most other fungal laccases are sensitive to and inhibited by

halide ions, with an IC_{50} of 0.02−1 600 mmol/L[35,41]. For basidiomycete laccases, this value does not usually exceed 100 mmol/L; for example, laccase from *Trametes villosa* shows an IC_{50} (Cl^-) of 40 mmol/L[42]. Thus, fungal laccases with halide tolerance have recently attracted much attention[43]. Like PIE5, a novel bacterial laccase-like enzyme, Lac1326, showed excellent tolerance to salt and maintained 204.6% and 167.2% of its original activity in the presence of 100 mmol/L and 500 mmol/L of NaCl, respectively. However, this enzyme showed the best catalytic activity in acidic conditions[44]. Enzymatic activity for another bacterial laccase, LacHa of *Halomonas alkaliantartica*, with an optimum pH and temperature at 7.5 and 45 ℃, respectively, was 1.5-fold increased by NaCl concentrations <500 mmol/L. The enzyme had comparably good stability in higher NaCl concentrations (ca. 60% in NaCl concentrations up to 1 000 mmol/L) but showed only around half of its potential activity at pH 8.0[45]. As far as we know, PIE5 is the first fungal laccase with halide tolerance ability and it is even stimulated by halide at alkaline conditions.

What is the molecular base of the excellent halide tolerance of *Cc*PIE5? Fungal laccases contain four catalytic Cu atoms in the protein (T1 Cu and the tri-nuclear Cu cluster, including T2 Cu, T3α Cu, and T3β Cu). Substrates are assumed to bind near the T1 Cu, which is known as the substrate binding and oxidation site[43]. It is widely accepted that chloride also binds near the T1 site and could act as a competitive inhibitor of electron donors by blocking the access of reducing substrates to the T1 Cu site of laccase or inhibiting the electron transfer at the T1 active site, thus inhibiting laccase activity, while halides with larger molecular radii are less efficient inhibitors (fluoride>chloride>bromide)[35]. In addition, in acidic conditions, chloride may bind to the T2/T3 copper cluster of laccase and affect electron transfer. However, the mechanism of laccase activity stimulation by chloride at alkaline conditions is poorly understood. Therefore, NaCl was used as the chloride donator to investigate the effect of chloride on *Cc*PIE5 activity at pH 8.0. Results showed that the activity stimulation effect of chloride on *Cc*PIE5 was substrate-dependent. For example, NaCl at a concentration of 2.0 mol/L stimulated laccase activity to 237%. By comparison, salt had no significant effect on *Cc*PIE5 activity with 2,6-DMP as the substrate. However, it inhibited the activity when using SGZ as a substrate, with an IC_{50} of 1 000 mmol/L (Figure 3B). Further experiments showed that the kinetic constants of *Cc*PIE5 changed in various chloride concentrations based on the Michaelis-Menten equation (Table 2). NaCl at low concentrations enhanced the substrate affinity and

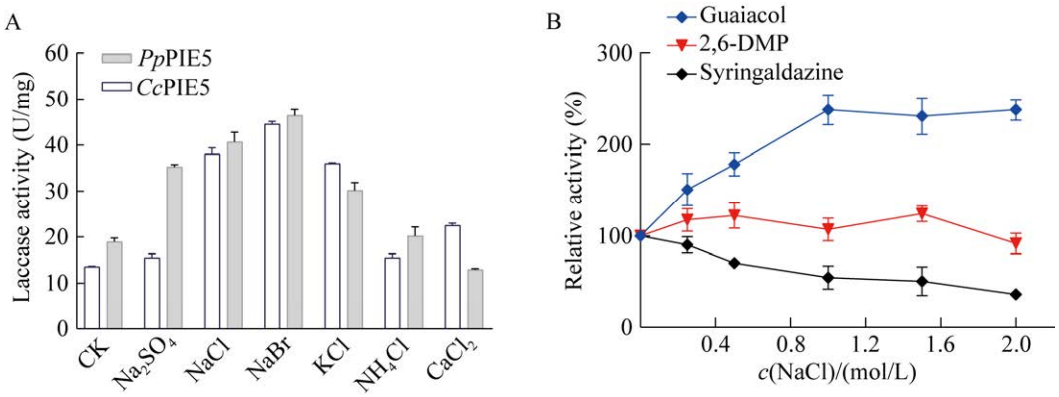

Figure 3 Chloride tolerance of PIE5. A: Effect of different ions at a concentration of 1.0 mol/L on *Pp*PIE5 and *Cc*PIE5 activities as measured with guaiacol. B: NaCl at different concentrations on *Cc*PIE5 activity with different phenolic compounds as substrates.

Table 2 Kinetic parameters of *Cc*PIE5 from FA2222 at different NaCl concentrations (n=3)

NaCl (mol/L)	k_{cat} (S^{-1})	K_m (mmol/L)	k_{cat}/K_m (mmol/(L·S))
0.0	133.67±9.37	4.37±0.77	30.59±5.24
0.2	92.73±7.55	1.71±0.58	54.20±2.87
0.4	89.75±5.90	1.11±0.32	80.88±4.59
0.7	89.03±6.91	0.94±0.29	94.71±6.34
1.0	87.63±3.40	0.93±0.15	94.31±3.56
1.5	68.26±3.67	0.48±0.12	141.52±5.68
2.0	58.72±2.64	0.65±0.13	90.91±3.93

the catalytic efficiency of the enzyme when using guaiacol as a substrate because the K_m and k_{cat} showed a downward trend as NaCl concentration increased to 1.5 mol/L.

Further experiments showed that the kinetic constants of *Cc*PIE5 changed in various chloride concentrations based on the Michaelis-Menten equation (Table 2). gradual increases in k_{cat}/K_m values of PIE5 toward guaiacol were observed as chloride increased from 0.0 mol/L to 1.5 mol/L, suggesting that chloride at relatively low concentrations can enhance the affinity of PIE5 to substrate, thereby stimulating the peroxidase activity. Based on the electron transfer theory, a proper substrate orientation and a proper distance between the substrate donor orbitals and the T1-His acceptor orbitals are essential to enhance the electron transfer rate[46]. Accordingly, the relevant active substrate conformations should be kept at a proper distance from T1 Cu[47]. Based on this theory, we hypothesized that NaCl might influence *Cc*PIE5 properties by binding to specific protein sites neat the T1 Cu site and causing subtle changes in local areas, thus enhancing the affinity of the substrate guaiacol to the T1 Cu site by providing a better conformation, shorting the distance between electron donor and acceptor, and finally boosting electron-proton transfer. On the other hand, substrates are different in molar volume (mv). For example, SGZ has an mv of 291.0 cm^3/mol, 2,6-DMP has an mv of 135.0 cm^3/mol, and guaiacol has an mv of 111.8 cm^3/mol. Thus, chloride could inhibit the activity of *Cc*PIE5 on substrates with a relatively large volume by disturbing their proper conformations at the substrate binding site. which showed the salt activation of PIE5 was substrate dependent, and therefore, indicated that different substrates interact with the enzyme at different regions. Thus, the interactions would be affected by the Cl^- ion bound to different sites. However, there were decreases in k_{cat}/K_m values of PIE5 toward guaiacol as chloride increased from 1.5 mol/L to 2.0 mol/L, indicating that chloride at high concentrations binds to the laccase domain and acts as a competitor of electron donor by blocking the access of substrate to the active site of PIE5 or reducing the electron transfer between them, therefore leading to inhibition of laccase activity[48].

2.4 Indigo carmine degradation by *Cc*PIE5 in salt environment

The predominant environmental concern associated with azo dyes arises from their inherent resistance to degradation, attributable to the presence of one or more azo groups (−N=N−)[49]. Azo dyes pose substantial environmental hazards, capable of modifying both the physical and chemical characteristics of soil, deteriorating aquatic ecosystems, and posing threats to surrounding flora and fauna. Even minute quantities of azo dyes in water are perceptible, adversely affecting the aesthetics, clarity, and gas solubility of the aquatic environment. This results in reduced light penetration, subsequently diminishing photosynthetic activity and inducing hypoxia, thereby disrupting the ecological balance of aquatic organisms. Indigo carmine is one of the most commonly used dyeing agents for food, denim, and polyester fibers[50]. Several laccase-based treatments of excessive concentrations of indigo carmine have been developed under acidic conditions and without high salt concentrations because of typical enzyme salt inactivation[51]. By comparisons, *Cc*PIE5 decolorized 43% of 100 mg/L indigo carmine within 2 h at pH 8.5 in the presence of 1.0 mol/L NaCl. With the addition of mediators vanillin, citronellal, SA, or ABTS, the decolorization

rate of *Cc*PIE5 increased significantly. SA was the optimum mediator for *Cc*PIE5. The decolorization rate reached over 90% under the initial reaction conditions (Figure 4A). The order of mediators that promoted the decolorization rate was as follows: SA>citronellal>ABTS>vanillin. By comparison, adding ferulic acid, vanillic acid, and SGZ repressed the decolorization rate (Figure 4A).

The conditions for indigo carmine decolorization were further optimized using a single-factor optimization strategy using SA as the mediator. *Cc*PIE5 showed high decolorization rates in a pH range of 8.0−9.5 and 50−80 ℃, with more than 70% of indigo carmine decolorized (Figure 4B, 4C), following the fact that the enzyme showed the optimum pH at alkaline conditions. *Cc*PIE5 could decolorize indigo carmine at low concentrations of mediator and enzyme. In the presence of 0.1 U/mL *Cc*PIE5 and 10 μmol/L SA, a decolorization rate of more than 90% was obtained after incubation for 2 h. A high concentration of mediator (30 μmol/L) slightly inhibited the decolorization rate. Further optimization of the incubation time showed that *Cc*PIE5 could decolorize (92.9±2.3)% of indigo carmine within 40 min (Figure 4D−4F).

Liquid chromatograph mass spectrometry (LC-MS) was employed to analyze the decolorization products of indigo carmine. Figure 5A depicts the electrospray ionization (ESI) mass spectrum of the untreated indigo carmine solution. The mass spectrum of the original dye solution displayed a primary peak at *m*/*z* 443 (Figure 5A), representing the singly charged anion of indigo carmine. Furthermore, an intense anion at *m*/*z* 210 was detected, corresponding to indigo carmine in its doubly charged ionic form (3-oxoindoline-5-sulfonate). Upon treatment with *Cc*PIE5, isatin 5-sulfonic acid (ISA) with a nominal mass of 227 (Figure 5B) was identified in its deprotonated form ([2-H]− of *m*/*z* 226). Another peak at *m*/*z* 244 was observed, corresponding to 4-amino-3

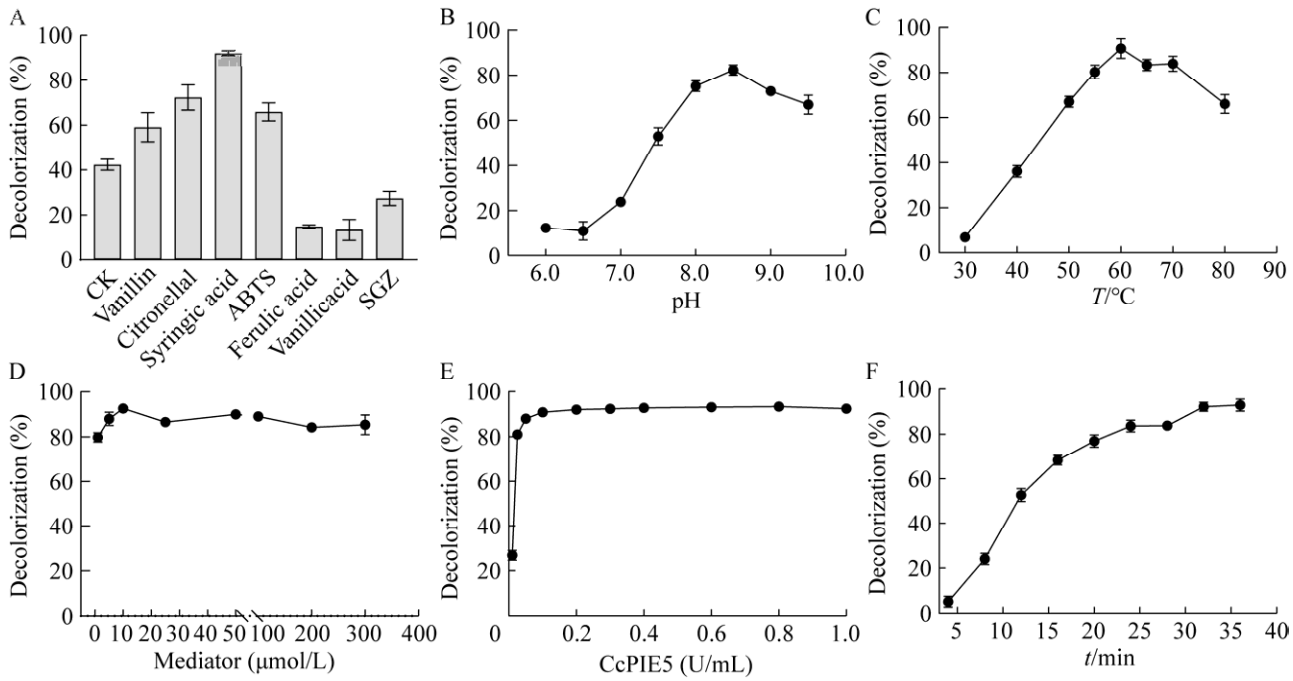

Figure 4 Optimization of indigo carmine decolorization conditions by *Cc*PIE5. A: Tests of different mediators at 0.2 mmol/L concentration in Tris-HCl buffer, pH 8.5 with 0.2 U/mL *Cc*PIE5. B−F: Effects of pH, temperature, SA dosage, *Cc*PIE5 dosage and incubation time on decolorization of indigo carmine by *Cc*PIE5 in presence of the best acting mediator SA.

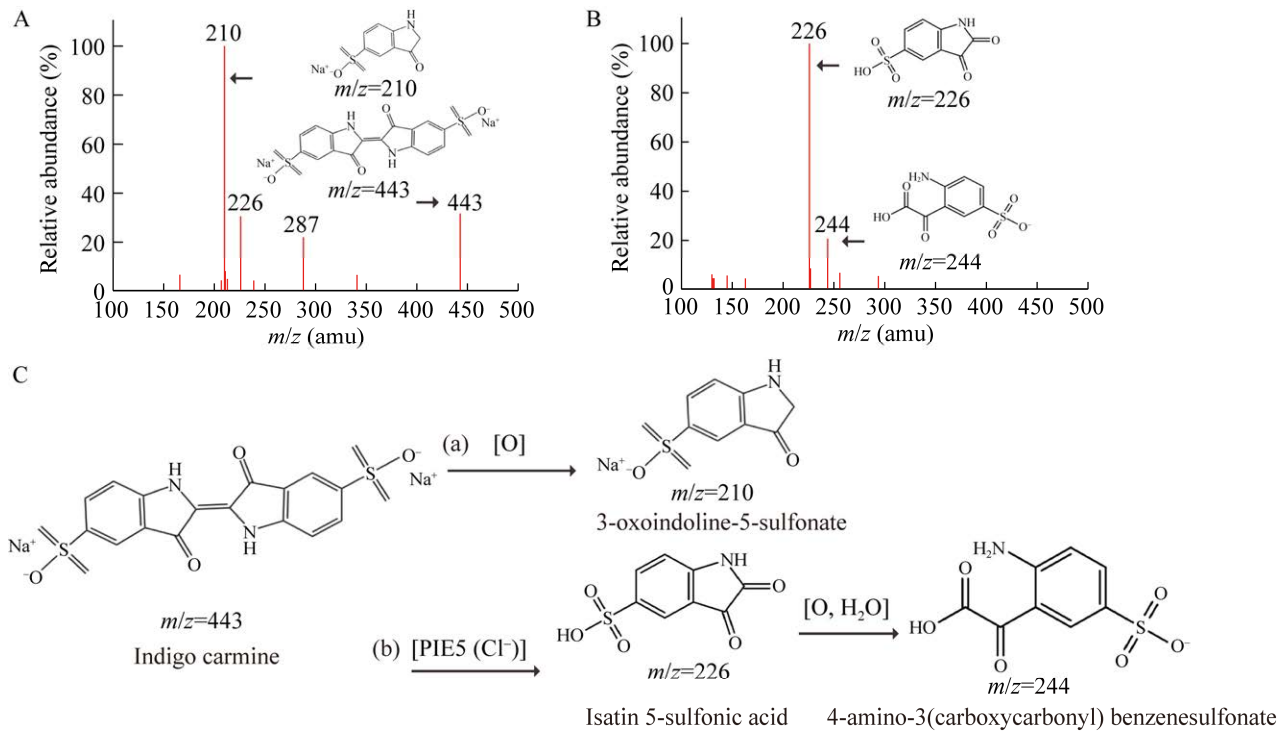

Figure 5 Indigo carmine end product determination. A: The mass spectra of indigo carmine before *Cc*PIE5 treatment, with main identified compounds with structural formulas marked. B: The mass spectra of indigo carmine after *Cc*PIE5 treatment, with main identified compounds with structural formulas marked. C: Proposed pathway of indigo carmine degradation by *Cc*PIE5. Natural degradation pathway (path a) and *Cc*PIE5 degradation pathway (path b) of indigo carmine; [O] refers to broken of carbon-carbon double bond by oxidation; [PIE5 (Cl^-)] refers to the oxidation of indigo carmine by *Cc*PIE5 without NaCl addition.

(carboxycarbonyl) benzenesulfonate, a derivative of ISA formed through straightforward chemical reactions[52]. As a result of the disruption of a conjugated system akin to indigo carmine, the solution rapidly decolorized following the addition of *Cc*PIE5. Examination of the indigo carmine dye structure in Figure 5C reveals two distinct degradation pathways. In path (a), (−N=N−) undergoes natural oxidative cleavage to yield 3-oxoindoline-5-sulfonate. In path (b), PIE5 expedites this process by catalyzing the insertion of oxygen atoms at the C2 and C2′ positions, resulting in the formation of a substantial quantity of ISA. ISA can undergo further oxidation to produce 4-amino-3(carboxycarbonyl) benzenesulfonate. Additionally, ISA naturally degrades to yield benzoic acid and aniline, which pose significantly lesser environmental harm[53].

3 Conclusions

An optimized fungal laccase Lcc9 mutant, namely PIE5, was successfully expressed in *C. cinerea*. The laccase activity in culture supernatant was (24.2±1.1) U/mL, and compared to *Pc*PIE5 produced in *P. pastoris*, *Cc*PIE5 did not change its biochemical properties after expression in *C. cinerea*. NaCl stimulated laccase activity at high concentrations by increasing the affinity of the substrate to the enzyme. *Cc*PIE5 decolorized more than 92.9% indigo carmine within 40 min at pH 8.5 in the presence of high salt, with ISA as the main product. Therefore, *Cc*PIE5 is best-suited in decolorizing dyes under

high temperatures and alkaline and salty conditions, and is a good candidate for specific applications in the environment and industry.

Author Contributions

WANG Xianhua, ZHU Xueling, LIN Jiayi, GU Xianfu, and PENG Qixia, performed the experiment and analysed the results. WANG Xianhua, XIAO Yazhong, Ursula Kües and FANG Zemin wrote the manuscript. XIAO Yazhong and FANG Zemin acquired funding. XIAO Yazhong Xiao, Ursula Kües and FANG Zemin conceptualized the project.

Competing Interests

The authors declare that they have no competing interest.

Data Availability

The data that has been used is confidential.

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