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Biodegradation and Decolorization of Crystal Violet Dye by Cocultivation with Fungi and Bacteria

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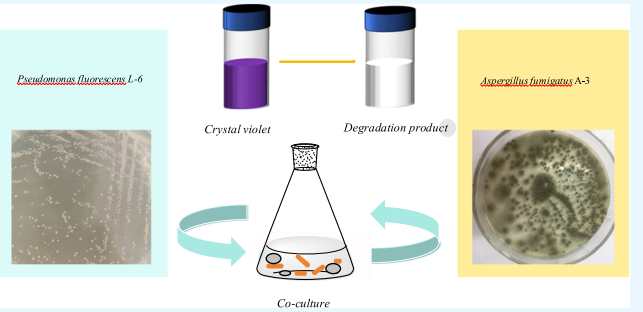
ABSTRACT: Microbial degradation of dyes is vital to understanding the fate of dyes in the environment. In this study, a fungal strain A-3 and a bacterial strain L-6, which were identified as *Aspergillus fumigatus* and *Pseudomonas fluorescens*, respectively, had been proven to efficiently degrade crystal violet (CV) dye. The decolorization of CV dye by fungal and bacterial cocultivation was investigated. The results showed that the decolorization rate of cocultures was better than monoculture (*P. fluorescens* in L-6 (PF), and that of *A. fumigatus* A-3 (AF)). Furthermore, enzymatic analysis further revealed that Lac, MnP, Lip, and NADH−DCIP reductases were involved in the biodegradation of CV dyes. UV−visible spectroscopy, Fourier transform infrared (FT-IR) spectroscopy, and gas chromatography−mass spectrometry (GC−MS) were used to examine the degradation products. GC−MS analysis showed the presence of 4-(dimethylamino) benzophenone, 3-dimethylaminophenol, benzyl alcohol, and benzaldehyde, indicating that CV was degraded into simpler compounds. The phytotoxicity tests revealed that CV degradation products were less toxic than the parent compounds, indicating that the cocultures detoxified CV dyes. As a result, the cocultures are likely to have a wide range of applications in the bioremediation of CV dyes.



1. INTRODUCTION

Crystal violet (CV) is a cationic triphenylmethane dye that is widely used in a variety of industrial applications. Some of these include dyeing textiles made from cotton, silk, wool, nylons, and polyacrylonitriles. Additionally, CV is also used in biological staining, veterinary medicine, and as a mutagenic and antibacterial agent.[1] Despite its various uses, CV is a recalcitrant chemical that resists environmental degradation, resulting in its long-term residue in wastewater and soil. Furthermore, even a small amount of CV can produce an intense color, and its dangerous effects on humans and the environment cannot be ignored. CV has been reported to be toxic to mammalian cells as well as mutagenic, carcinogenic, and mitogenic in its toxicity,[2,3] highlighting the need for an effective degradation technique. Given the potential risks of CV on human health and the environment, it is imperative to develop an efficient method for its degradation, which can reduce its toxicity and minimize its impact on the environment.

The discharge of textile dye wastewater can pose serious health risks to the ecosystem, and unfortunately, a considerable amount (10−15%) of dye wastewater is directly discharged without any treatment.[4] Synthetic dyes, which are commonly used in the textile industry, are highly resistant to light, temperature, and microbial attack, making it challenging to decolorize them.[5,6] A number of methods, including biodegradation,[7] adsorption,[8] photocatalytic degradation,[1] electro-Fenton process,[9] and advanced oxidation processes (AOPs), have been attempted to degrade synthetic dyes, such as CV.[10] However, it has been found that physical or chemical treatment methods, such as adsorption, precipitation, chemical degradation, and photodegradation, are costly, inefficient, and environmentally unfriendly. One promising approach that has emerged in recent years is biological cocultivation technology that employs lignin-degrading enzymes to treat wastewater contaminated with synthetic dyes. Since lignocellulose, the primary component of plant cell walls, has a highly complex structure, multiple enzymatic systems are needed to break it down and depolymerize it.[11] The lignin-degrading enzyme system includes laccase (Lac), lignin peroxidase (LiP), and manganese peroxidase (MnP), among which Lac is a green "catalyst" used to remediate wastewater containing dyes. These enzymes are crucial in reducing the concentration of lignin in wastewater and accelerating its biodegradation.[12−14] Among these enzymes, LiP and MnP have gained increasing popularity in degrading refractory dyes and wastewater treatment because of their higher efficiency to oxidize dyes than Lac.[15,16] Due to

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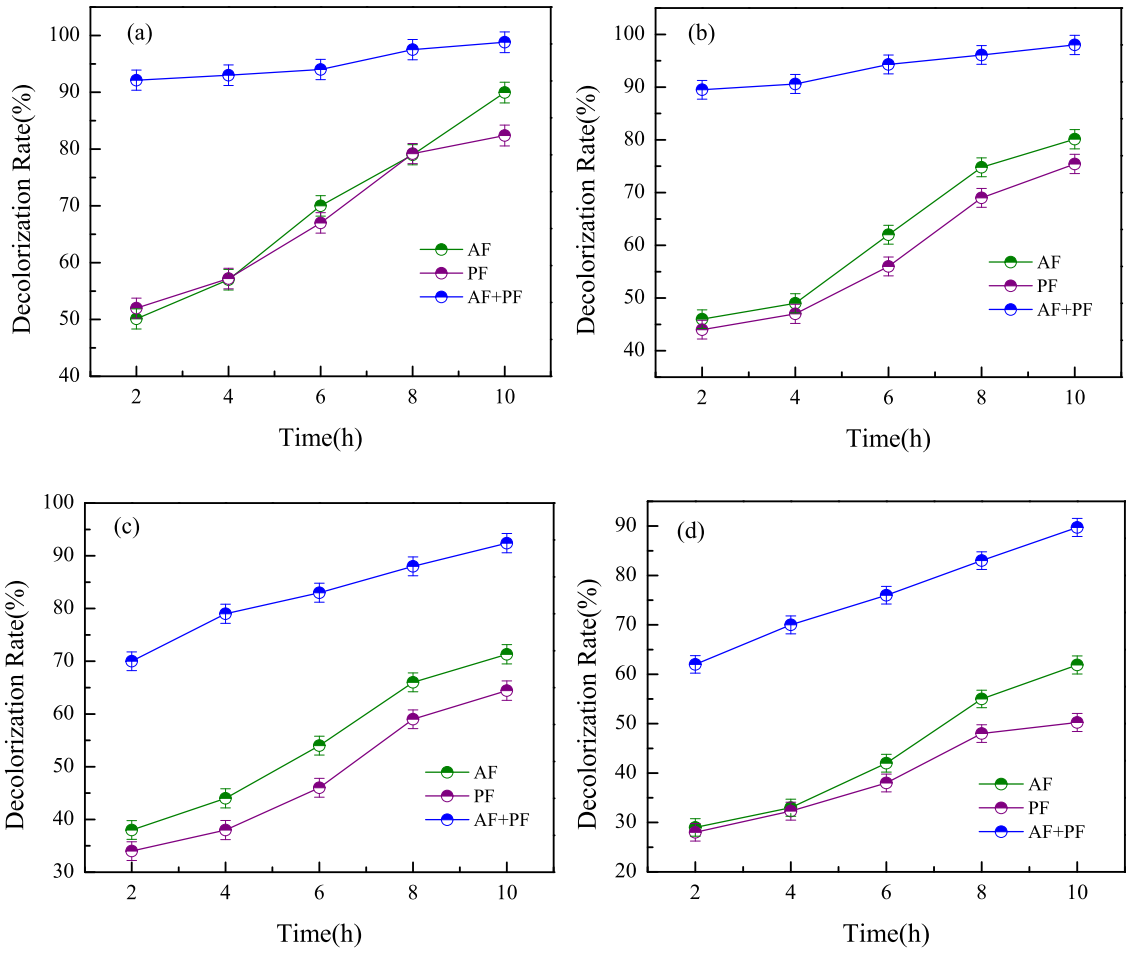

Figure 1. Effects of mono- and coculture on dye decolorization of CV at different concentrations: (a) 50 mg/L; (b) 100 mg/L; (c) 150 mg/L; and (d) 200 mg/L.

limited resources and space, microorganisms present in the natural environment are known to interact with each other in various ways, including cross-species and cross-genera induction.[17] These interactions usually involve competition, which can result in the production of various biological activities with antimicrobial properties, such as enzymes and biosurfactants.[18,19] Some bacteria have been discovered to produce more bioactive chemicals and enzymes when they contact with other strains.[20] These interactions can be investigated by coculturing two or more species in a laboratory setting.[21]

Microorganism coculture provides various advantages over pure cultures, including greater variety, such as increased diversity, production of more active components in secondary metabolites, increased enzyme activity, and improved contaminant degradation.[22−24] Microbial coculturing has attracted extensive attention in the development of biological control agents, wastewater treatment, and the fermentation of products.[25−27] It has been reported that when two strains are cocultured together, they exhibit an interaction in a complex manner, such as parasitic, antagonistic, or synergistic.[28] Depending on the combination of microbial strains and their corresponding circumstances, the studies showed that the coculture process induced high expression of some degradative genes and key enzymes,[23] increased the synthesis of ligninolytic enzymes, and increased the dye's decolorizing ability.[29] MnP and LiP have been reported as the main enzyme involved in dye degradation.[30] Besides, Lac has also been identified as the major enzyme responsible for dye decolorization in cocultures.[31]

In this study, fungi and bacteria were cocultured to degrade and decolorize CV. This study was aimed: (i) to isolate and screen the CV degrading strains, (ii) to study the decolorization effect of monoculture and coculture on CV dye, (iii) to analyze the degradation products and the degradation pathway by UV−vis spectrophotometry (UV−vis), Fourier transform infrared (FT-IR) spectroscopy, and gas chromatography−mass spectrometry (GC−MS), (iv) to determine the enzyme activity during CV degradation, and (v) to evaluate the phytotoxicity of CV dye and degradation products.

2. RESULTS AND DISCUSSION

2.1. Isolation and Identification of CV Degradation Strains. Several strains were screened with lignin enzyme activity and dye decolorizing ability, among which strains A-3 and L-6 had the highest enzymatic activities and CV decolorizing ability. Therefore, strains A-3 and L-6 were selected for further study. The 16S rDNA and ITS analysis indicated that strain L-6 belonged to the *Pseudomonas fluorescens*, while A-3 belonged to the *Aspergillus fumigatus*.

The phylogenetic tree was constructed by the neighbor-joining method, as shown in Figure S1.

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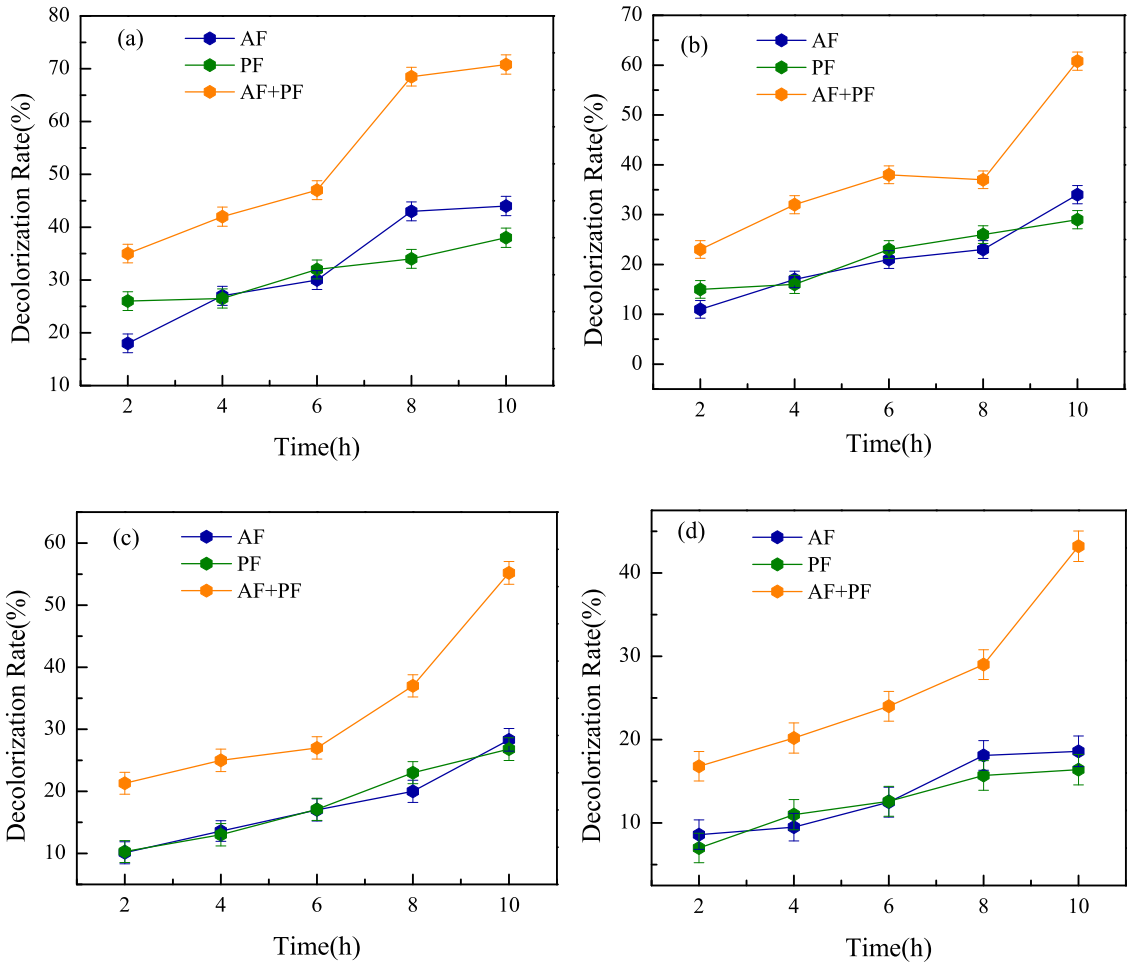

Figure 2. Effects of enzyme extract on dye decolorization of CV at different concentrations: (a) 50 mg/L; (b) 100 mg/L; (c) 150 mg/L; and (d) 200 mg/L.

2.2. Decolorization Effect of Mono- and Coculture on Different Dye Concentrations. For decolorization studies, different concentrations (50, 100, 150, and 200 mg/L) of CV dye were cultured with the inoculum of monoculture and coculture. As demonstrated in Figure 1, dye doses of 50, 100, 150, and 200 mg/L used in coculture showed the potential to decolorize CV by 100, 99.99, 92.39, and 89.73%, respectively. The decolorization rates of AF were 89.95, 80.13, 71.32, and 61.89%, respectively, while those of PF were 82.38, 75.43, 65.43, and 50.25%, respectively. Using different dye concentrations, the decolorization rate of CV increased gradually after 10 h, and the decolorization rate of CV decreased with the increase of dye concentration. The decolorization rate at low concentrations was higher than that at a high gradient.

The coculture showed a significantly higher decolorization of CV dye compared to monocultures AF and PF. Coculture of the fungus *Penicillium citrinum* WXP-2 and the bacterium *Citrobacter freundii* WXP-9 showed a synergistic effect compared with monoculture.[35] Similarly, when the strains were cocultured, such as *Enterobacter cloacae*, *Bacillus subtilis*, *E. cloacae*, and *B. subtilis*, the result indicated more effective decolorization of dye compared to single cultures.[36,37]

The cocultures exhibited a noticeable increase in CV decolorizing activity, which can be attributed to increased laccase and peroxidase synthesis. This enhanced enzymatic production in cocultured microorganisms is a result of interactions between different species and/or genera.[47] The cocultures are superior to monocultures in terms of adaptability to complex and changeable environmental circumstances. This is mostly owing to their ability to produce a broad spectrum of enzymes, utilize intermediate metabolites for further mineralization, and facilitate the transformation of contaminants into nontoxic compounds.[47]

2.3. Decolorization Effect of Enzyme Extract on Different Dye Concentrations. The study aimed to investigate the effect of crude enzyme extracts of AF, PF, and AF + PF on the decolorization of CV at different concentrations (50, 100, 150, and 200 mg/L). The result presented in Figure 2 showed that the decolorization efficiency of the enzyme extract of AF + PF cocultures was higher than AF and PF cultures. Specifically, after 10 h, the enzyme extract of AF + PF cocultures obtained decolorization efficiencies of 70.8, 60.8, 55.2, and 43.2%, at CV concentrations of 50, 100, 150, and 200 mg/L, respectively. In comparison, enzyme extracts from AF and PF cultures decolorized at lower rates, with 44.3, 35.6, 28.3, and 18.6% for AF and, 37.4, 30.8, 26.8, and 16.4% for PF, respectively.

These results suggest that the enzyme extracts may play an important role in the decolorization of CV, with the enzyme extract of the AF + PF coculture demonstrating remarkable efficiency. Our findings are consistent with those of previous studies on *Ischnoderma resinosum*, which demonstrated similar

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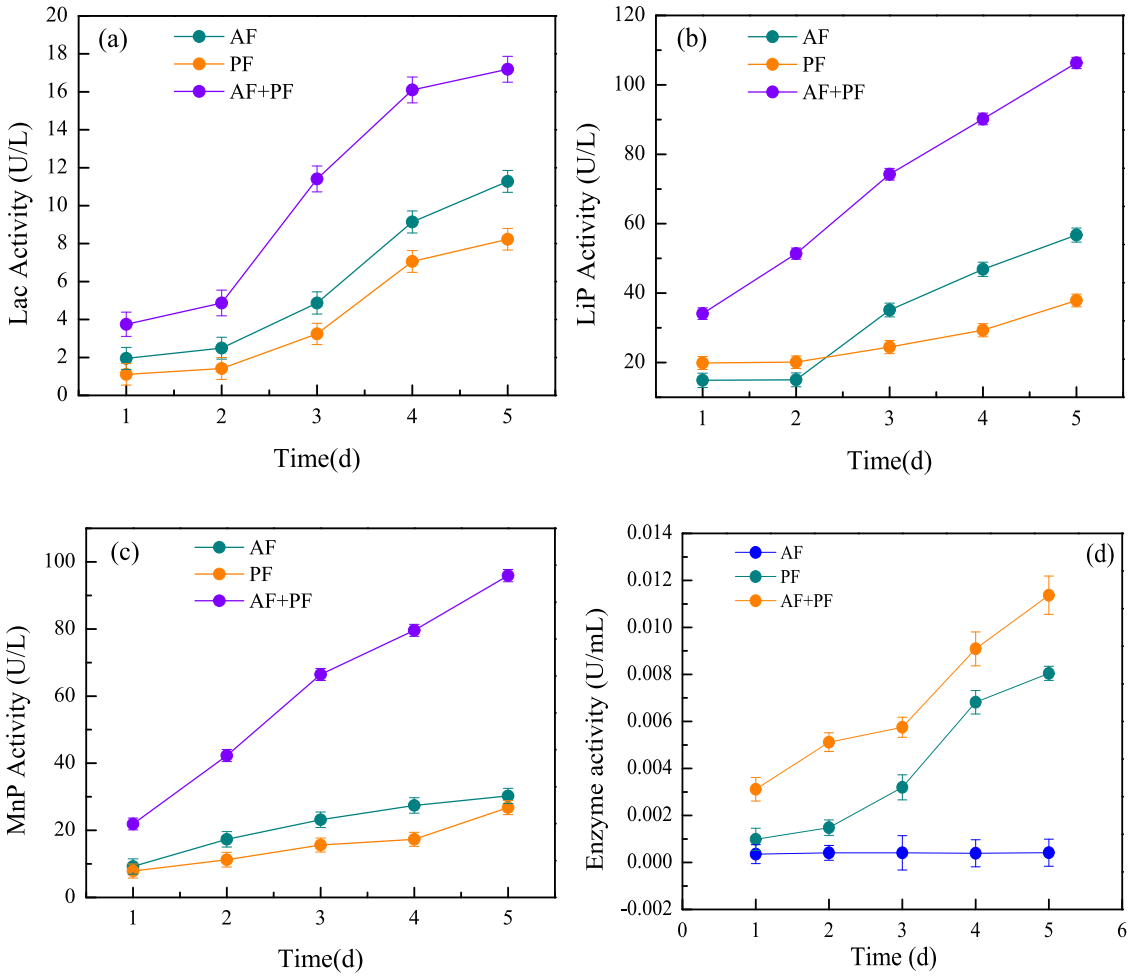

Figure 3. Enzyme activity in decolorization of CV: (a) Lac; (b) LiP; (c) MnP; and (d) NADN−DCIP.

effectiveness of CV decolorization.[38] It has also been observed that lignin-degrading enzymes produced by fungus and bacteria play a role in the decolorization and degradation of textile dyes.[39] However, the study discovered that the strains in the coculture method lacked the potential to efficiently exert their synergistic impact in increasing enzyme production. Furthermore, some researchers have suggested that single strains may be a more preferable option for dye decolorization compared to mixed cultures.[40,6]

2.4. Enzyme Activity in Dye Decolorization. The laccase activity of monoculture and coculture was evaluated, as shown in Figure 3. The results indicated that laccase activity increased gradually with increasing fermentation time up to the fifth day of incubation in both monoculture and coculture. Figure 3a indicates on the fifth day of incubation, the highest laccase activity (17.19 U/L) was recorded in the coculture (AF + PF), compared to 11.28 and 8.23 U/L in the AF and PF monocultures, respectively. As a result, coculture has a positive effect on laccase synthesis, resulting in higher enzymatic activity. Similarly, peroxidase activity also gradually increased in their profile following incubation. Results in Figure 3b showed that after 5 days of incubation, the coculture had the greatest LiP activity of 106.32 U/L, while AF and PF monocultures had 56.73 and 37.89 U/L, respectively. As shown in Figure 3c, coculture had the maximum MnP activity at 95.89 U/L, while AF and PF had 30.25 and 26.78 U/L, respectively. For NADH−DCIP reductase, the enzyme activity of coculture was significantly higher than those of AF and PF monocultures.

It is worth noting that the monocultured strains were not effective in lignin degradation. However, the coculture of highly efficient degradation strains played a synergistic role, resulting in a higher degradation ability. Likewise, Kadam et al.[41] reported that the cocultures of Pseudomonas sp. and *Aspergillus ochraceus* produced higher laccase and MnP than that of monoculture.

Because of the synergistic effects of enzymes produced by different strains in various combinations, the cocultures were considered excellent dye decolorizers.[22,42] Lignin-degrading enzymes have been successfully used in the textile industry and have interesting industrial applications.[43] Therefore, when bacteria and fungi were cocultured, their lignin peroxidase, manganese peroxidase, and laccase showed increased enzymatic activity, suggesting that the microbial coculture system has great potential for bioremediation of synthetic dye contamination.

2.5. Characterization of Intermediate Products. *2.5.1. UV−Vis Spectroscopy.* This study investigated the degradation of CV dye by coculture using UV−vis spectroscopy. The degradation was monitored by observing a reduction in the absorbance of value (λ_{max}) of the dye at different time intervals (control, 4, 8, and 12 h) using UV−vis spectrophotometry. A previous study has demonstrated that biodegradation and biosorption are the primary causes of dye

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removal by biological processes.[44] During biodegradation, the main absorption peaks disappear, or new absorption peaks appear in the visible region. Conversely, if dye decolorization was caused by absorption, all absorption peaks in the UV−visible region will decrease synchronously. The results presented in Figure 4 showed that the main characteristic

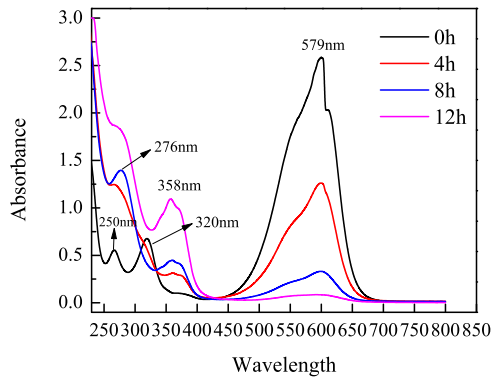

Figure 4. UV−vis spectra of CV at different times.

absorption peaks of CV were 579, 320, and 250 nm. These peaks disappeared within 12 h, and new absorption peaks appeared at 358 and 276 nm. To observe the color of microbial cells before and after dye decolorization, in terms of monocultures, bacteria showed no color change before and after decolorization, while both the cells of the cocultures and single fungi also showed color after decolorization. According to UV−vis spectrum analysis, bio-decolorization of CV may be caused by both biodegradation and biosorption.

2.5.2. FT-IR Spectroscopy. The FT-IR spectrum of the CV dye before and after degradation is shown in Figure 5. The

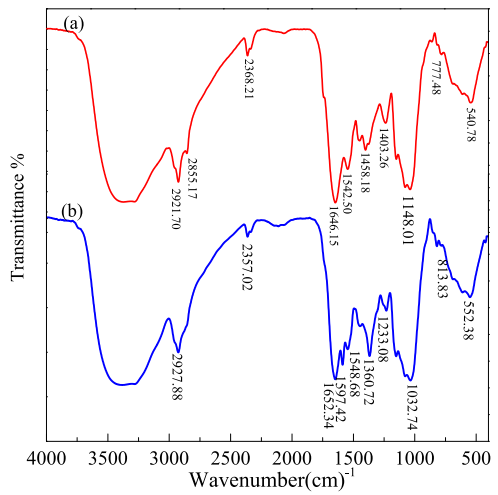

Figure 5. FT-IR spectra of CV (a) before and (b) after degradation.

spectra reveal the appearance of new peaks at 1597.42, 1360.72, and 1233.08 cm^{-1} after degradation. The peak at 1597.42 cm^{-1} is attributed to the stretching vibration of the C═O groups,[2] whereas the absorption peak at 1360.72 cm^{-1} could be due to the −OH groups of the alcohols (R−OH) or phenols (Ar−OH).[7] The stretching vibration of the aromatic amine's $-NH_2$ group is responsible for the absorption peak at 1233.08 cm^{-1} These changes in the infrared spectrum suggest that CV was degraded, and new metabolites were formed. The functional groups −OH, C═O, and $-NH_2$ were found in the metabolites identified after CV degradation.

2.5.3. GC−MS. Decolorization and biodegradation of dyes are based on enzymes synthesized by microorganisms. The degradation mechanism of various dyes is mainly based on the action of enzymes, such as azo reductase, Lac, MnP, LiP, NADH−DCIP reductase, etc. Although different enzymes may have different effects on dyes.[48] Lac is copper-containing polyphenol oxidoreductase that can catalyze the oxidation of various substituted phenols and nonphenolic compounds,[49] and consequently plays an important role in different biological treatment processes.[50] Lac has a great degradation effect on many aromatic compounds. MnP can oxidize a variety of aromatic compounds as well as degrade some substances that are difficult to biodegrade.[51] LiP can achieve C−C bond cleavage, benzyl alcoholization, hydroxylation, aromatic ring cracking, and demethylation.[52] LiP has a significant degradation effect on azo dyes, heterocyclic dyes, polymer dyes, and triphenylmethane dyes.

The degradation metabolites of CV were identified and analyzed by gas chromatography−mass spectrometry. Four possible intermediates were detected as follows: 4-(dimethylamino) benzophenone, 3-dimethylaminophenol, benzyl alcohol, and benzaldehyde. The degradation products of CV were detected by GC−MS, similar to that mentioned in refs 45,2. The degradation products of CV are shown in Table 1. The GC−MS spectra of CV degradation products are shown in Figure S2.

Based on the degradation products and combined with the enzymes, a possible degradation pathway was proposed. Under the action of the oxidase, the C═N and C═C double bonds in CV were oxidatively cleaved to form the intermediate metabolites [*N*,*N*-dimethylaminophenyl] [*n*-methylaminophenyl] benzophenone and 3-dimethylaminophenol; then, the metabolite [*N*,*N*-dimethylaminophenyl] [*n*-methylaminophenyl] benzophenone was demethylated to produce 4-(dimethylamino)benzophenone, which process may involve the LiP; the C═O double bond of 4-(dimethylamino)-benzophenone breaks to form 3-dimethylaminophenol and benzaldehyde. The benzaldehyde was further reduced to benzyl alcohol. The degradation of CV may be a multistep process, beginning with the cleavage of the phenolic group by lignin enzymes followed by oxygenase oxidation, and depending on different degrading microorganisms or the enzymes used, then CV was degraded into small-molecule compounds by the action of enzymes. The results suggested that coculturing can be an effective method for wastewater treatment and biodegradation processes.

Compared with the reported CV degradation products, the degradation of CV by *Agrobacterium radiobacter* has resulted in the metabolites: *N*,*N*,*N*′,*N*″-tetramethylpararosaniline, [*N*,*N*-dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone, *N*,*N*-dimethylaminobenzaldehyde, 4-methyl amino phenol, and phenol; as analyzed by gas chromatography−mass spectroscopy (GC/MS), the degradation products obtained were similar to those in ref 53.[53]

It is good to note that the degradation of CV into low-toxicity substances is a positive result as this reduces the harmful impact of the dye on the environment. The proposed degradation pathway provides insight into the mechanism involved in the degradation of CV by coculture as shown in Figure 6. These findings are useful for optimizing the process of dye degradation and developing more efficient and

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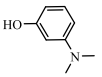
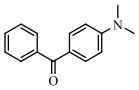
Table 1. Chemical Structures of CV Degradation Products

No.	RT (min)	Molecular weight	*m/z*	Molecular formula	Chemical name	Chemical structures
1	7.659	106	107	C_7H_6O	Benzaldehyde	
2	9.606	108	109	C_7H_8O	Benzyl alcohol	
3	17.311	137	138	$C_8H_{11}NO$	3-Dimethylaminophenol	
4	25.546	225	226	$C_{15}H_{15}NO$	4-(Dimethylamino)benzophenone	

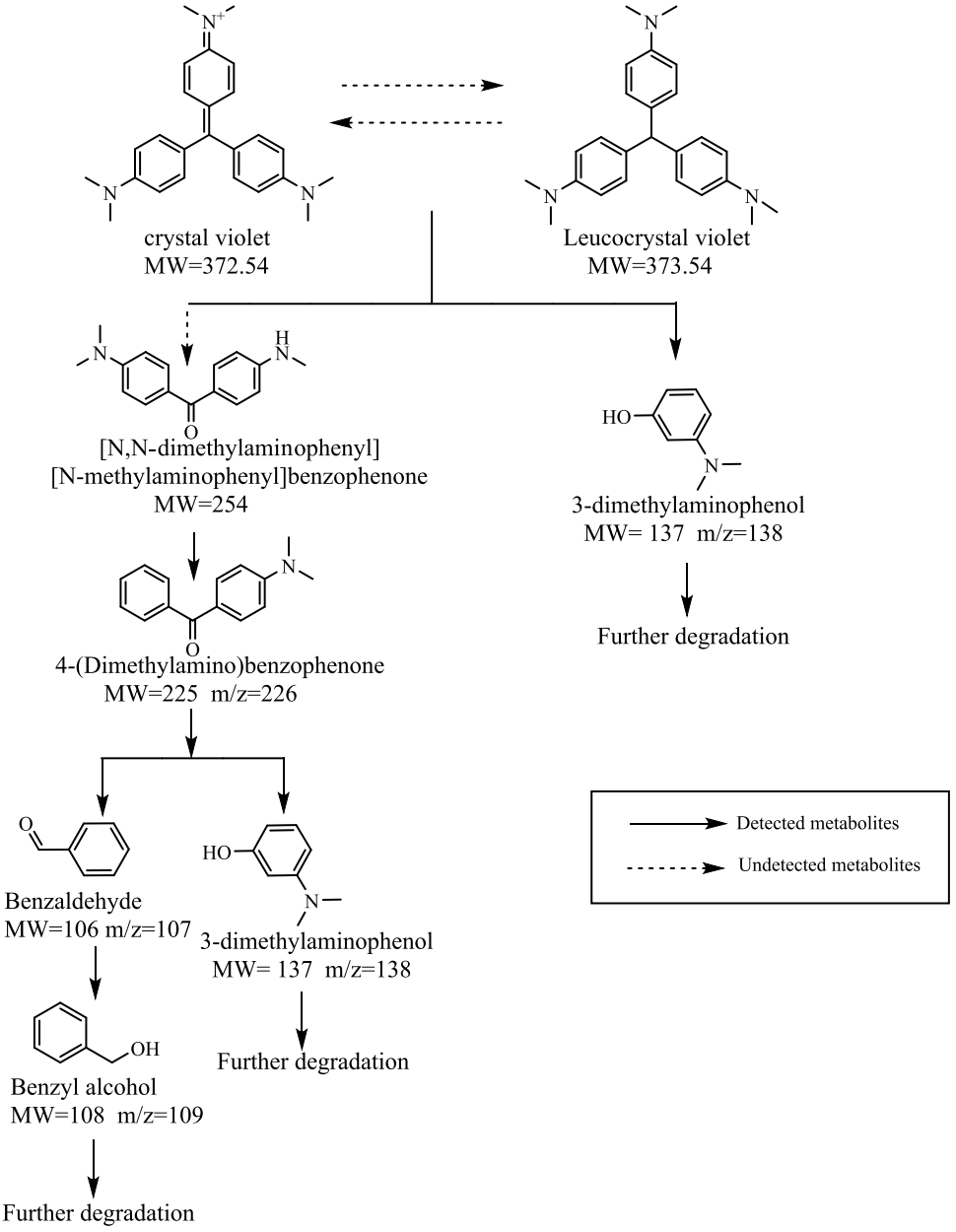


Figure 6. Proposed degradation pathway of CV.

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Table 2. Phytotoxicology of CV and Its Degradation Products[a]

	T. aestivum			*V. radiata*		
samples	plumule (cm)	radical (cm)	germination (%)	plumule (cm)	radical (cm)	germination (%)
distilled water	9.68 ± 0.71[a]	6.36 ± 1.45[a]	100 ± 2.81[a]	3.51 ± 1.09[a]	5.23 ± 1.54[a]	98 ± 0.92[a]
degradation products	7.88 ± 1.09[b]	4.11 ± 0.89[b]	86 ± 3.23[b]	2.85 ± 1.26[b]	4.45 ± 1.03[b]	85 ± 1.03[b]
CV	4.71 ± 1.01[c]	1.16 ± 0.60[c]	42 ± 2.74[c]	1.63 ± 1.35[c]	2.86 ± 1.84[c]	51 ± 1.19[c]

[a]Note: Lowercase letters represent significant differences between the treatments' means ($^*P \leq 0.05$).

sustainable methods for treating dye-contaminated wastewater. Overall, this research demonstrates the potential of using microbial coculture for the effective degradation of dyes and the importance of studying the mechanisms and pathways involved in this process.

2.6. Phytotoxicity Evaluation. The phytotoxicology of CV and its degradation products is shown in Table 2. The results of phytotoxicity showed that the biodegraded metabolites of CV were less harmful than the parent dye. CV at a concentration of 100 mg/L significantly inhibited the germination of *Triticum aestivum* and *Vigna radiata* by 42 and 51%, respectively, but the inhibition was significantly lower for the degradation products. The increased seed germination in the biodegraded metabolites is less toxic than the parent dyes, thus providing evidence for the hypothesis that the degradation of CV causes a significant reduction in toxicity of CV against bacteria, which may be due to the degradation of CV to low-toxicity substances. These findings are consistent with refs 7,34,46, which demonstrated a decrease in toxicity of CV after degradation, as evidenced by improved seed germination rates and enhanced survival of first-instar Artemia larvae. Overall, this study provides evidence that the degradation of CV by coculture may be a promising approach for removing this dye from wastewater, as it leads to the production of less toxic compounds that are beneficial to both bacteria and plants.

3. CONCLUSIONS

In this study, the CV decolorizing strain was isolated and identified. Cocultivation with *P. fluorescens* and *A. fumigatus* has a stronger decolorization effect on CV dye than that with individual bacteria or fungi. This efficient degradation ability is likely due to the synergistic effect of laccase, manganese peroxidase, lignin peroxidase, and NADH−DCIP reductase. In addition, based on the intermediates identified formed during the degradation process by GC−MS, we proposed a pathway for CV degradation by coculture. Furthermore, phytotoxicity experiments revealed that CV was transformed into less harmful metabolites. These findings suggested that cocultures have great potential for practical application in CV dye wastewater treatment. Although the mechanism of CV biodegradation by the strain was analyzed and the degradation pathways were proposed, the molecular mechanism underlying the superior decolorizing performance and high efficiency of cocultures remains unclear. Therefore, further research is needed to investigate the reasons behind the high decolorizing efficiency of coculture.

4. MATERIALS AND METHODS

4.1. Microorganisms and Materials. *A. fumigatus* A-3 (Genbank no. MG183670) and *P. fluorescens* L-6 (Genbank no. MG183671) were used in this study and isolated from the textile wastewater in Lanzhou, China.

CV (triphenylmethane dye, λ_{max} = 579 nm) as a model dye was purchased from Shanghai Sangon Biotech Company, China. All chemicals used in this study were of analytical grade.

4.2. Isolation and Screening of CV Decolorizing Strain. The isolation and identification of bacterial strains A-3 and L-6 were carried out in accordance with the literature.[32,33]

The textile wastewater sample was collected from a sewage treatment plant located in Lanzhou City, Gansu Province. To isolate the strains for efficient decolorization of CV dye, 10 mL of wastewater was added to a flask of 100 mL of Luria−Bertani (LB) and potato dextrose agar (PDA) modified with CV dye (100 mg/L) and incubated at 30 °C and 160 rpm for 48 h. Then, 1 mL of the culture solution was diluted and plated on LB-CV and PDA-CV agar plates, respectively. Different bacteria with clear colorless rings around their colonies indicating decolorization of the CV dye were selected and further screened based on their ability to decolorize and tolerate the highest CV dye concentrations when grown in LB-CV and PDA-CV dye agar plates. Finally, a bacterial strain (L-6) and a fungal strain (A-3) were chosen based on their growth performance and potential for CV dye decolorization and used in further experiments.

4.3. Identification of CV Degrading Bacteria. The genomic DNA of strain L-6 was extracted using a Rapid Bacterial Genomic DNA Isolation Kit, purchased from Shanghai Sangon Biotech Co., Ltd., China. The 16S rRNA gene was amplified by using universal primers (27F) 5′-AGAGTTTGATCC TGGCTCAG-3′ and (1492R) 5′-TACGGCTACCTTGTTACGACTT-3′. The polymerase chain reaction (PCR) program was as follows: 1 cycle at 94 °C for 3 min, 40 cycles of 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 45 s, and a final extension of 72 °C for 5 min.

The Rapid Fungi Genomic DNA Isolation Kit was used to extract genomic DNA of strain A-3. The ITS gene was amplified using 1 μL of genomic DNA as template DNA and universal primers (ITS1 F) 5′-TCCGTAGGT-GAACCTGCGG-3′ and (ITS4 R) 5′-TCCTCCGCTTATT-GATATGC-3′. The procedure for PCR is initial denaturation at 94 °C for 4 min followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 45 s at 56 °C, extension for 90 s at 72 °C and final extension for 5 min at 72 °C.

The PCR products were sequenced and blasted with sequences in the NCBI database GenBank. The phylogenetic analysis was performed using MEGA 6.0.

4.4. Mono- and Coculture. Preparation of spore suspension: the *A. fumigatus* (A-3) were cultivated at 30 °C for 3 days on potato dextrose agar plate; after incubation, the fungal colonies were harvested. Sterile water was added to the PDA agar plate containing *A. fumigatus* A-3, and then the suspension was collected into a sterile Eppendorf tube to obtain a high-concentration spore suspension. Sterile water was added to dilute the spore suspension to a spore concentration of approximately 1×10^6 spores/mL.

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Preparation of seed solution: single colonies of *P. fluorescens* (L-6) were inoculated in Luria−Bertani (LB) liquid medium and cultured at 37 °C, 160 rpm for 12 h, and the obtained culture solution was used as the seed solution.

Preparation of cocultures: 1% (V/V) A-3 spore suspension and 3% (V/V) seed inoculum of PF were inoculated to 250 mL Erlenmeyer flasks with 100 mL of decolorization medium composed of sucrose (10 g/L), NH_4Cl (2 g/L), KH_2PO_4 (2 g/L), $MgSO_4{\cdot}H_2O$ (0.5 g/L), and then cultured at 30 °C,160 rpm for 5 days to obtain the cocultures (AF + PF).

4.5. Decolorization of CV by Crude Enzyme Extract. To prepare the crude enzyme extract, the cocultures were inoculated in 250 mL Erlenmeyer flasks containing 100 mL of sterilized decolorization medium amended with 100 mg/L filter sterilized CV dye. The flasks were incubated at 30 °C, 160 rpm for 5 days, and the samples were collected at different times. A sample was centrifuged at 12,000*g* 4 °C for 10 min to obtain the supernatant, which was used as crude enzymes to determine enzyme activity.

4.6. Determination of Enzyme Activity. Laccase:[31] 2 mL of acetic acid−sodium acetate buffer solution (200 mmol/L, pH = 5), 0.5 mL of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) solution (0.5 mmol/L), and 0.5 mL of crude enzyme solution were added to the reaction system at 28 °C for 10 min, and the change in absorbance at 420 nm was measured. One enzyme activity unit was defined as the amount of enzyme oxidizing 1 mmol of ABTS per minute.

Lignin peroxidase:[32] 1.5 mL of 250 mmol/L tartaric acid−sodium tartrate buffer solution with pH = 3, 1 mL of 15 mmol/L veratrol solution, and 0.4 mL of crude enzyme solution were added to the reaction system. Finally, 0.1 mL of 20 mmol/L H_2O_2 solution was added to initiate the reaction. The reaction was conducted at 30 °C for 2 min, and the absorbance was measured at 310 nm. One enzyme unit (U) is defined as the amount of enzyme required to oxidize 1 μmol of veratrol per minute.

Manganese peroxidase:[30] 3.4 mL of 200 mmol/L, pH 4.5 acetic acid−sodium acetate buffer solution, 0.1 mL of 1.6 mmol/L $MnSO_4$ solution, and 0.4 mL of crude enzyme solution were added to the reaction system. The reaction was started by adding 0.1 mL of a 1.6 mmol/L H_2O_2 solution and continued at 37 °C for 3 min. The change in absorbance was measured at 240 nm. One enzyme unit (U) was defined as the enzyme required to oxidize 1 μmol of Mn^{2+} per minute to become Mn^{3+} quantity.

The enzyme activity (U/L) of Lac, MnP, and LiP was calculated as follows

$$\mathrm{U/L} = \frac{\Delta A}{\min} \times \frac{V \times 10^6}{\varepsilon \times v \times L} \tag{1}$$

where ΔA is the change in absorbance for the sample; V is the total volume of the reaction mixture (mL); L is the path length (cm); and v is the volume of enzyme solution (mL).

NADH−DCIP reductase activity was measured as described in ref 54. 50 mM phosphate buffer solution (pH 7.4), 0.05 mM DCIP and 0.05 mM NADH as substrate, and 0.8 mL of enzyme. The enzyme activity was obtained by measuring the change at 590 nm ($\varepsilon_{590\,\mathrm{nm}}$ = 19 $\mathrm{nm^{-1}\ cm^{-1}}$). All enzyme determinations were performed in triplicate, and the average value was used to calculate enzyme activity units. One unit of enzyme activity is the amount of enzyme capable of oxidizing 1 μmol of substrate/min.

NADH−DCIP reductase activity is calculated according to the following formula:

$$\frac{\left(\frac{\Delta A}{\min_a} - \frac{\Delta A}{\min_b}\right) \times V}{\varepsilon \times d \times \vartheta} \tag{2}$$

where $\frac{\Delta A}{\min_a}$ is the change in absorbance per minute for the sample; $\frac{\Delta A}{\min_b}$ is the change in absorbance per minute for the blank; V is the total volume of the reaction mixture (mL); d is the path length (cm); and υ is the volume of enzyme solution (mL).

4.7. Dye Degradation Experiment. The residual dye concentration was obtained using a UV−vis spectrophotometer (Shimadzu UV 1800) at 579 nm, and the percentage decolorization was determined using the following formula:

$$D = \frac{C_0 - C_t}{C_0} \times 100\% \tag{3}$$

where D is the degradation of CV (%), C_0 (mg/L) is the absorbance value of the initial dye concentration, and C_t (mg/L) is the absorbance value of the final dye concentration in samples after decolorization. Samples were diluted prior to measurement ensuring that the absorbance showed values <1.0.

4.8. Characterization of Intermediates. The cocultures were inoculated at a concentration of 10% (V/V) into decolorizing medium (CV, 100 mg/L) and incubated at 30 °C for 12 h. After decolorization, the sample solution was centrifuged with 10,000*g* for 5 min at 4 °C. The supernatant was collected to extract metabolites and used for UV−vis analysis, and the bacterial cells obtained were used for FT-IR analysis. A UV−vis spectrophotometer was used to determine the CV and its degradation solution at full wavelength (200−800 nm).

After freeze-drying, the bacterial cells were further processed to form a KBr powder film, which was then analyzed by a Fourier transform infrared spectrometer for spectral analysis. The infrared spectrometer was programmed to scan the KBr powder film over the range of 400−4000 $\mathrm{cm^{-1}}$ with a resolution of 2 $\mathrm{cm^{-1}}$.

The supernatant was collected after degradation, and an equivalent volume of dichloromethane (high-performance liquid chromatography (HPLC) grade) was extracted. The extract was dehydrated with Na_2SO_4 and evaporated at 45 °C in a rotary evaporator under vacuum. The HPLC grade dichloromethane was used to dissolve the degradation product and filter through a 0.22 μm filter membrane. The samples were then analyzed using gas chromatography−mass spectrometry (Agilent) to determine the level of degradation products.

GC−MS conditions: HP-5MS gas chromatography column (30 m × 0.25 mm × 0.25 μm), injection volume 1 μL, injection port temperature 220 °C, using splitless injector (injector temperature 200 °C), helium as carrier gas at the flow rate of 1 mL/min. The column temperature was set to 50 °C (5 min); 50−140 °C (10 °C/min, retention time: 2 min); 140−240 °C (10 °C/min, retention time: 5 min).

MS conditions: ion source electron ionization (EI), ion source temperature of 250 °C, and mass scanning range 35−600 m/z.

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4.9. Phytotoxicity Evaluation. A phytotoxicity test was performed to evaluate the toxicity of CV and its degraded products. The seeds (*T. aestivum* and *V. radiata*) used in the phytotoxicity evaluation were purchased from Gansu Green Longyao Seed Industry Co., Ltd. The supernatant of the degradation solution was poured into 90 mm Petri dishes for 12 h and covered with double-layer sterile filter paper. The seeds of *T. aestivum* (30 seeds per plate) and *V. radiata* (20 seeds per plate) were placed on the filter paper and watered by 10 mL of CV solution (100 mg/L) or its degradation products solution (100 mg/L) per day. The distilled water was used as control. The experiment was conducted in triplicate for each seed, and the plates were placed in an incubator at 25 °C and 70% relative humidity with a 12:12 light−dark cycle. The germination, length of plumule, and radicle of *T. aestivum* and *V. radiata* were recorded after 5 days. The phytotoxicity was expressed in the form of equation as follows:

$$\text{germination (\%)} = \frac{\text{no.of seeds germinated}}{\text{no. of seeds sowed}} \times 100\% \quad (4)$$

4.10. Statistical Analysis. All experiments were performed in triplicate. The results obtained for each set of experiments are expressed as the mean and standard deviation. Data were analyzed by one-way analysis of variance (ANOVA) with the Tukey−Kramer multiple comparisons test. Readings were considered significant when $P \leq 0.05$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c06978.

Phylogenetic tree of strain A-3 and L-6 based on ITS and 16S rRNA sequences (Figure S1) and GC−MS spectra of CV degradation products (Figure S2) (PDF)

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Notes

The authors declare no competing financial interest.

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