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Complex Laccase-Oxidoreductase Mixture: Effective for Oxidative Decolorization and Detoxification of Dyes and Complex Effluent from Textile Mill

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Abstract Effluent discharged from textile industries, particularly the denim dyeing units containing indigo carmine/crystal violet/highly dimethyl-aminated crystal violet, is a huge threat to flora and fauna in that area. These effluents are highly toxic, mutagenic, and linked to prevalence of cancer in these areas. Since no single enzyme is capable of breaking down these different chemical moieties, this study involved production of complex oxidoreductases on thermally extracted aqueous extract of wheat bran. The extracellular filtrate was rich in several oxidoreductases, particularly laccase (88.64 U g^{-1} wheat bran) and manganese peroxidase (20.13 U g^{-1} wheat bran). Addition of 2,6-dimethyl aniline further increased the laccase production to 153.76 U g^{-1} wheat bran. The culture filtrate produced on wheat bran alone could marginally (< 20%) decolorize (except reactive orange 16 or RO16 which was not acted upon) the tested dyes at dosage of 500 U L^{-1}. Addition of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) or ABTS during the decolorization process increased decolorization to more than 70% for Kiton blue A, acid violet 17, and reactive black 5. Nearly 60% decolorization was achieved for RO16 by culture filtrate produced on induced medium in the presence of ABTS. Treatment of the combined effluent, containing derivatives of crystal violet, resulted in nearly 90% decolorization with concomitant removal of dimethyl-amino units and accumulation of *o*-dimethyl-amino dimethyl aniline (m/z 164) indicating an oxidative breakdown pathway which is reverse of the chemical synthetic route of these dyes. The phyto-detoxification and reduction of mutagenicity accompanied the degradation process.

Keywords Semi-solid cultivation · Laccase · Bioremediation · Crystal violet · Effluent treatment

1 Introduction

Textile industries are a key driver of economic growth in several Southeast Asian countries. It has emerged as a major job-creating sector and accounts for huge export earnings, thus contributing to GDP value. On the other hand, the impact of textile industries on the environment is a cause of concern and needs to be addressed on a priority basis. Of the many sectors, the effluents discharged from the denim dyeing units pose a threat to the local agricultural lands as the effluents are discharged into the irrigation canals without any treatment (https://timesofindia.indiatimes.com/city/delhi/dye-another-day/articleshow/84251466.cms).

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The dyes have also been linked to prevalence of cancer in the nearby areas. Some azo dyes are found to be toxic, carcinogenic, and mutagenic (Fernandes et al., 2015; Ayed et al., 2017). Carcinogenicity of azo dyes is due to release of benzidine which induces human and animal tumors (Chung 2016). Furthermore, defiling with other substances used during dyeing is also suspected to add to the toxicity of some dyes (Prival et al., 1984). Several azo dyes, such as reactive black 5 (RB5, a diazo dye), reactive orange 16 (RO16), reactive violet 5 (RV5), triarylmethane dyes such as Kiton blue A (KBA), and acid violet 17 (AV17), are extensively used in the dyeing industry. In addition to these, crystal violet (CV), a triarylmethane dye, and its dimethyl-amino $(CH_3)_2NH_2$-derivatives are extensively used in the denim dyeing industries due to color fastness and adsorption onto the cotton fibers. While the contribution of the dyes to textile industry is enormous, a safe way to eliminate these from the environment is a top priority.

In our previous studies (Vats and Mishra, 2017; 2018), it was demonstrated that a mixture of oxidoreductases produced by the basidiomycete *Cyathus bulleri* on wheat bran (WB) was effective in decolorizing RB5, RO16, RV5, KBA, and AV17, but the extent of decolorization was low until the strong laccase mediator 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) or ABTS was added to the reaction mixture. CV and its derivatives are derived from condensation of dimethyl aniline with phosgene (Gessner and Meyer, 2000) followed by further condensation with dimethyl aniline. CV can also be synthesized by condensation of formaldehyde or 4-dimethylamino-benzaldehyde with dimethyl aniline (US Patent EP1309662B1) followed by further condensation with dimethyl aniline and oxidation. Derivatization of CV by addition of alkyl, chloro, and dialkyl amino groups results in the formation of a variety of dyes (Gessner and Meyer, 2000) that are extensively used in the textile industry. Long-term exposure to CV showed it to be a mutagen as well as a carcinogen (Rehman et al., 2017). CV has also been reported to cause painful sensitization to light and is responsible for causing perpetual injury to the conjunctiva and cornea (Mittal and Roy 2021; Puneetha et al., 2021). Sepia shell-based composite (Elwakeel et al., 2020) as well as ball-milled hickory wood biomass (Yang et al., 2022) have been successfully used for sorption of CV as well as for treatment of complex effluents (Elwakeel et al., 2020). The major drawback here is that there is transfer of the dye from one phase to another. Processes such as Fenton and photo-Fenton oxidation have been found to be effective (Rehman et al., 2018) on CV, but like other advanced oxidation processes, are expensive to operate on large scale.

Treatment involving the use of biological agents (such as whole cells, enzymes thereof, or plants) has emerged as a very effective method eliminating both cost and accumulation of solid sludge (Bhatia et al., 2017). Laccases, peroxide-dependent enzymes, and several aryl-alcohol oxidases produced by white-rot fungi on natural lignocellulosic substrates are effective on dyes, but their effectiveness has not been demonstrated on effluents. A complex mixture of enzymes also ensures production of hydrogen peroxide (by the action of aryl-alcohol oxidases), which is essential for the activity of other ligninolytic enzymes (Hernández-Ortega et al., 2012). The high chemical oxygen demand (COD), biological oxygen demand (BOD), alkaline pH, and complexity of dyes in the textile wastewaters makes it arduous for a single enzyme system to remove color and toxicity, and hence, treatment by a mixture of enzymes, produced economically on cheaper substrate, would be highly desirable. The white-rot basidiomycete *C. bulleri* has been reported (Vats and Mishra, 2017; 2018) to produce a potent mixture of enzymes on wheat bran (WB), orange peels, and wheat straw. The culture filtrate as well as the laccase purified from culture filtrate of WB grown fungus (Vats and Mishra, 2018) was reported to be highly effective in decolorizing several dyes and effluent containing a simple triarylmethane dye. In this study, we report the effectiveness of culture filtrate, produced on two different media, viz., WB and 2,6-dimethyl aniline (DMA) containing WB extract, in decolorization of several azo, triarylmethane, and anthraquinone dyes. The culture filtrate was also evaluated for decolorization and degradation of CV containing effluent paving way for economical and environmentally friendly agents for remediation in the textile sector.

2 Materials and Methods

2.1 Chemicals, Dyes, and Textile Effluent

ABTS, 2,6- dimethoxy phenol (DMP), 2,6-dimethyl aniline (DMA), and veratryl alcohol were procured from Sigma-Aldrich Chemicals Pvt. Ltd. Five dyes, namely, reactive violet 5 (RV5), Kiton blue A (KBA), acid violet 17 (AV17), reactive black 5 (RB5), and reactive orange

16 (RO16), were obtained from Department of Textile and Fiber Engineering, IIT Delhi. Effluent was obtained from a denim dyeing industry in Ghaziabad, U.P.

2.2 Strains

White-rot basidiomycete, *Cyathus bulleri* Brodie DAOMC 195062, was used and maintained on malt extract agar (MEA) plates at 28 °C. Freshly grown fungus grown on MEA plates for 5 to 6 days was used as an inoculum.

2.3 Substrate and Culture Conditions

WB, procured from a local flour mill, was used as the solid substrate. Five grams of WB was taken in an Erlenmeyer flask and 100 mL of distilled water was added to make the substrate wet. It was followed by autoclaving for 20 min at 15 psi and 121 °C. A total of eight flasks were set for fungal growth. Laccase inducer, DMA (Eggert et al., 1996), was added to four of the flasks. Four mycelial plugs (Ø ~5 mm from freshly grown cultures) were used for inoculation. Cultures were incubated at 28 °C under static conditions (Vats and Mishra, 2017). Harvesting of the cultures was done on 12th day. Culture filtrate was sieved through a muslin cloth and further centrifuged at 8000 g for 20 min to remove any leftover mycelia. The mycelium-free culture filtrate served as the source of the oxidoreductases.

2.4 Measurement of Protein Content and Ligninolytic Enzyme Activities in the Culture Filtrate

Total protein content in the culture filtrate was determined using Bradford's reagent (BioRad). The assay was executed as per manufacturer's protocol given in the instruction manual of quick start Bradford's protein assay. Standard plot of BSA was used for measuring the protein concentration.

Biochemical assays of laccase, manganese peroxidase (MnP), lignin peroxidase (LiP), aryl alcohol oxidase (AAO), and lytic polysaccharide mono-oxygenase (LPMO) were carried out in the uninduced and the induced culture filtrates. Activity of laccase was determined by assessing the increment in ABTS* radical, which absorbs at 420 nm (ε420 36 mM^{-1} cm^{-1}) (Eggert et al., 1996). LiP assay was carried out with veratryl alcohol as a substrate (Camarero et al., 1999) with externally added H_2O_2. Enzyme activity was measured through generation of veratraldehyde which shows absorbance at 310 nm (ε310 9.3 mM^{-1} cm^{-1}). The MnP activity was determined using DMP as a substrate (Wariishi et al., 1992). Enzyme activity was determined by measuring change in absorption of DMP at 469 nm (ε469 4.9 mM^{-1} cm^{-1}). A colorimetric assay was set up to determine the activity of LPMO where DMP was used a substrate (Wang et al., 2018a). Activity of AAO was spectrophotometrically assayed using veratryl alcohol (Okamoto and Yanase, 2002). Enzyme activities are reported in U mL^{-1}, where 1 U is expressed as 1 µmol of product made in a minute under assay conditions. For the effluent (the combined effluent), COD, total dissolved solids (TDS), total soluble solids (TSS), total solids (TS), and pH were determined using standard procedures (Rice et al., 2012).

2.5 Dye Decolorization Studies

Stock solutions (1000 ppm) were prepared for RV5, KBA, AV17, RB5, and RO16 in double distilled water. Dye decolorization was carried out in 48-well plates with the mycelium-free culture filtrate from uninduced and the induced cultures at two laccase equivalent dosages viz. 100 U L^{-1} and 500 U L^{-1} of culture filtrates (at equal volumes) at dye concentration of 50 ppm. In a parallel study, the same reactions were carried out in the presence of ABTS (100 µM). The plates were shaken at 100 rpm at 25 °C. After 30 h, the O.D. of the samples was determined at the λmax of the respective dyes in a Perkin Elmer multimode plate reader. Percentage decrease in absorption maximum of various dyes (RV5—λmax 550 nm, KBA—λmax 640 nm, AV17—λmax 530 nm, RB5—λmax 590 nm, and RO16—λmax 490 nm) was used to compute percent dye decolorization (D) = $[(I - F)/I] \times 100$, where I is the initial absorbance and F is the final absorbance at the λmax of the dye.

2.6 Electron Spray Ionization-Mass Spectrometry (ESI-MS) Analysis of the Effluent

The effluent from the denim dyeing industry was treated with the culture filtrate obtained from the induced culture. A fixed volume of culture filtrate (laccase equivalent of 1000 U L^{-1}) was added to 50

mL of the combined effluent, both in absence or presence of ABTS, and the reaction incubated at room temperature (~27 °C) under shaking conditions under dark. The reaction was quenched after 12 h and 24 h in a boiling water bath and samples stored at 4 °C. The centrifuged samples were injected into an ESI-MS instrument (AB Sciex, USA), and the spectrum was observed in the positive ion mode. The operating conditions were as follows: ion spray voltage of 5000 V; nebulizer gas at 20 lb $in.^{-2}$; curtain gas at 25 lb $in.^{-2}$; declustering potential at 60 V; focusing potential at 265 V; and flow rate of 5 µL min^{-1} as described previously (Kenzom et al., 2014). The spectra were obtained in a mass range (m/z) from 100 to 1100 amu. The expected product structures were put forward with the aid of mass fragmentation tool (ChemDraw Pro 8.0), and these were then verified with the m/z values obtained from the ESI-MS study.

2.7 Phytotoxicity Studies

The toxicity of the effluent was evaluated by measuring seed germination of *Vigna radiata* in which both untreated and the treated effluent (24 h treatment) were used. The experiments were conducted in triplicates. At least 15 seeds were kept in a petri plate over a Whatman filter paper disk soaked with untreated and the treated effluents. The filter disk soaked in distilled water was used as a control. The plates were incubated undisturbed in an incubator at 28 °C for 24 h (Di Salvatore et al., 2008). Root length was measured with the help of a thread and scale. Percent toxicity was calculated as follows: {(root length control − root length test sample)/root length control} × 100.

2.8 *Salmonella* Mutagenicity Test

The tester strain *Salmonella typhimurium* TA98 was obtained from the gene bank at Institute of Microbial Technology (IMTECH), Chandigarh (India). The tester strain genotype was checked by biotin and histidine dependence, *uvr*A/B, *rfa* mutation, and R-factor plasmid pKM101 (ampicillin resistance) and was confirmed immediately after receiving the cultures. The untreated and the treated effluent were used for testing mutagenicity at two concentrations (undiluted and two times diluted). The culture was grown in 20 mL medium containing 16.2 mL water, 0.4 mL of 50 X Vogel-Bonner Medium E, 1 mL of 40% glucose, 0.2 mL of sterile histidine (2g 4000 mL^{-1} water), 0.12 mL of 0.5 mM biotin, and 0.063 mL of sterile ampicillin (8 mg mL^{-1} of 0.02 N NaOH). It was incubated for 10–12 h at 37 °C in shaking condition for enough aeration for 10^9 bacterial cells. This fresh culture (0.1 mL) was mixed with 0.2 mL of His/Bio solution and 0.1 mL of test sample and incubated at 37 °C for 90 min. A negative control was also set up in which instead of the test sample, autoclaved distilled water was added. This was mixed with 1.8 mL of molten top agar (43–48 °C) in a sterile test tube. The top agar consisted of 0.6% agar and 0.6% NaCl. This aliquot was mixed thoroughly and poured over minimal glucose agar plates. After hardening of top agar, the plates were incubated for 24–48 h at 37 °C. The spontaneous revertants and the number of revertant colonies were counted in each plate. All tests were run in duplicates.

3 Results and Discussion

3.1 Culture Conditions

Fungus harvested on the 12^{th} day showed even growth on the solid substrate. Total protein content was measured in the culture filtrates. Total protein of 2.65 mg g^{-1} and 5.5 mg g^{-1} WB was obtained in the uninduced and the induced cultures, respectively, indicating that DMA induced additional enzyme production. Laccase, MnP, LiP, AAO, and LPMO activities in the uninduced culture filtrate were (per g WB) 88.64 U, 20.13 U, 2.4 U, 7.36 U, and 33.28 U, respectively. In the induced culture, laccase activity was enhanced to 153.76 U g^{-1} WB whereas no increase was seen for MnP, LiP, AAO, and LPMO activities which were (per g WB) 21.28 U, 2.72 U, 7.84 U, and 35.36 U, respectively (Fig. 1). The different components in the WB (cellulose, hemicellulose, pectin) all induce a number of carbohydrate active enzymes as well as the lignin degrading enzymes, particularly, laccase. A detailed analysis of the transcriptome of WB grown cultures indicated (Vats and Mishra, 2021) a large repertoire of oxidoreductases that can be effective on dyes as well as complex textile effluents. Increased laccase activity in presence of DMA confirmed that it is a strong inducer of laccase as reported in several earlier studies (Salony et al., 2006; Valle et al., 2015; Wu et al., 2018). It was also concluded to augment

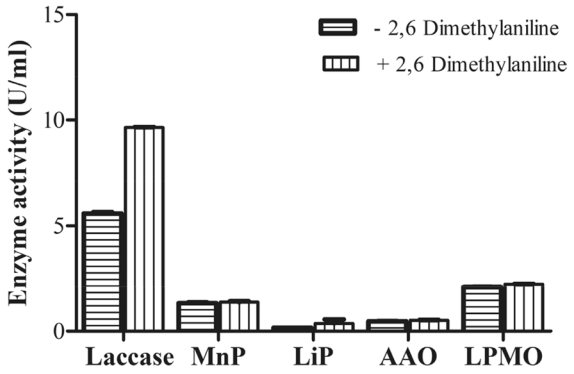

Fig. 1 A profile of different enzyme activities in the culture supernatant of *C. bulleri* grown in aqueous extract (100 mL) of wheat bran (5 gm). Activities are shown in absence or presence of 2,6-DMA

the dye degrading activity of the crude culture filtrate as laccases have been implicated to work effectively on several dyes (Bilal et al., 2019). Both the culture filtrates were evaluated for their ability to decolorize several categories of dyes.

3.2 Decolorization of Different Categories of Dyes by Culture Filtrates Obtained on Uninduced and Induced Cultures

Decolorization was carried out with culture filtrate obtained from the uninduced and the induced culture, both in absence and presence of ABTS. Supplementary Table S1 shows the percent decolorization of different dyes under different conditions. As the results indicate (Fig. 2A), when the culture filtrate from uninduced WB grown fungus was used, the decolorization of dyes was from ~4 (RV5) to ~20% (AV17) as the laccase equivalent dosage was raised to 500 U/L. In case of AV17, lower dosage was also effective as it has been previously reported that laccase of *C. bulleri*, is, in general, effective on triarylmethane dyes and decolorizes it by successive removal of methyl groups (Chhabra et al., 2009). No effect was observed at low dosage on RV5, KBA, and RO16, the latter being highly recalcitrant to enzymatic action (Przystas et al., 2012; Ledakowicz and Paździor, 2021). While bacterial and fungal biomass have been reported to be effective in removal of color of RO16 (Svobodová et al., 2007; Telke et al., 2009; Bedekar et al., 2014), the major problem with this approach is that the bacterial and the fungal culture growth is inhibited on longer incubation rendering the process ineffective. As opposed to the whole biomass treatment, the mixture of oxidoreductases (including laccases) produced on DMA-supplemented WB grown cultures, as seen in the present study, appears to be a more effective way for remediation of this dye. This enzyme formulation is highly stable when tested over a period of 30 days. Also, the culture filtrate obtained from the induced culture decolorized all the dyes to

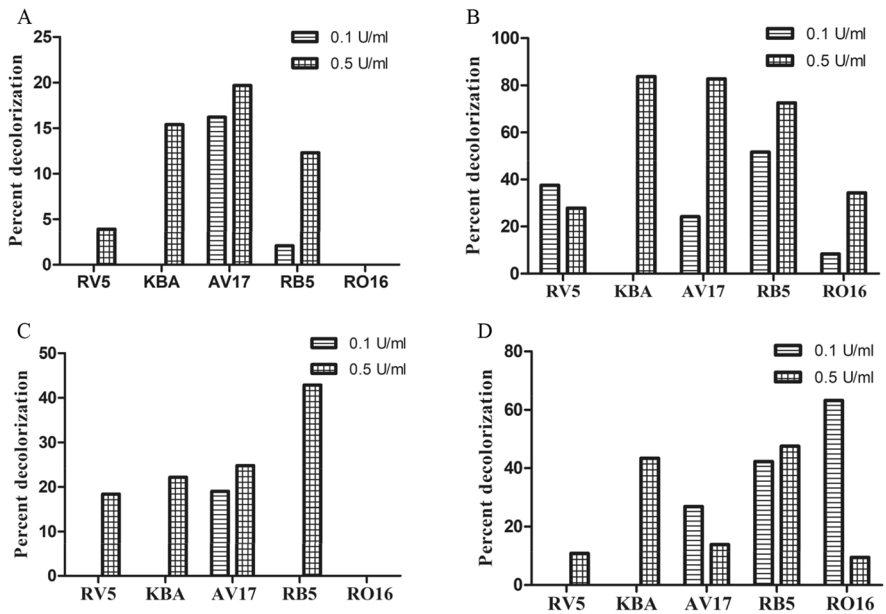

Fig. 2 Percent decolorization of dyes with the culture filtrate obtained from uninduced culture (**A** without ABTS and **B** with ABTS). **C**, **D** Percent dye decolorization with the culture filtrate obtained from induced culture (**C** without ABTS and **D** with ABTS)

a higher extent (from ~18 for RV5 to 45% for RB5) (Fig. 2C) when compared to that with the uninduced culture and this effect was attributed to higher activity of laccase. RB5 is an azo dye reported to be generally recalcitrant, and it is important to note that the crude culture filtrate can work effectively on this dye.

Regardless of cultivation conditions (whether DMA was added or not), addition of ABTS resulted in enhancement of decolorization of all dyes. For instance, in the uninduced cultures, percent decolorization of RV5 was ~4% which was increased to ~30–40% depending upon the laccase dosage. Between 70 and 90% decolorization was achieved when ABTS was added to the reaction carried out by the uninduced culture. Decolorization was reduced when ABTS was added to the culture filtrate produced on the induced medium. Thus, the results suggest that the culture filtrate produced on uninduced WB is sufficient to carry out decolorization. It was also noticed that decolorization of RO16 did not occur till ABTS was added and low dosage of laccase was effective when culture filtrate from induced culture was added. Induction of some specific laccase isoform was probably responsible under these conditions. Higher laccase equivalents under these conditions had inhibitory effect on the decolorization process.

3.3 Treatment of Complex Effluent with the Crude Culture Filtrate

The COD, TDS, TS, and TSS of the effluent were determined to be 727.2 ± 2 mg L^{-1}, 1758 ± 10 mg L^{-1}, 2742 ± 8 mg L^{-1}, and 984 ± 8 mg L^{-1}, respectively, and the pH was around 7. Based on the MS data, the dye was concluded to be CV which was derivatized with dimethyl-amino groups. The first-order mass spectrum of the untreated effluent (Fig. 3A) showed successive peaks with a difference of 44 m/z, viz. 419, 463, 507, 551, 595, 639, 683 727, 771, 815, 859, etc., which is due to addition or removal of dimethyl-amino groups. Addition of dimethyl-amino group to the aromatic rings is well known in synthetic chemistry. In fact, CV can be synthesized by several methods (Gessner and Mayer, 2000) such as (i) condensation of dimethyl aniline and phosgene to make, first, 4,4′-(Bis(dimethyl-amino) benzophenone) or Michler's ketone (an intermediate important for synthesis of several dyes and pigments) which cannot be isolated. Further condensation with various anilines and other derivatized anilines results in the formation of CV. It can also be synthesized as a one-pot reaction by condensation of dimethyl aniline with formaldehyde to form 4,4′-methylene bis (N, N-dimethyl aniline) followed by reaction with dimethyl aniline and concomitant oxidation in air in the presence of (dihydro-dibenzo-tetra aza-annulene) iron and chloranil. Several other metal compounds and nitrous gases can also be used as oxidation catalysts. CV has also been reported (US Patent EP1309662B1) to be produced by condensation of 4-dimethyl-amino-benzaldehyde with dimethyl aniline (2 mol equivalent) or from 4,4′-bis (dimethyl-amino) diphenylmethanol with dimethyl aniline (1 mol equivalent). The resulting leuco base is further oxidized. Extensive derivatization of CV, by addition of alkyl groups and diamino alkyl groups, has also been reported (US Patent EP3429900) indicating that addition of dimethyl-amino groups to the triarylmethane moiety is chemically feasible. The effluent when treated (for 24 h) with culture filtrate (equivalent to 1000 U L^{-1} of laccase) obtained from uninduced culture of *C. bulleri* showed m/z peaks characteristic of dimethyl-amino derivatives of CV with lower intensity of smaller m/z moieties (m/z 551, 507, etc.) (Fig. 3B). This indicated successive removal of dimethyl-amino groups, reverse of the synthetic steps, and points to the collective action of oxidoreductases that can bring about successive removal of the functional group of m/z 44. Increased intensity of a peak at m/z of 164.09 indicated action of enzymes leading to formation of dimethyl-amino derivative of dimethyl aniline. A major peak with m/z of 140 was also seen and concluded to be formed by removal of dimethyl groups from the product at m/z of 164.09. Since the MS was operated in positive ion mode, extra protonation of the moiety can occur (Mazumdar and Banerjee, 2012). Longer incubation (10 days) resulted in lowering the peaks at high m/z (Fig. 3D) of 727.45, 683.42, 639.40, 595.37, etc. and formation of low molecular derivatives. Increased intensity of peak at m/z of 164.09 was seen indicating accumulation of dimethyl-amino derivative of dimethyl aniline.

Incubation of the effluent with the uninduced culture filtrate for 12 h in the presence of ABTS resulted in rapid degradation of moieties of high molecular weight deceasing their relative intensities (m/z

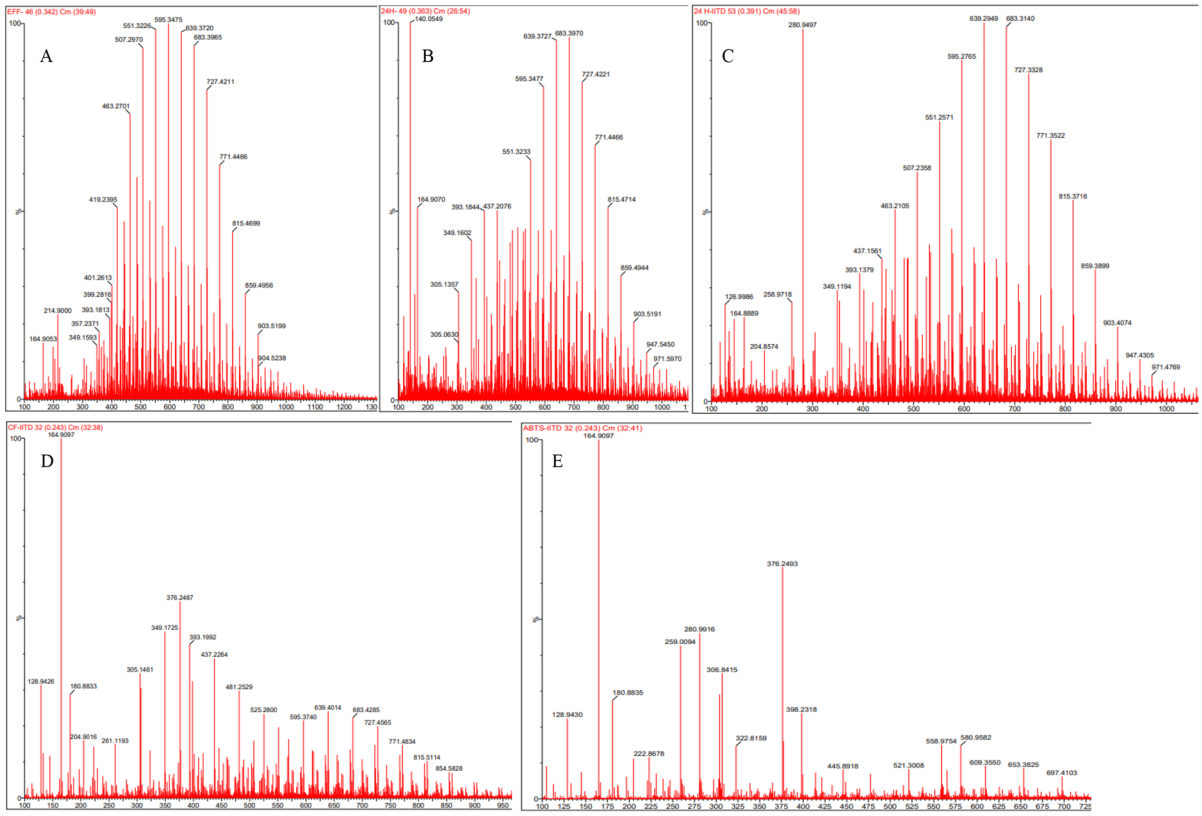

Fig. 3 First-order mass spectrum of different samples. **A** Untreated effluent, **B**. 24 h treated effluent, **C** 24 h treated effluent in presence of ABTS, **D** Effluent treated for 10 days, **E** effluent treated for 10 days in presence of ABTS

of 577.29, 533.27) with concomitant appearance of smaller products (Fig. 3C) at m/z of 280.95, 178.96, and 136.90 which were missing when the treatment was carried out in the absence of ABTS (Fig. 3B). This was attributed to the formation of carbinol form of the dye, which forms in the presence of ABTS (Vats and Mishra, 2018) and initiates rapid breakdown of the triaryl moiety. Formation of a compound with m/z of 280.95 and several others (at m/z of 164.09) through degradation of the carbinol form of derivatized CV is shown in Fig. 4. Longer incubations resulted in extensive degradation of the dye and accumulation of moieties with m/z of 164.9 and 128.94 (Fig. 3E). Although the structure of the moiety at 164.9 could be explained based on the known chemistry of laccase, the origin of the moiety at 128.94 could not be explained. It may arise due to removal of dimethyl-amino group followed by addition of protons of the moiety at 164.09. Supplementary Table S2 shows relative intensities of several peaks present in the effluent and treated sample in absence and presence of ABTS. On longer incubations, the prominent peak at 376 m/z (Fig. 3D, E), depicting protonated CV, was reduced both in the absence and in the presence of ABTS. Other peaks were reduced to very low intensities.

3.4 Proposed Pathway of Degradation of Derivatized Crystal Violet

Fig. 4 shows the proposed degradation pathway of molecular species at m/z 595 (found to be most abundant in the effluent) and m/z of 727. The formation of smaller molecules can be explained by proposing successive de-dimethylamination of the parent dye, due to action of laccase (and other oxidoreductase) present in the culture filtrate. In presence of ABTS, the major peaks observed were of m/z values of 136, 178, 280, 437, 533, etc. These arise from degradation of the carbinol form of the dye. This would result

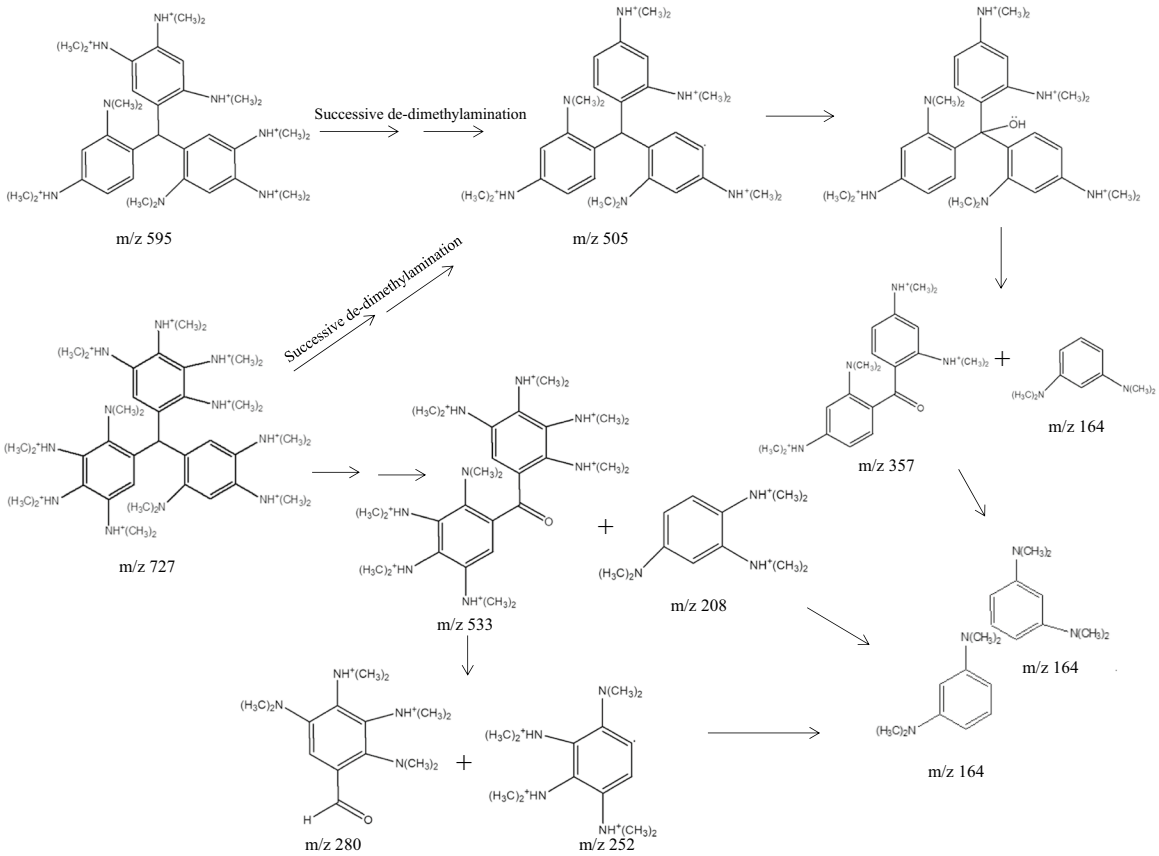

Fig. 4 Degradation pathway of derivatized crystal violet (m/z 727, m/z 595) in the effluent

in the formation of a molecule at m/z 357 and m/z of 533 which were detected in the mass spectrum. It has been proposed (Vats and Mishra, 2018) that the degradation of the triarylmethane dye proceeds via formation of carbinol form of the dye. Generally, carbinol form is formed in presence of the mediator which is further oxidized in presence of purified laccase. Since the culture filtrate contains a mixture of enzymes, including AAO, internal supply of hydrogen peroxide, and other low molecular weight mediators can occur (Wiberth et al., 2018; Pawlik et al., 2022). This can be used by the MnP and LiP. Hence, even in the absence of an external mediator, carbinol formation can occur, and further oxidation can take place explaining the occurrence of the m/z 164 peak. It is also important to note that such oxidative reactions, involving removal of dimethyl-amino groups, have been shown chemically (Paras et al., 2009), and it can be concluded that the laccases (and other oxidoreductases) present in the culture filtrate can carry out this reaction.

3.5 Phytotoxicity and Mutagenicity of the Untreated and Treated Effluent

In order to evaluate the toxicity of the dyes, the effluent, and the metabolites generated after biological treatment of the effluent, several methods involving growth of microbial (bacterial, yeast), mammalian cell lines, or germination and growth of seeds (phytotoxicity test) have been developed as they aid in detection of toxicity of intermediary products formed in biodegradation (Brusick 1987; Maila and Cloete 2005; Mazzeo et al., 2018). Since the effluent investigated in the present study was released in to irrigation canals, phytotoxicity of the untreated and the treated effluent was investigated. The results (Fig. 5) indicated that the untreated effluent was phytotoxic and reduced the germination of the *V. radiata* seeds by 30%.

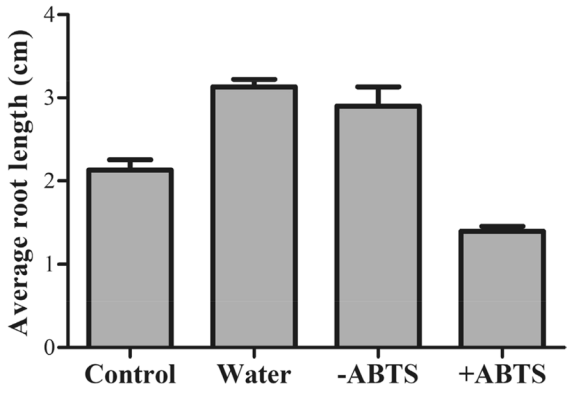

Fig. 5 Average root length (in cm) of seeds of *Vigna radiata* before and after treatment of the effluent. Control is the seed length in untreated effluent

Effluent treated with the culture filtrate obtained from WB grown fungus could reduce the phytotoxicity by more than 90%. However, the effluent treated in presence of ABTS was found to be more toxic indicating a major drawback of including synthetic mediators in the treatment of dyes and effluents. The toxicity of ABTS is widely reported in mediator-assisted laccase decolorization processes (Malarczyk et al., 2009). Apart from phytotoxicity, mutagenicity was evaluated by Ames' test and indicated the untreated effluent to be highly mutagenic based on a large number of revertants obtained. Treatment of the effluent by crude culture filtrate at a dosage of 1000 U L^{-1} (laccase equivalent) resulted in reduction in the number of revertants (10–12) indicating reduction in mutagenicity. Addition of ABTS during the treatment also supported lower number of revertants indicating that ABTS was not mutagenic.

4 Conclusions

Enzyme mixture produced by *C. bulleri* on WB was found to be potent with laccase being the predominant one among the assayed enzymes. Addition of DMA resulted in nearly 2-fold increase in laccase activity which increased dye decolorization of all tested dyes. The culture filtrate obtained from the uninduced culture was effective in treatment of effluent containing highly modified CV, indicating it to be very effective in breakdown of CV by successive removal of dimethyl-amino groups. Interestingly, the highly derivatized CV dye used in the present study is chemically synthesized using catalysts and a number of toxic starting materials such as phosgene, Michler's ketone. Removal of successive dimethyl-amino groups by the oxidoreductases also indicates the possibility of reassembling these on to a phenolic compound and thus providing an alternate route to synthesis of dyes. The treatment resulted in reduction of phytotoxicity by 90%. *Salmonella* mutagenicity tests showed that mutagenicity of the dyes and the effluent was reduced by several folds after treatment with the culture filtrate. This study also suggests that such biotransformation work and metabolite identification studies are urgently required to fully evaluate the potential of fungal crude enzyme mixtures on treatment options of effluents. While using the fungi directly may not be feasible as fungal growth is retarded in presence of complex effluents, treatment with crude culture filtrates is possible. This is the first report of treatment of complex effluent and reactions of removal of dimethyl-amino groups from triarylmethane dyes.

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Author Contribution Aakanksha Ahlawat: conceptualization, experimental work, data analysis, mass spectrometry, and first draft. Saroj Mishra: conceptualization, planning, resources, data analysis, and editing and preparation of the final draft.

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Data Availability The raw data used and/or analyzed in this study are available with the corresponding author and can be supplied on reasonable request.

Declarations

Ethics Approval and Consent to Participate Not applicable

Consent for Publication All authors have agreed to submit the paper.

Competing Interests The authors declare no competing interests.

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