Decolorization and Biotransformation of Triphenylmethane Dye, Methyl Violet, by *Aspergillus* sp. Isolated from Ladakh, India

Kumar, C. Ganesh[1]*, Poornima Mongolla[1], Anver Basha[1], Joveeta Joseph[1], V.U.M. Sarma[2], and Ahmed Kamal[1]

[1]*Chemical Biology Laboratory, and* [2]*Natural Products Laboratory, Indian Institute of Chemical Technology, Uppal Road, Hyderabad 500 607, India*

Methyl violet, used extensively in the commercial textile industry and as a biological stain, is a hazardous recalcitrant. *Aspergillus* sp. strain CB-TKL-1 isolated from a water sample from Tsumoriri Lake, Karzok, Ladakh, India, was found to completely decolorize methyl violet within 24 h when cultured under aerobic conditions at 25°C. The rate of decolorization was determined by monitoring the decrease in the absorbance maxima of the dye by UV–visible spectroscopy. The decolorization of methyl violet was optimal at pH 5.5 and 30°C when agitated at 200 rpm. Addition of glucose or arabinose (2%) as a carbon source and sodium nitrate or soyapeptone (0.2%) as a nitrogen source enhanced the decolorization ability of the culture. Furthermore, the culture exhibited a maximum decolorization rate of methyl violet after 24 h when the C:N ratio was 10. Nine *N*-demethylated decolorized products of methyl violet were identified based on UV–visible spectroscopy, Fourier transform infrared (FTIR), and LC–MS analyses. The decolorization of methyl violet at the end of 24 h generated mono-, di-, tri-, tetra-, penta-, and hexa-*N*-demethylated intermediates of pararosaniline. The variation of the relative absorption peaks in the decolorized sample indicated a linear decrease of hexa-*N*-demethylated compounds to non-*N*-demethylated pararosaniline, indicating a stepwise *N*-demethylation in the decolorization process.

Keywords: *Aspergillus*, decolorization, dyes, methyl violet, *N*-demethylation, biotransformation

A large number of chemical dyes is used in the textile industry and the effluents generated by these industries contain a significant percentage of the unutilized dye, which is a cause of concern of environmental health. The

*Corresponding author
Phone: +91-40-27193105; Fax: +91-40-27193189;
E-mail: cgkumar@iict.res.in; cgkumar1@rediffmail.com

available wastewater treatment facilities are often unable to remove commercial dyestuffs from contaminating wastewaters. Aesthetic merit, gas solubility, and water transparencies are affected by the presence of dyes, even in small amounts. Methyl violet (*N,N,N',N',N'',N''*-hexamethyl pararosaniline; MV, synonym of crystal violet) is a triphenylmethane dye, which is extensively used in the textile industries for dyeing nylon, silk, wool, and cotton; as a biological stain; and in paper printing [5]. It is used in human and veterinary medicine as an antifungal agent for dermatological infections and also in oral medication for treatment of pinworms and other tropical diseases [6]. MV has been classified as a hazardous recalcitrant and shows poor biodegradation. Conventional wastewater treatment systems are unable to effectively remove MV from the wastewaters, resulting in its dispersal into the environment [19]. Another alarming factor is that MV and some triphenylmethane dyes are reported to be potent carcinogens [10]. Many processes including chemical oxidation and reduction, physical precipitation, and flocculation, photolysis, adsorption, electrochemical treatment, advanced oxidation, and reverse osmosis have been investigated for the removal of MV from wastewater [4]. However, these technologies are costly, little adaptable to a wide range of dye wastewaters, and relatively inefficient in removal, which leads to waterways [12, 24]. The biological processes are considered promising and have attracted increased attention since they are cost-effective and environment-friendly, and produce less sludge as compared with physical and chemical treatment methods.

Research on fungal decolorization of dye wastewater has been growing intensively and different fungal genera have been investigated [12]. Very few reports exist on the biodegradation of triphenylmethane dyes by fungi, such as *Phanerochaete chrysosporium* [5], *Cyathus bulleri* [26], and *Trametes versicolor* [6], and bacteria such as *Bacillus subtilis* [28], *Enterobacter cloacae* [14], *Pseudomonas mendocina* [23], *Stenotrophomonas maltophila* [15],

Sphingomonas paucimobilis [9], and *Rhizobium radiobacter* [20]. However, owing to the diversity, concentration, and composition of the dyes and other chemicals in textile effluents, it is necessary to find suitable fungal species that are able to decolorize and degrade these dyes efficiently. In addition, the biodegradation of MV in fungal systems is not clearly elucidated. In the present study, we demonstrate a stepwise *N*-demethylation of MV by an *Aspergillus* sp. isolated from a water sample from Tsumoriri Lake, Karzok, Ladakh, India, which efficiently decolorized MV within 24 h under optimized culture conditions.

MATERIALS AND METHODS

Chemicals Used
Chemicals like Brilliant green (λ_{max} 630), Methylene blue (λ_{max} 665), Auramine O (λ_{max} 434), Rhodamine B (λ_{max} 543), Methyl violet (λ_{max} 584), Toludiene blue (λ_{max} 626), Amaranth (λ_{max} 510), and Indigo carmine (λ_{max} 610) used in this study were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. All other chemicals used were of analytical grade and of the highest purity available. The microbiological media were procured from Himedia Laboratories Pvt. Ltd., Mumbai, India.

Isolation of Dye-Decolorizing Fungi, Growth Conditions, and Storage of Fungi
One gram of the soil or water sample collected from different biosphere zones of a cold desert, Ladakh, India, was suspended in 99 ml of sterile distilled water and mixed on a vortex shaker. About 100 µl from each of these diluted samples (1:1,000) was spread plated on sterile Petri dishes containing 15–18 ml of potato dextrose agar (PDA) or Rose Bengal chloramphenicol agar (RBCA). The plates were incubated at 28°C for 4–5 days until fungal growth of the colonies was fully formed. The fungi were purified by repeated subculturing to get pure cultures and were maintained on PDA slants.

Among these cultures, the isolates that initially decolorized Rose Bengal present in the RBCA medium were further tested for their ability to decolorize MV. The screening for dye decolorization was carried out in a formulated liquid medium containing (g/l) glucose, 20; sodium nitrate, 0.6; KH_2PO_4, 1, and $MgSO_4 \cdot 7H_2O$, 5. The experiments were performed in liquid cultures under aerobic conditions of pH 5, temperature of 25°C, and agitation at 150 rpm for 4 days, after which the dye at a concentration of 10 mg/l was added and further incubated for 24 h. The medium was then filtered through Whatman filter paper No. 1 and the residual dye in the cell-free supernatant (2 ml) was extracted with an equal volume of 1-butanol. Decolorization of the dye was determined by monitoring the decrease in absorbance at the wavelength maximum for each dye by UV–visible spectroscopic analysis. Decolorization activity was calculated based on the modified method of Sani and Banerjee [22]: Decolorization (%) = [(initial absorbance)–(observed absorbance)/(initial absorbance)]×100. All decolorization experiments were performed in duplicate. The isolate, CB-TKL-1, which exhibited the strongest ability to degrade MV, was used in further experiments to assess the ability of this strain to decolorize other textile dyes, namely, Amaranth, Brilliant green, Methylene blue, Auramine O, Rhodamine B, Toludiene blue, and Indigo carmine.

Optimization of Conditions for Decolorization of Methyl Violet
Since MV was decolorized to the maximum when compared with other tested dyes, parameter optimization studies for MV decolorization with respect to pH, temperature, agitation, carbon and nitrogen sources, and dye concentration were performed in a minimal medium containing (g/l): KH_2PO_4, 1 and $MgSO_4 \cdot 7H_2O$, 5. The influence of pH and temperature on rate of decolorization was studied under pH conditions ranging from pH 3.0 to 7.0 and temperature conditions ranging from 20°C to 40°C. Agitation effect was studied under conditions of 100 to 250 rpm and also under static conditions. Various carbon sources examined included glucose, fructose, maltose, lactose, sucrose, xylose, arabinose, sorbitol, mannitol, and starch at a concentration of 2% (w/v), whereas nitrogen sources included inorganic sources like ammonium nitrate, ammonium sulfate, and sodium nitrate and organic sources like yeast extract, beef extract, malt extract, peptone, tryptone, soyapeptone, soyameal, casein, and urea at a concentration of 0.2% (w/v). Furthermore, the effects of glucose and sodium nitrate were studied individually at various concentrations ranging from 5–50 g/l and 0.5–5 g/l, respectively, when supplemented in the basal medium. The C:N ratio on the rate of decolorization was examined by addition of glucose and sodium nitrate as carbon and nitrogen sources, respectively, at the same time in the basal medium. In this regard, glucose (20 g/l) was used and the amount of sodium nitrate was varied to attain the desired C:N ratio. The dye concentrations tested were 5, 10, 15, 20, 25, and 30 mg/l. Experiments were carried out in 250-ml baffled flasks containing 100 ml of medium. After inoculation with 1% spore inoculum (concentration of 1×10^9 spores) of 5-days-grown culture, the flasks were incubated at 25°C for 5 days on a rotary shaker at 150 rpm. After fermentation, the medium was filtered through Whatman filter paper No. 1 and the cell-free supernatant (2 ml) was extracted with an equal volume of 1-butanol and used for determining the decolorization activity. All assays were done in duplicates and the presented results are average values of two independent experiments.

UV–Visible and Fourier Transform Infrared (FT–IR) Spectral Analyses
Decolorization was monitored by UV–visible spectroscopic analysis on a UV–visible spectrophotometer (JASCO V650; Tokyo, Japan), and the changes in the absorption spectrum in the wavelength range of 200–700 nm were recorded. FT–IR analysis of the decolorized products of MV and that of control dye (MV) were monitored on a Thermo-Nicolet Nexus 670 FT–IR spectrophotometer (Thermo Fisher Scientific Inc., Madison, WI, USA) using KBr pellets, and spectra were collected at a resolution of 4 cm⁻¹ in the wave number region of 400–4,000 cm⁻¹.

LC–MS Analysis
The amounts of residual MV in the culture medium at the end of the various culture periods were determined by HPLC. The intermediate metabolites of MV decolorized were separated by silica gel (100–200 mesh size) column chromatography and then analyzed on a LC–MS equipped with an ESI interface. The LC–MS analysis was performed on the Agilent 1100 series LS/MSD Trap SL (Agilent Technologies, Germany) interfaced with a diode array detector (Model G1315B, Agilent Technologies, Germany) controlled with the Agilent LC/MSD Trap software (version 4.2). The instrument was equipped with a Nova-Pak HR, 6 µm, 3.9×300 mm, C_{18} column (Waters, Milford, MA, USA). The analytes were eluted with

a mobile phase of methanol:water (1:1) at a flow rate of 1 ml/min. The column eluate was introduced into the ESI (Agilent Technologies, Germany) source of the MS operated in the positive-ion mode with fullscan spectra over the *m/z* scan range of 50–600. Interface conditions for optimal ionization were a dry gas temperature of 250°C, a high purity (99.99%) nitrogen dry gas of 9.0 l/min, nitrogen nebulizer pressure of 35.0 psi, and a dwell time of 200 ms. Data acquisition and data processing were performed with the Agilent Chemstation software (version A.09.01).

RESULTS

Isolation and Characterization of Strain CB-TKL-1
In total, 155 fungal strains were isolated from different biosphere zones of a cold desert, Ladakh, India. Among these, one promising isolate, CB-TKL-1, isolated from a water sample collected from Tsumoriri Lake, Karzok, Ladakh, India, exhibited maximum decolorization activity of MV and other tested dyes. Based on cultural and morphological characteristics of spore and hyphae by lactophenol blue staining, the strain CB-TKL-1 was identified as *Aspergillus* sp. There have been a few reports on the biodegradation of MV by some fungi such as *Phanerochaete chrysosporium* [5] and *Cyathus bulleri* [26], an actinomycete, *Nocardia corallina* [29], and bacteria such as *Bacillus subtilis* [28], *Enterobacter cloacae* [14], *Pseudomonas mendocina* [23], *Stenotrophomonas maltophila* [15], *Sphingomonas paucimobilis* [9], and *Rhizobium radiobacter* [20]. However, the present study is the first report on *Aspergillus* sp. having the ability to degrade MV. However, it has been reported that different *Aspergillus* sp. are able to degrade other dyes like Reactive blue, Acid red, Orange II, Drimarene blue, Sulfur black, and Malachite green [1, 2, 21].

Optimization of Culture Conditions for Decolorization of Methyl Violet by *Aspergillus* sp.
Preliminary experiments indicated that *Aspergillus* sp. strain CB-TKL-1 was capable of decolorizing all the dyes tested (Fig. 1) within 7 days. Among the dyes decolorized, the strain CB-TKL-1 was more effective against the *N*-alkylated triarylmethane dyes (methyl violet and brilliant green) followed by thiazin dyes (methylene blue and toludiene blue) over others. MV was degraded more than 95% after 24 h of cultivation, and thereafter only a slight change in the rate of decolorization was observed. In contrast, Auramine O was not a good dye for *Aspergillus* sp. strain CB-TKL-1, and was only degraded to about 46% after 7 days. Based on the optimization experiments, the pH conditions needed for maximum decolorization of MV by *Aspergillus* sp. strain CB-TKL-1 was observed to be a pH range of 5.0 to 6.0 with an optimum pH of 5.5 (Supplementary data; Fig. S1). However, a gradual decrease in decolorization rate was noticed at pH values of less than 5.0 and more than 6.5. Maximum decolorization of MV
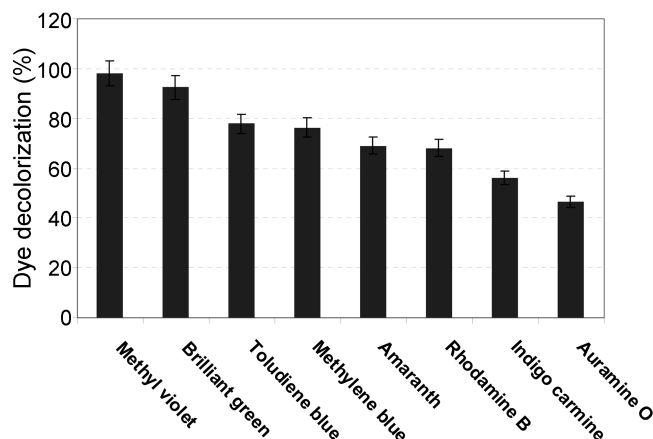

Fig. 1. Decolorization of different dyes (10 mg/l) by *Aspergillus* sp. strain CB-TKL-1.

was demonstrated at a temperature range of 25°C to 35°C and a temperature of 30°C was chosen as optimum (Supplementary data; Fig. S2). At temperatures above 35°C and below 25°C, a decrease in decolorization rate was observed. *Aspergillus* sp. strain CB-TKL-1 is strictly aerobic and the dye decolorization rate increased with shaking, and maximum decolorization occurred at 200 rpm (Supplementary data; Fig. S3). Among the carbon sources tested, glucose was observed as the most effective carbon source, followed by arabinose (Supplementary data; Fig. S4). The effect of different nitrogen sources on MV decolorization indicated that sodium nitrate was the most effective nitrogen source, followed by soyapeptone and yeast extract (Supplementary data; Fig. S5). When glucose and sodium nitrate were supplemented individually at various concentrations ranging from 5–50 g/l and 0.5–5 g/l, respectively, in the basal medium, the maximum decolorization rate of MV was observed on supplementation of 20 g/l (w/v) glucose (Supplementary data; Fig. S6) and 2 g/l (w/v) sodium nitrate (Supplementary data; Fig. S7), respectively. Moreover, the *Aspergillus* sp. strain CB-TKL-1 recorded a maximum
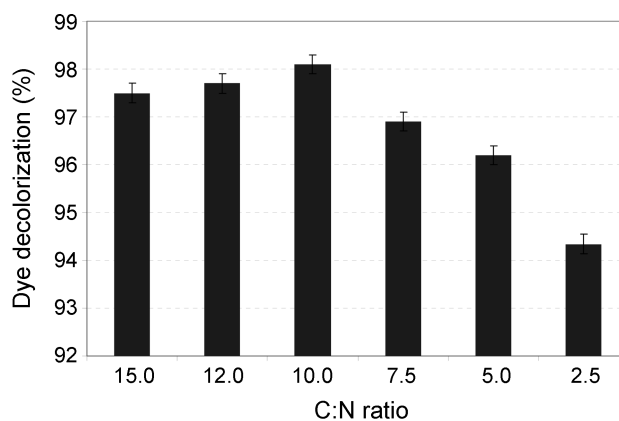
Fig. 2. Effect of C/N ratio on the decolorization of methyl violet by *Aspergillus* sp. strain CB-TKL-1.
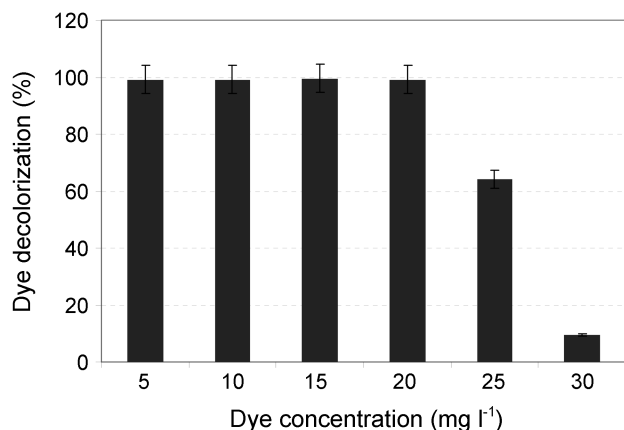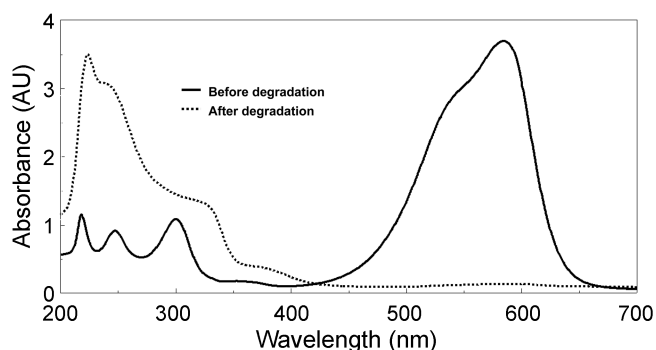
Fig. 3. Effect of initial concentration of methyl violet on the decolorization by *Aspergillus* sp. strain CB-TKL-1 in a minimal medium.
Conditions: pH-5.5; temperature=30°C; agitation=200 rpm; spore concentration =1×10^9 spores/ml.

decolorization rate of MV after 24 h when the C:N ratio was 10 (Fig. 2). The maximum decolorization rate of MV by *Aspergillus* sp. was observed with the addition of 20 mg/l of MV in the medium, whereas higher concentrations of MV inhibited the growth and decolorizing ability of *Aspergillus* sp. (Fig. 3).

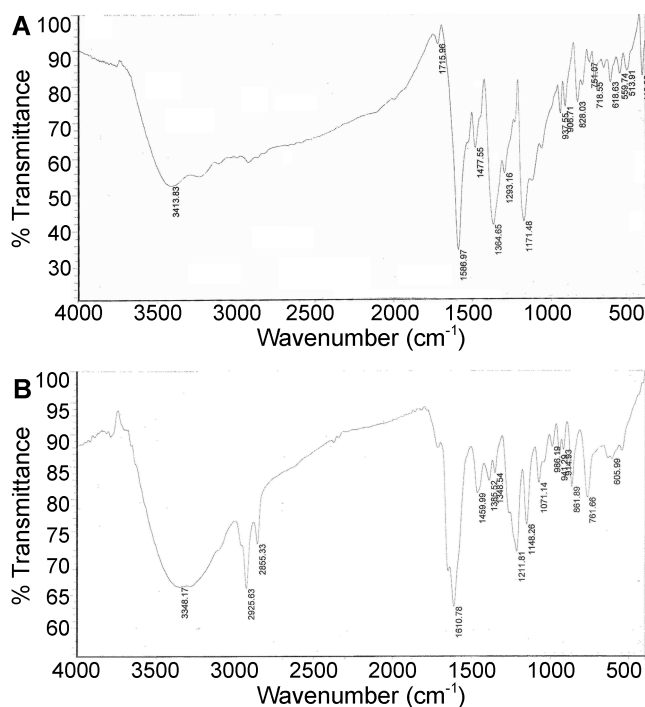**Analysis of Methyl Violet Decolorized Products**

UV–visible analysis (200–800 nm) of the supernatants of batch cultures at different time intervals indicated decolorization and a decrease in the MV dye concentration. MV exhibits a major absorption peak at λ_{max} 592 nm with three minor peaks in the range of 200–350 nm in the UV–visible spectrum. After aerobic treatment, the intensity of the major peak decreased remarkably owing to decolorization, and an above 3-fold increase in the absorption peak was detected at around λ_{max} 256 nm, which was consistently visible in the spectra for all the treated samples, which may be due to the formation of intermediates due to the aerobic decolorization of MV. The MV peak observed at λ_{max}

592 nm at 0 h decreased without any shift in the λ_{max} up to complete decolorization of the medium after 24 h (Fig. 4). In an earlier study, it was reported that dye decolorization by bacteria or fungi could plausibly be due to the adsorption of dye by the microbial cells or due to biodegradation [3]. In the case of adsorption, the UV–visible absorption peaks decrease proportionately to each other, whereas in the case of biodegradation, there is a complete disappearance of the major visible light absorption peak or a new peak appears, as observed in our study and shown in Fig. 4.

The FT–IR spectra of the control MV (Fig. 5A) and the 24 h dye decolorized sample (Fig. 5B) showed a distinct variation in the fingerprint wave number region of 1,500–500 cm^{-1}, which indicates the decolorization of MV by *Aspergillus* sp. CB-TKL-1. In the FT–IR spectrum of the control MV (Fig. 5A), specific peaks to the fingerprint wave number region of 1,500–500 cm^{-1} represents the mono-substituted and para-disubstituted benzene rings, whereas the presence of a wave number peak at 1,586 cm^{-1} corresponds to the C=C stretching of the benzene ring. Moreover, the peak at 1,171 cm^{-1} supports for the aromatic C–N stretching vibrations. The characteristic peak at 3,413 cm^{-1} signifies the presence of a free -NH$_2$ group corresponding to amine antisymmetric stretching vibrations. The peaks at 1,715 and 1,477 cm^{-1} represent the C=C bend and the OH bend, respectively. The C–O stretch corresponds to the peak at 1,171 cm^{-1}, whereas the peaks at 751 and 718 cm^{-1} represent the symmetric bending of the benzene ring

Fig. 4. UV–visible spectra of methyl violet before and after decolorization by *Aspergillus* sp. strain CB-TKL-1.

Fig. 5. FT–IR spectra of (**A**) methyl violet and (**B**) methyl violet after decolorization by *Aspergillus* sp. strain CB-TKL-1.

hydrogens. In comparison, the FT–IR spectrum of the decolorized products of the MV (Fig. 5B) showed characteristic peaks at 3,348 and 2,925 cm^{-1}, which are attributed to the stretching vibrations of the hydroxyl group and C–H asymmetric stretching, respectively. The major adsorption band at 1,610 cm^{-1} indicates the N–H bending vibrations, whereas the peaks at 1,459, 1,385, and 1,071 correspond to the sideways ring stretch, -CH$_2$ scissoring bend, and =CH$_2$ out-of-plane twist, respectively. The absorption bands below 900 cm^{-1} represents, the aromatic nature of the decolorized products. Furthermore, the absence of peaks at 718, 618, and 559 cm^{-1} suggests the loss of the ring.

LC–MS Analysis of the *N*-Demethylation Products of Decolorized Methyl Violet

The decolorized products formed by *Aspergillus* sp. CB-TKL-1 after a 24-h culture period were analyzed by LC–ESI–MS (data not shown). The ESI–MS peak 1 was identified as methyl violet and peaks 2–10 as other intermediate metabolites. The *N*-demethylated intermediates, *N*,*N*-dimethyl-*N'*,*N'*-dimethylpararosaniline (DDPR), *N*,*N*-dimethyl-*N'*-methylpararosaniline (DMPR), and *N*,*N*-dimethylpararosaniline (DPR), were found to be more polar and were eluted earlier than that of the *N*,*N*-dimethyl-*N'*-methyl-*N''*-methylpararosaniline (DMMPR), *N*-methyl-*N'*-methyl-*N''*-methylpararosaniline (MMMPR), and *N*-methyl-*N'*-methylpararosaniline (MMPR) species. Furthermore, the presence of two *N*-methyl groups indicates that they are stronger auxochromes as compared with the *N*,*N*-dimethyl or amino groups, so we envisaged that the maximal absorption of DDPR, DMPR, and DPR intermediates would occur at wavelengths shorter than the band positions of the DMMPR, MMMPR, and MMPR species. A similar phenomenon was observed in the case of photodegradation of sulforhodamine-B under visible irradiation [8, 27].
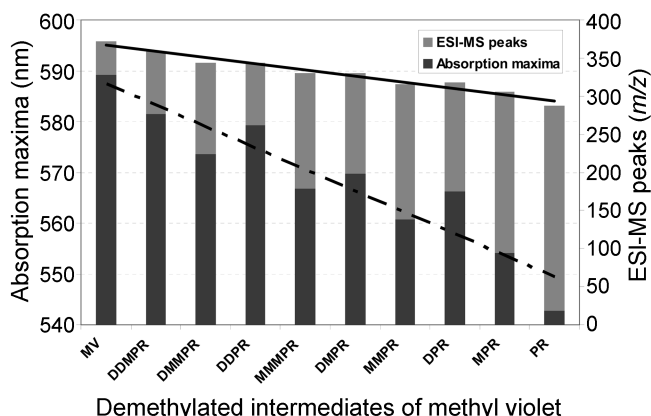
Methyl Violet Decolorization Pathway by *Aspergillus* sp. Strain CB-TKL-1

Based on a systematic identification of the primary and secondary metabolites, the metabolic pathway for MV decolorization in *Aspergillus* sp. strain CB-TKL-1 was elucidated. The MS analysis confirmed that components 1 (*m/z*=371.6), 2 (*m/z*=358.2), 3 (*m/z*=344.1), 4 (*m/z*=344.1), 5 (*m/z*=330.1), 6 (*m/z*=330.2), 7 (*m/z*=316.1), 8 (*m/z*=318.3), 9 (*m/z*=305.2), and 10 (*m/z*=287.7) represent methyl violet, DDMPR, DMMPR, DDPR, MMMPR, DMPR, MMPR, DPR, MPR, and PR, respectively (data not shown). Each of the three side chains of the MV dye molecule has two dimethyl groups (compound 1, Fig. 6). DDMPR (compound 2, Fig. 6) is obtained by the removal of one methyl group from the MV molecule; DMMPR, DDPR, MMMPR, DMPR, MMPR, and DPR (compounds 3–8, Fig. 6) correspond to three pairs of isomeric molecules with 2 to 4 fewer methyl groups than the MV dye molecule. The
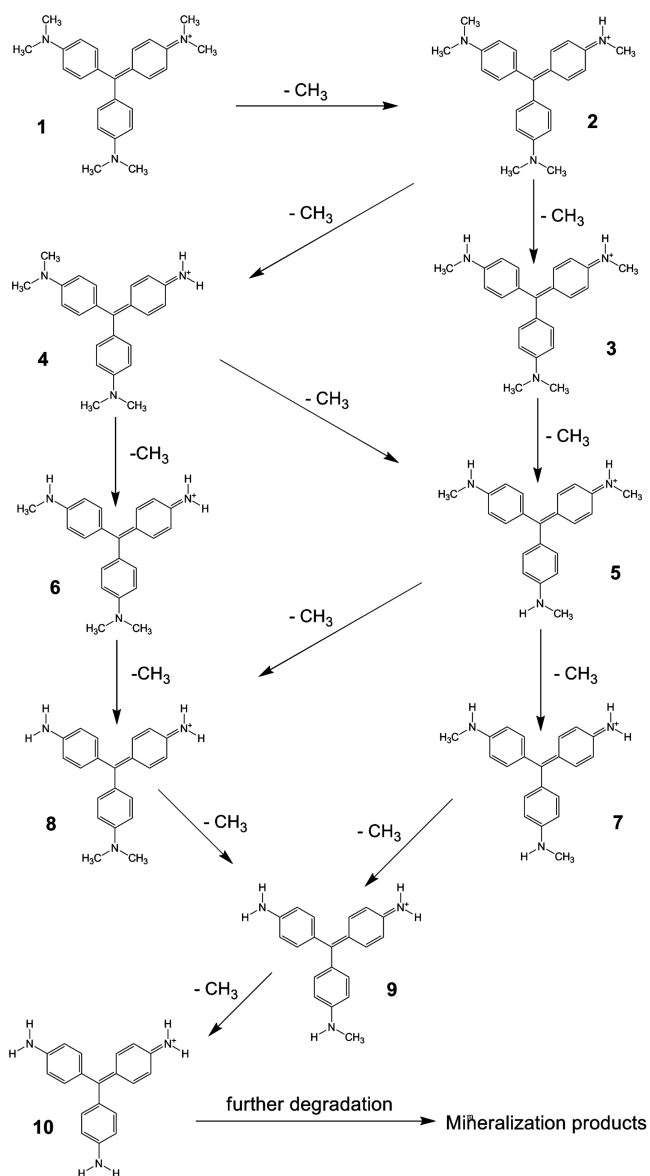
Fig. 6. Proposed *N*-demethylation pathway for decolorization of methyl violet by *Aspergillus* sp. strain CB-TKL-1, with the identification of different degradation intermediates by HPLC–ESI–MS.

removal of a methyl group from two different sides of the MV molecule results in the formation of DMMPR (compound 3, Fig. 6), whereas the other corresponding isomer in this pair, DDPR (compound 4, Fig. 6), is generated by the removal of two methyl groups from the same side of the MV structure. In the second pair of isomers, the removal of three methyl groups from each side of the MV molecule results in the formation of MMMPR (compound 5, Fig. 6), whereas DMPR (compound 6, Fig. 6) is produced by the removal of two methyl groups from one side of the MV structure and an additional methyl group is removed from the other side of the MV structure. In the third pair of

Fig. 7. The ESI−MS peaks and absorption maxima of *N*-demethylated intermediates produced by decolorization of methyl violet by *Aspergillus* sp. strain CB-TKL-1.

MV=*N,N,N′,N′,N″,N″*-hexamethylpararosaniline; DDMPR=*N,N*-dimethyl-*N′,N′*-dimethyl-*N″*-methylpararosaniline; DMMPR=*N,N*-dimethyl-*N′*-methyl-*N″*-methylpararosaniline; DDPR=*N,N*-dimethyl-*N′,N′*-dimethylpararosaniline; MMMPR=*N*-methyl-*N′*-methyl-*N″*-methylpararosaniline; DMPR=*N,N*-dimethyl-*N′*-methylpararosaniline; MMPR=*N*-methyl-*N′*-methylpararosaniline; DPR=*N,N*-dimethylpararosaniline; MPR=*N*-methylpararosaniline; PR= pararosaniline.

isomers, the removal of two methyl groups from one side of the MV structure and one methyl group from each of the other two sides of the MV structure produces MMPR (compound 7, Fig. 6), whereas DPR (compound 8, Fig. 6) is formed through the removal of two methyl groups from two different sides of the MV molecule. Compounds 9 (MPR) and 10 (PR) have one and zero methyl groups, respectively. Based on these results, the proposed decolorization pathway is shown in Fig. 6. The summary of results obtained from the LC−ESI−MS spectra is given in Fig. 7, which clearly suggests the stepwise decolorization of MV through *N*-demethylation.

DISCUSSION

Earlier studies on MV biodegradation demonstrated that this triphenylmethane dye is relatively resistant to microbial biodegradation in different environments and in wastewater treatment facilities. The inability of many bacteria to degrade MV has been attributed to the fact that this dye is very toxic to many microorganisms [17, 18]. Our results suggest that MV is not toxic to *Aspergillus* sp. strain CB-TKL-1 at 20 mg/l and that this fungus is able to decolorize MV. Michaels and Lewis [18] showed that among field-collected samples, between 6% and 28% of the bacterial colonies isolated from various environmental sources possessed the ability to decolorize MV. However, these authors stated that transformation of MV by these bacteria appeared to be slow when compared with the rate of transformation of other dyes. They also stated that none of

the triphenylmethane dyes in their study were degraded to undetectable levels. Biodegradation of triarylmethane dyes by fungi has received less attention than that accorded to biodegradation by bacteria. However, Kwasniewska [16] showed that oxidative red yeasts like *Rhodotorula* sp. and *Rhodotorula rubra* readily degraded MV to undetectable levels. In these studies, neither H_2O_2 nor ascorbic acid decolorized MV.

In the present study, the decolorization activity of *Aspergillus* sp. strain CB-TKL-1 was significantly enhanced by the addition of 0.2% sodium nitrate. These results corroborate the observations of Jain *et al.* [13], who found that without a nitrogen source in the culture medium, only 35% decolorization was found after four days of incubation, but on addition of 0.5% nitrogen source, the organism showed some amount of dye decolorization. Addition of glucose had a positive effect on the efficiency of *Aspergillus* sp. in the present study and was critical to initiate the fungal growth and the decolorization process [25]. Since pH was found to be one of the critical environmental factors to affect MV biodegradation [18], we examined the effect of pH on biodegradation of this dye. The degradation occurred only in slightly acidic conditions; under our experimental conditions, the optimum pH for MV decolorization by *Aspergillus* sp. was found to be 5.5 and the decolorization was inhibited at a pH higher than 6.5. In the present study, *Aspergillus* sp. strain CB-TKL-1 decolorized the dye up to 20 mg/l of MV, beyond which the fungal growth was inhibited and the rate decreased. El-Naggar *et al.* [11] earlier reported that maximum decolorization of MV in an air bubble bioreactor by a *Pseudomonas aeruginosa* was at a concentration of 15 mg/l. Beyond this concentration, the decolorization was reported to be reduced drastically.

Bumpus and Brock [5] demonstrated that *Phanerochaete chrysosporium* degraded MV, and they identified only three degradation products (*viz.*, *N,N,N′,N′,N″*-penta-, *N,N,N′,N″*-tetra-, and *N,N′,N″*-tri-methylpararosaniline) when cultured under nutrient nitrogen-limiting conditions. Our study clearly indicates that the decolorization of MV by *Aspergillus* sp. CB-TKL-1 occurs in a stepwise pattern, and *N*-demethylation appears to be the dominating mechanism in decolorization. Cha *et al.* [7] earlier reported that the cytochrome P450 system is involved in *N*-demethylation of the triphenylmethane dye malachite green to leucomalachite green and also converted the *N*-demethylated products to *N*-oxidized metabolites. Furthermore, the decolorization pathway of MV by *Aspergillus* sp. CB-TKL-1 was different when compared with that of *Bacillus subtilis* and *Nocardia corallina*, whose major biodegradation products were 4,4′-*bis*-dimethylamine benzophenone (Michler's ketone) and α-dimethylaminophenol, although no additional metabolites were reported [28, 29].

To summarize, we have demonstrated that *Aspergillus* sp. strain CB-TKL-1 is capable of decolorizing MV used

in the dyeing industry to the extent of 20 mg/l, and the process appears to be a successive *N*-demethylation of the *N*-methylated triphenylmethane dye. A proposed pathway of the metabolite formation is given in Fig. 6. Further examination of the process is needed to identify the interacting molecules, if any, involved in the process. Our paper presents for the first time data on the decolorization and *N*-demethylation of MV by *Aspergillus* sp., and the proposal of a *N*-demethylation pathway for MV is also suggested. Further studies are in progress.

Acknowledgments

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