ORIGINAL PAPER

Effectual decolorization and detoxification of triphenylmethane dye malachite green (MG) by *Pseudomonas aeruginosa* NCIM 2074 and its enzyme system

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Abstract Malachite green (MG) a complex and resonance-stabilized triphenylmethane (TPM) textile dye, resistant to transformation, was decolorized using *Pseudomonas aeruginosa* NCIM 2074. The bacteria decolorized MG (50 mg l^{-1}) completely within 5 h into simple metabolic intermediates in aerobic condition at pH 7 and temperature $35 \pm 3°C$ with 53.23% of the COD reduction. Induction in the activities of MG reductase, laccase, and aminopyrine *N*-demethylase were observed during MG decolorization suggesting these enzymes were involved in the decolorization process. The products after decolorization were examined by UV–Vis, IR spectroscopy, TLC, and HPLC. MG was enzymatically reduced to leucomalachite green (LMG), and further sequential enzymatic reaction converted LMG into *N*-demethylated and *N*-oxidized metabolites, including primary and secondary arylamines. The final product formed in this pathway was benzophenone characterized using GC-mass spectroscopy. The cytotoxicity and phytotoxicity study revealed the transformation of MG into non-toxic product by *P. aeruginosa* NCIM 2074.

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Introduction

The dyestuff usage has been increasing day by day because of tremendous increase of industrialization and man's urge for color (Daneshvar et al. 2007) Since 1856, over 105 different dyes have been produced worldwide with an annual production of over 7×10^5 metric tons (Ali 2010). This rapid industrialization results in the discharge of large amount of waste to the environment, which in turn creates more pollution, affecting photosynthetic activity in aquatic life by reducing light penetration and may also be toxic to organisms due to the presence of aromatics, metals, chlorides, etc. (Kalyani et al. 2009). To depollute the dye wastewater, various methods including adsorption, chemical precipitation and flocculation, photolysis, chemical oxidation and reduction, electrochemical treatment, and ion pair extraction have been used (Wang et al. 2009). All these chemical or physical–chemical methods possess significant differences in color removal results, volume capability, operating speeds, and capital costs (Forgacs et al. 2004). As available alternative, biological processes have received increasing interest owing to their low cost, effectiveness, ability to produce low sludge, and environmental benignity (Jadhav et al. 2010). Biological processes have the potential to convert or degrade the pollutant as water, carbon dioxide, and various salts of inorganic nature (Dhanve et al. 2009). The isolation of potent species and thereby degradation is one of the interests in the

biological aspect of effluents treatment (Jadhav et al. 2010). Recently, a number of studies have focused on biodegradation of dye in wastewaters. A wide variety of microorganisms (bacteria, fungi, algae, and yeast) are reported to be capable of decolorizing a wide range of dyes (Forgacs et al. 2004).

The triphenylmethane dye malachite green (MG) is abundantly available dye widely used in the textile industry for coloring nylon, wool, silk, leather, and cotton (Murugesan et al. 2009). It is also used widely as the most efficacious antifungal agent in the fish-farming industry (Daneshvar et al. 2007). Though the use of this dye has been banned in several countries and is not approved by the US Food and Drug Administration, it is still being used in many parts of the world because of its low cost, ready availability, and efficacy (Chen et al. 2009). The solution containing MG discharged into receiving streams will affect the aquatic life causing detrimental effects in liver, gill, kidney, intestine, and gonads (Chen et al. 2010). Similarly, MG is highly toxic to mammalian cells; it promotes hepatic tumor formation in rodents and also causes reproductive abnormalities in rabbits (Fernandes et al. 1991; Rao 1995). Several MG-decolorizing microorganisms have been reported from microalgae (Daneshvar et al. 2007; Tsai and Chen 2010), yeast (Jadhav and Govindwar 2006), fungus (Eichlerova et al. 2006), and bacteria (Sani and Banerjee 1999; Deng et al. 2008). The biochemical mechanism underlying the decolorization of MG has been elucidated in fungi (Cha et al. 2001), but not in bacteria. Recently, Chen et al. (2010) have evaluated MG decolorization and primary biodegradation pathway by *Pandoraea pulmonicola* YC32 using a batch and continuous system. Biochemical studies of the decolorization process indicate that laccase (Murugesan et al. 2009), lignin peroxidase (Papinutti and Forchiassin 2004) from fungi, and reductase (TMR) from bacteria are involved in the enzymatic decolorization of the MG (Jang et al. 2005; Gomare et al. 2009). In the genus *Pseudomonas*, *P. aeruginosa* has been shown to decolorize MG (Lin et al. 2004), but the enzymes associated with the decolorization or degradation of MG have not yet been investigated.

There are few studies in the literature on the evaluation of the hazards of textile dyes using plants bioassays. Plant bioassays techniques are more sensitive and simpler than other methods used to detect the cytotoxic and genotoxic environmental pollutants (Kalyani et al. 2009; Jadhav et al. 2010). This technique is advantageous because plant systems are less expensive and less time consuming than mammalian systems, with similar chromosomal morphology toward mammals, showing similar response to mutagens (Radic et al. 2010). The phytotoxic or genotoxic effects produced by untreated dyeing effluents are the result of a combination of several factors, rather than one.

These factors include the presence of carcinogenic amines, toxic heavy metals, pentachlorophenol, chlorine bleaching, halogen carriers, free formaldehyde, biocides, fire retardants, and softeners. All these factors have been shown to have inhibitory effects (Moawad et al. 2003). The evaluation of textile effluent toxicity by chemical characterization and biological testing is therefore extremely important for screening the suitability of untreated and treated dyeing effluents for land application.

The purpose of this study was to investigate various enzymes involved in the biological MG degradation pathway and metabolites formed after degradation. In addition, phytotoxicity and cytotoxicity of the treated and nontreated MG were assessed.

Materials and methods

Microorganism and culture condition

Pseudomonas aeruginosa NCIM 2074 was obtained from the National Centre for Industrial Microorganism (NCIM), Pune, India and was maintained on nutrient agar slants at 4°C. Subcultures were routinely made every 15 days. This strain was aerobically (120 rpm) grown at 35°C for 24 h in NB medium (beef extract 0.1%, yeast extract 0.2%, peptone 0.5%, and NaCl 0.5% at 30°C, pH 7.0).

Dyes and chemicals

Malachite green was obtained from Sd Fine Chemicals Limited (Biosar, India). 2,2′-Azinobis (3-ethylbezthiazoline-6-sulfonate) ABTS, NADH, and aminopyrine were obtained from Sigma-Aldrich, USA. All the chemicals used were of the highest purity available and of the analytic grade.

Effect of physicochemical parameter

Decolorization of MG by *P. aeruginosa* NCIM 2074 was studied at various pH values (4-10) and temperatures f(5-45°C).

Decolorization experiments

A loopful of microbial culture (*P. aeruginosa* NCIM 2074) was cultivated in aerobic condition (120 rpm) for 24 h at 35°C in 250 ml Erlenmeyer flask containing 100 ml media. Decolorization experiments were performed by the addition of MG 50 mg l^{-1} to 24-h-old cultures of *P. aeruginosa* NCIM 2074. Aliquots of 3 ml was withdrawn at different time intervals, centrifuged at 7,000 rpm for 15 min to separate the bacterial cell mass. Decolorization

of the dye was analyzed using UV–Vis spectrophotometer (Hitachi U 2800, Tokyo, Japan) at 618 nm. The chemical oxygen demand (COD) was determined by the open reflux/ titrimetric method (Kalyani et al. 2009). All decolorization experiments were performed in three sets, and the decolorization activity is expressed in terms of the percentage decolorization as follows:

$$\text{Decolorization } (\%) = \frac{(Ai) - (At)}{(Ai)} \times 100$$

where A_i was the initial absorbance, and A_t is the absorbance at incubation time t.

Bacterial growth was measured by estimating the intracellular protein content. Cell pellet was boiled in 1 M NaOH for 15 min and protein content measured by Lowry method (Lowry et al. 1951). The relation between protein concentration and OD_{620} was 1.0 $OD_{620} = 425$ mg l^{-1}.

To study the effect of media composition on decolorization of MG, minimal salt medium having composition (g l^{-1}): NH$_4$Cl: 0.50, MgSO$_4$·7H$_2$O: 0.20, KH$_2$PO$_4$: 0.40, and FeSO$_4$·2H$_2$O: 0.10 along with different carbon/nitrogen sources such as, glucose (1%), starch (1%), sucrose (1%), yeast extract, peptone (2%), and combination of yeast extract with different sugars of above mentioned concentration was used.

Preparation of cell free extract

The bacterial cells grown in the nutrient broth at 30°C for 24 h, considered as control, were centrifuged at 7,000 rpm for 20 min. These cells (75 mg ml^{-1}) were suspended in a potassium phosphate buffer (50 mM, pH 7.4) and sonicated (Sonics-vibracell ultrasonic processor, Newtown, USA) keeping sonifier output at 50 amp and giving seven strokes each of 30 s, with 3-min interval at 4°C. The homogenate was centrifuged at 8,000 rpm for 20 min, and supernatant was used as a source of crude enzyme. Similar procedure was used to quantify enzyme activities during the dye decolorization at different time intervals (1, 3 and 5 h).

Enzyme assays

The laccase and MG reductase assays were performed as reported earlier (Gomare et al. 2009). Enzyme activities were calculated using extinction coefficient of oxidized ABTS (3.6×10^4 M^{-1} cm^{-1}) at 420 nm and of MG (148,900 M^{-1} cm^{-1}) at 618 nm. Aminopyrine N-demethylase activity was determined by measuring formaldehyde, liberated using Nash reagent (Kalyani et al. 2008). All the assays were carried out at room temperature using UV–Vis spectrophotometer (Hitachi U-2800, Japan).

Analytical procedure

The decolorization was monitored using UV–Vis spectroscopy analysis (Hitachi U 2800, Japan). After decolorization, bacterial biomass was removed by centrifugation, and supernatant was extracted with ethyl acetate (five times, each time with 100 ml). The ethyl acetate extracts were then dried over anhydrous Na$_2$SO$_4$ and evaporated in vacuo. The dried sample was dissolved in 10 ml of HPLC grade methanol for analyses by FTIR, HPLC, and GC-mass spectroscopy.

Degradation products were also analyzed by thin-layer chromatography (TLC) with silica gel 60 F254 (Merck, Mumbai, India) developed using methanol: acetic acid: water (10:2:4 v/v/v). Reverse-phase HPLC was performed with Waters 2690 series component system equipped with a UV–Vis detector. Samples were resolved on a RP-C18 guard column (4.6×250 mm; particle size, 5 mm) to detect MG and its biodegraded metabolites. The metabolites were eluted at a flow rate of 1.0 ml min^{-1} with a isocratic solvent system (solvent A, 50 mM ammonium acetate, pH 2.5, 40%; solvent B, methanol, 60%) for 10 min and detection wavelengths of 618 and 366 nm.

FTIR analysis of biodegraded MG was carried out using Perkin Elmer 783 Spectrophotometer using control dye. The FTIR analysis was done in the mid IR region of 400–4,000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr. Pellets formed were fixed in sample holder, and the analysis was carried out.

The identification of metabolites formed during decolorization was carried using an Agilent 5973 gas chromatography coupled with mass spectroscopy. The gas chromatograph was equipped with HP-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 μm). The GC conditions were as follows: initially, the oven temperature was maintained at 60°C for 2 min, and then it was increased to 280°C at a rate of 10°C min^{-1}. The helium carrier gas flow rate was 1.0 ml min^{-1}. The temperature of the MS transfer line was 280°C; ionization was performed by electron ionization at 70 eV. The identification of biodegraded metabolites were achieved by comparing the retention time of authentic sample (benzophenone) and the mass spectra of the samples with standard library (NIST 147).

Toxicity study

Cytotoxicity

For this study, 100, 300, and 500 ppm concentrations of the MG dye and decolorized metabolites (extracted after 5 h decolorization) were used. Healthy onion bulbs of *Allium cepa* were purchased from a local market. The roots were grown in tap water under laboratory conditions. When the

radical roots had protruded beyond the basal plate by at least 2 cm, the roots were then treated with different concentrations of dye and extracted metabolites for 72 h. Only healthy and newly emerged roots were selected for uniformity in size and shape and use for the test solution. For cytogenetic analysis, the *A. cepa* root tips were cut and subsequently fixed in a freshly prepared mixture of absolute ethanol and acetic acid (3:1, v/v). To remove the fixative solution, the fixed root tips were washed in distilled water three times and transferred to 70% alcohol and stored in refrigerator until use. The root tips were macerated in a solution of 1 N HCl at 60°C followed by three baths of 2 min in distilled water. Excess water was removed by filter paper and root tips were stained with (2%) propano-orcein. After staining, roots were rinsed with distilled water until complete removal of the excess reagent. Slides were prepared with a drop of acetic acid (45%), and later covered with cover slips, and analyzed under the light microscope. Cytogenetic analysis consisted of the mitotic index (MI), the proportion of mitotic phases, and scoring of aberrant cells. Mitotic index was calculated as the percent ratio of dividing cells and total number of scored cells. The percentage of each type of aberrant cell, such as c-mitosis, laggards, chromosome breaks, anaphase bridges, stickiness, and micronuclei was calculated according to previously described methods (Carita and Marin-Morales 2008; Jadhav et al. 2010).

Phytotoxicity

The phytotoxicity study was carried out to assess the toxicity of MG. The obtained final product was dissolved in the water to form a concentration of 500 ppm. The study was carried out (at room temperature) using *Vigna sinesis* and *Vigna aconitifolia* (10 seeds of each) by adding separately 10-ml sample of MG (500 ppm) and its degradation products (500 ppm) per day. Control set was carried out using water at the same time. Germination (%) and length of plumule and radical was recorded after 7 days.
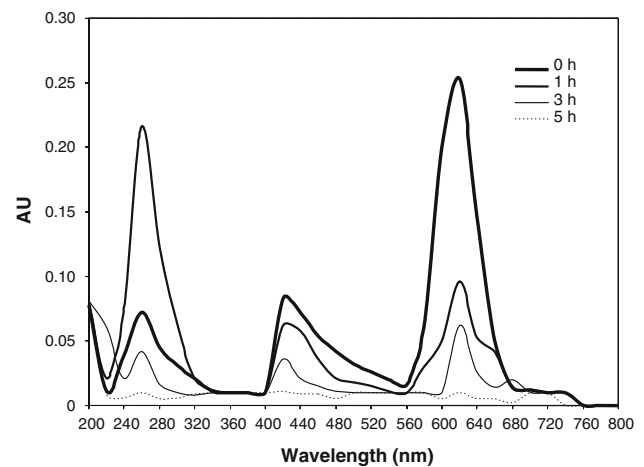
Statistical analysis

Data were analyzed by One-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test.

Results and discussion

### Characteristics of microbial decolorization and COD reduction

The biodegradation of the MG dye was monitored by UV–Vis analysis. Figure 1 illustrates the typical UV–Vis

Fig. 1 UV–Vis spectra of MG (50 mg l^{-1}) biodegraded by scans *P. aeruginosa* NCIM 2074 containing 50 mg l^{-1} at different time period. pH 7; temperature 35°C

spectra of the control MG (0 h) and biodegraded sample at various time periods (1, 3, and 5 h). The peaks observed (264, 428, and 618 nm) at initial time (0 h) decreased without any shift in λ_{max} up to complete decolorization of medium. According to (Asad et al. 2007), decolorization of dyes by bacteria could be due to adsorption by microbial cells or to biodegradation. In the case of adsorption, the UV–Vis absorption peaks decrease approximately in proportion to each other, whereas in biodegradation, either the major visible light absorbance peak disappears completely, or a new peak appears. The spectrum of MG in visible region exhibits a main peak with maximum absorbance at 618 nm, and it disappears completely after 5 h. To ensure that the decrease in absorbance was due to biodegradation and not caused by pH change or physical adsorption, we determined the effect of pH over the range pH 4–10. The absorbance of MG was not affected by pH over the range tested. The study of live versus inactivated cells proved that only live bacterial cells were able to decolorize the dye where as inactivated cells were unable to do so. Consequently, according to the above results, the color removal by *P. aeruginosa* NCIM 2074 strain largely attributed to biodegradation, and was not due to physical change or adsorption.

To investigate the effect of pH on dye decolorization, the initial pH of the medium was adjusted in the range from 4.0 to 10.0. The result revealed that the *P. aeruginosa* NCIM 2074 was capable of decolorizing MG over a pH range of 4.0–10.0. Dye decolorization was increased with increasing pH concentration. The maximum decolorization of about 100% was observed at pH 7.0, above which the percent decolorization rate was slightly decreased. The previous report shows that pH between 6.0 and 8.0 was optimum for decolorization of TPM and azo dyes by *Pseudomonas* sp. (Mali et al. 2000). The strain decolorized
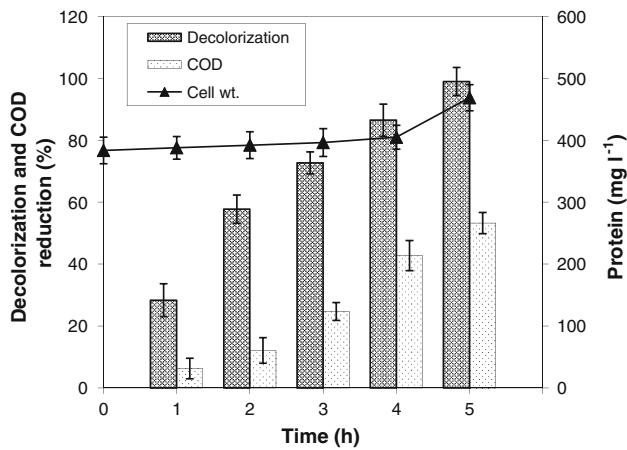
Fig. 2 Reduction of COD and color with time by *P. aeruginosa* NCIM 2074. pH 7; temperature 35°C

the MG dye in a wide range of pH, a desirable characteristic, i.e., in contrast with common decolorizing bacteria that have a narrow pH range (Moosvi et al. 2005).

The maximum decolorization (100%) of MG by bacterium *P. aeruginosa* NCIM 2074 was observed at 35°C. The percent decolorization rate was decreased with decrease in temperature to 25°C 48% decolorization was observed. The strain showed decolorizing ability of about 65% when temperature increased to 45°C but the percent decolorization reduced when temperature was further increased to 50°C. The similar result was observed for mixed bacterial culture JW-2 (Moosvi et al. 2007). The ability of this strain to decolorize the MG dye under a broad range of pH and temperature suggested that this strain could be useful in practical dyeing of textile wastewater. Figure 2 shows the percentage removal of COD with percent decolorization and cell growth. The observed decolorization was very fast, i.e., 100% and the COD removal (53.23%) within 5 h at given concentration.

Effect of medium composition

The preliminary studies revealed that the dye could not be used as the sole carbon or nitrogen source by the organism. Dyes are deficient in carbon content and biodegradation without any extra carbon source is found to be very difficult (Moosvi et al. 2007). The effects of different co-substrates were evaluated for MG decolorization at 50 mg l^{-1} concentration. Simple substrates like glucose, starch, acetate, ethanol, and more complex ones, such as yeast extract and peptone have been used for dye decolorization under methanogenic conditions (Rajaguru et al. 2000; Singh et al. 2007). In this study, maximum decolorization (91.71%, with cell growth 478 mg l^{-1}) and minimum decolorization (5%, with cell growth 109 mg l^{-1}) was observed in yeast extract and MSM medium, respectively (Table 1).

Table 1 Decolorization of MG (50 mg l^{-1}) by *P. aeruginosa* NCIM 2074 grown in minimal salt media (MSM) supplemented with co-substrates at 37°C under shaking condition

S. no.	Carbon and nitrogen	Decolorization (%)	Growth (protein mg l^{-1})
1	MSM	05.24 ± 1.75	109 ± 3.54
2	MSM + glucose	15.31 ± 1.89	286 ± 4.89
3	MSM + starch	29.72 ± 2.03	226 ± 8.43
4	MSM + sucrose	35.30 ± 1.76	258 ± 6.12
5	MSM + yeast extract	91.71 ± 2.65	478 ± 11.86
6	MSM + peptone	27.1 ± 1.56	412 ± 11.24
7	MSM + NH$_4$Cl	22 ± 1.56	176 ± 3.73
8	MSM + urea	52 ± 2.62	216 ± 4.23
9	MSM + glucose + yeast extract	63.06 ± 2.65	342 ± 7.23
10	MSM + starch + yeast extract	32.23 ± 2.11	368 ± 8.15
11	MSM + sucrose + yeast extract	36.93 ± 1.91	402 ± 10.25

According to reports, YE have been shown to be the preferred co-substrate for decolorization of dyes (Joshi et al. 2008; Telke et al. 2008). The metabolism of YE is considered essential to the regeneration of NADH that act as the electron donor for various bond reductions in dye (Telke et al. 2008). In this study, starch, glucose, and peptone were found to be poor co-substrates as compared to YE. It has been reported that, decolorization of many azo dyes by *Pseudomonas luteola* was inhibited by in the presence of the glucose (Chang et al. 2001; Bor-Yann 2002). In order to check the influence of other co-substrate on decolorization pattern of MG, the yeast extract in MSM was supplemented in combination of different sugar (Table 1).

Identification of enzyme activities possibly involved in MG degradation

The many oxidative and reductive enzymes are involved in the degradation of various textile dyes. The involvement of various oxidase, reductase, and demethylase enzymes in decolorization of textile dyes have been reported earlier (Parshetti et al. 2010; Patil et al. 2010). It is almost impossible to assay the activities of all the relevant enzymes. Instead, we decided to concentrate on the most important enzyme which would control the degradation of MG. The laccase, MG reductase, and aminopyrine N-demethylase enzyme activities were recorded during the time course of MG degradation (Table 2). Induction in the laccase, reductase, and aminopyrine N-demethylase were observed during the time course of MG decolorization under the specified conditions.

Table 2 Intracellular enzyme activities in of *P. aeruginosa* NCIM 2074 cells in induced state (at 1, 3, and 5 h during decolorization) compared to cells in control

Enzyme	Control	1 h	3 h	5 h
Laccase[a]	0.834 ± 0.01	1.84 ± 0.04***	1.12 ± 0.03***	0.956 ± 0.01
MG-reductase[a]	7.34 ± 0.18	10.85 ± 0.27***	18.57 ± 0.32***	8.27 ± 0.21
Aminopyrine *N*-demethylase[b]	1.14 ± 0.44	3.21 ± 0.54*	4.12 ± 0.94**	6.12 ± 0.94***

Values are mean of three experiment $\pm$ SD significantly different from the control at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, by ANOVA with Tukey–Kramer comparison test

[a] Enzyme activity—U mg of protein^{-1} min^{-1}

[b] nmol of formaldehyde released mg of protein^{-1} min^{-1}

Biodecolorization and biodegradation analysis

Analysis of the MG biodegradation was done using TLC, HPLC, FTIR, and GCMS. In TLC, four bands with R_f values 0.91, 0.86, 0.81, and 0.36 were obtained in the degraded MG medium, while only a single band with R_f value 0.72 was obtained in the control indicating the possible biodegradation of MG.

The change of functional groups between MG and metabolites was given by comparison of FTIR spectra of MG with metabolites obtained during degradation period. FTIR spectrum of control MG displayed specific peaks in the fingerprint region (1,500–500 cm^{-1}) for mono-substituted and para-disubstituted benzene rings which are supported by a peak at 1,585 cm^{-1} for C=C stretching of benzene rings (Fig. 3a). Along with these peaks for aromaticity, a peak at 1,170 cm^{-1} for the –C–N stretching vibrations and a peak at 2,923 cm^{-1} for C–H stretching of asymmetric –CH$_3$ group allow the perception of chemical structure of MG. FTIR spectrum of degradation products extracted after 5 h displayed peak at 2,926 cm^{-1} for C–H stretch and at 1,786 cm^{-1} for C=O stretching supported by another peak at 1,670 cm^{-1} (Fig. 3b), whereas, the peak at region (1,500–500 cm^{-1}) for disubstituted benzene derivative indicates aromatic nature of metabolites. Since the FTIR spectroscopy gives better perception of change in chemical structure and functional groups, biodegradation of MG by the *P. aeruginosa* NCIM 2074 was confirmed.

The HPLC analysis of dye sample collected at the beginning showed one major peak at retention time 4.54 min (Fig. 4a). As, the decolorization progressed the appearance of additional peaks were observed because of degradation of parent dye (after 5 h) with one minor peak at the retention time of 5.70 min and three major peaks at retention times of 3.93, 3.15, and 2.84 min (Fig. 4b).

Although several TPM dye-decolorizing microorganisms have been reported (Papinutti and Forchiassin 2004; Jadhav and Govindwar 2006; Daneshvar et al. 2007; Deng et al. 2008), the enzymes and the pathway which are responsible for the decolorization or degradation of TPM dye by bacteria have never been described. In order to verify degradation
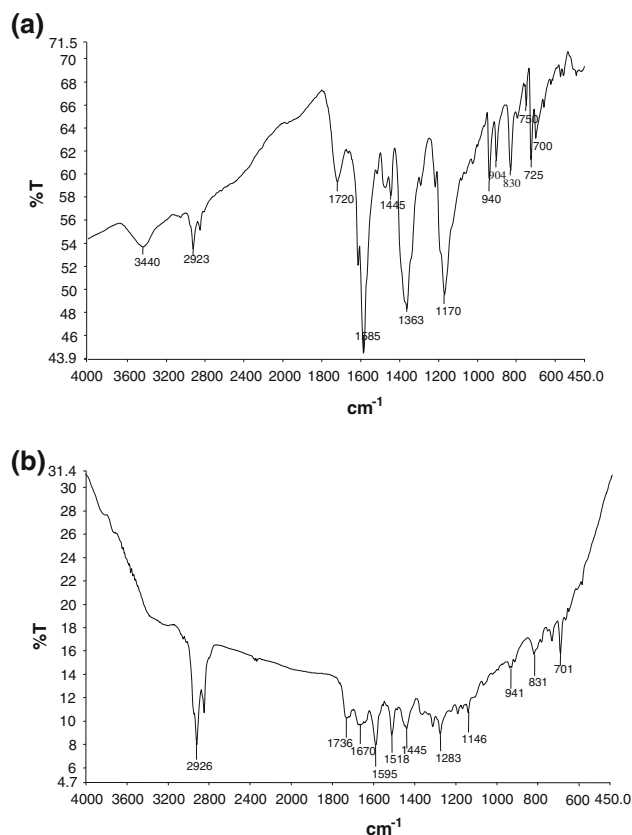

Fig. 3 HPLC elution profile of control MG (**a**), and its degradation products 5 h (**b**)

products formed during MG decolorization by *P. aeruginosa* NCIM 2074, the GC/MS analysis was carried out for various time periods of decolorization, which revealed the presence of several peaks. To eliminate the possibility of dye degradation during GC/MS analysis, the standard of MG was analyzed as well. MG could not be detected by GC/MS after incubating for 5 h, suggesting the high biodegradation efficiency of MG by the strain. The retention times (Total ion chromatograph) of various degradation products after 5 h of degradation are shown in Fig. 5. The components eluted having different retention times were subjected to mass spectrometry and identified by interpretation of their
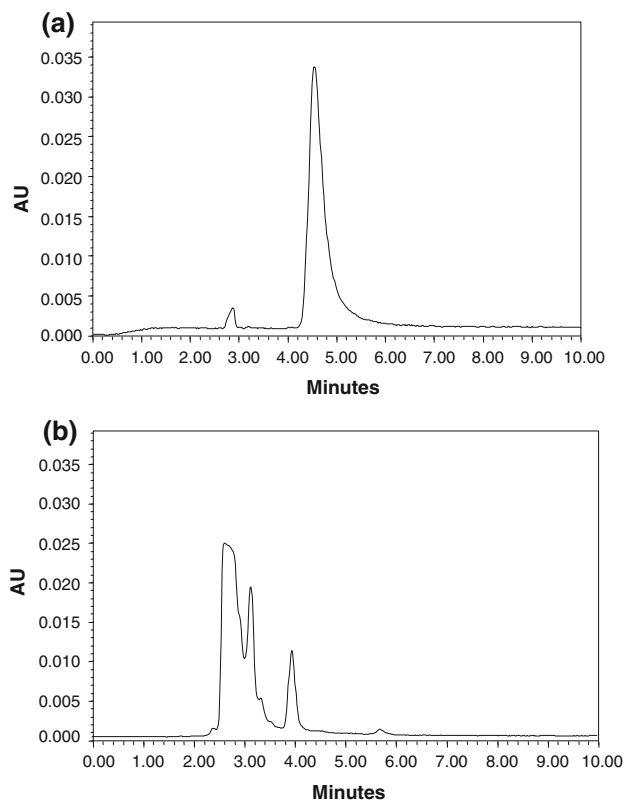
Fig. 4 FTIR spectra of control MG (**a**), and its degradation products 5 h (**b**)
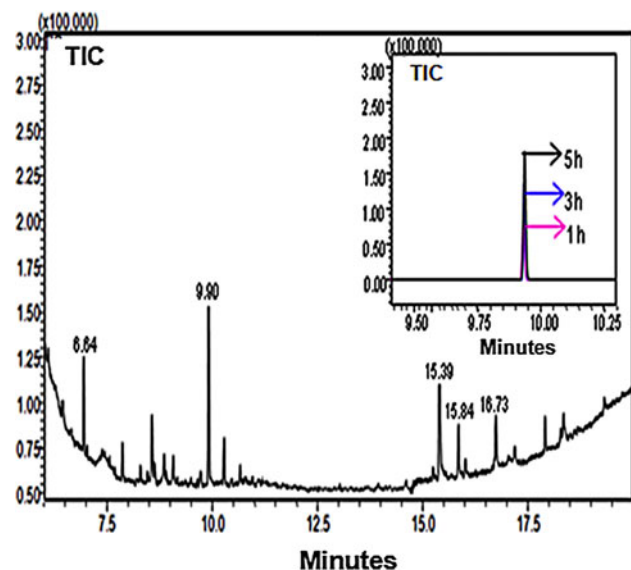
Fig. 5 Typical GC/MS total ion chromatogram (TIC) for the extracted MG metabolites after 5-h incubation. *Inset* Accumulation of bezophenone at different time periods

fragment ions in the mass spectra. The chromatographic data suggest that the intensity of entire components except benzophenone (retention time 9.66) reduced after 5 h of decolorization. The mass spectra of degradation products are

shown in Table 3. Based on the time for decolorizing the parent compound and forming intermediate compounds, MG split into leucomalachite green (LMG) (at m/z 330), (4-(4-aminophenyl) (phenyl) methyl) N–N-dimethylaniline) (at m/z 301), and 4-dimethylamino (at m/z 225), aniline (at m/z 93). These intermediate compounds might be accumulated to their highest concentration, and then started to transform as the decolorization progressed. LMG was the first intermediate to be detected after 1-h incubation and accumulated to its highest concentration [(Fig. 1) shown as the peak 270 nm] after another 4-h incubation it is then transformed to other compound(s) mentioned above. Further, after complete decolorization, accumulation of benzophenone (at m/z 181) considered as one of the final products in MG decolorization was observed in GC analysis over a period of time (Fig. 5 inset).

On the basis of the experimentally identified intermediate species, various enzyme induction and earlier reported studies (Table 4), a possible reaction mechanism for degradation of MG is proposed (Fig. 6). According to the proposal, our assumption that the reductase catalyzed initially as reductive cleavage resulted in the protonated molecules for LMG (at retention time 16.73 min) supports the reductive cleavage of MG. The similar initial reduction of MG to LMG was observed using intestinal bacteria (Henderson et al. 1997). Further demethylation of the LMG by aminopyrine N-demethylase resulted in di-desmethyl derivative (4-(4-aminophenyl) (phenyl) methyl) N–N-dimethylaniline) with retention time of 15.84 min. Based on previous reports (Cha et al. 2001; Chen et al. 2010), these peaks correspond to LMG (m/z 330) and its di-desmethyl derivative (m/z 301). The oxidative attack on this phenyl moiety by laccase produced 4-dimethylamino benzophenone (at retention time 15.39 min) accompanied by release of aniline (at retention time 6.64 min). Further demethylation of 4-dimethylamino benzophenone by laccase or aminopyrine N-demethylase resulted in the postulated intermediate 4-amino benzophenone, which was subjected to further deamination to yield benzophenone (at retention time 9.90 min) as a final product in this pathway.
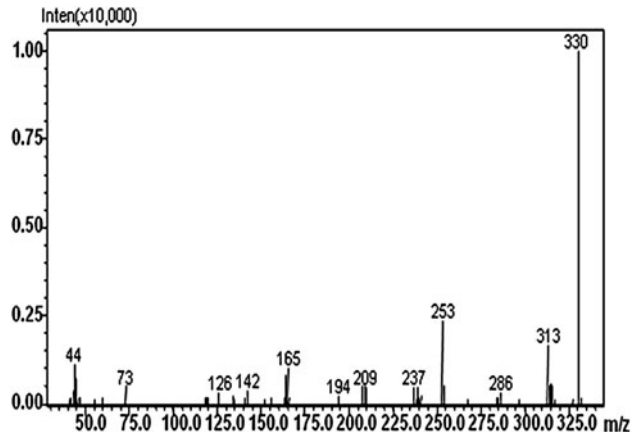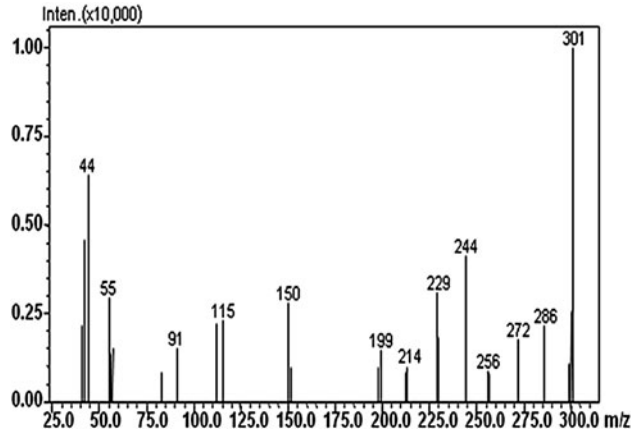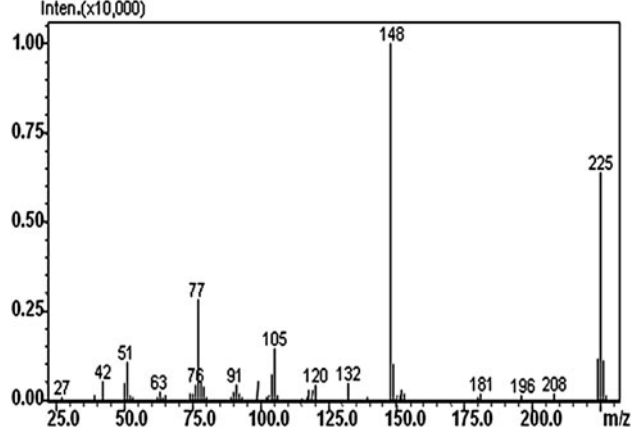
As shown in Fig. 6, reductive transformation of MG to LMG and its further demethylation and oxidation were also observed. Based on analysis of metabolites in MG degradation by *P. aeruginosa* NCIM 2074, new metabolites of MG were found, and a novel pathway for MG degradation was proposed.
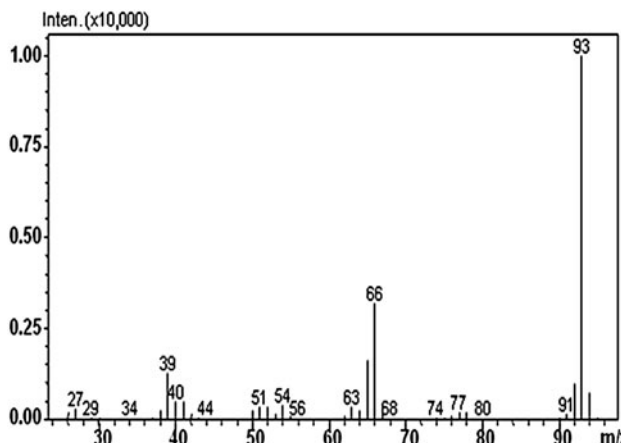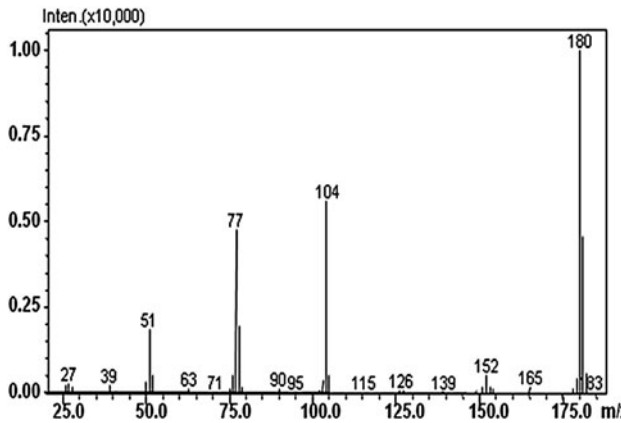
Toxicity study

Cytotoxicity

The toxicity of dyes is the main environmental concern, and so it was decided to evaluate the toxicity of the MG

Table 3 GC-MS data for metabolites obtained after biodecolorization of MG

Metabolites	Rt. time (min)	M_w (m/z)	Mass spectrum
LMG	16.73	330	
(4-[4-aminophenyl) (phenyl) methyl] N–N-dimethylaniline)	15.84	301	
4-dimethylamino benzophenone	15.39	225	

Table 3 continued

Metabolites	Rt. time (min)	M_w (m/z)	Mass spectrum
Aniline	6.64	93	
Benzophenone	9.90	181	

Table 4 The metabolites generated during biodegradation MG dye by different bacteria

Microorganism	Metabolites generated during decolorization	References
Escherichia coli ATCC 25922	LMG	Henderson et al. (1997)
Cunninghamella elegans	LMG, mono-desmethyl, di-desmethyl, tri-desmethyl, and tetra-desmethyl derivatives	Cha et al. (2001)
Acremonium kiliense	Desmethyl-MG, di-desmethyl-MG	Youssef et al. (2008)
Brevibacillus laterosporus MTCC 2298	Tetradesmethyl LMG, [4-(1-cyclohexyl)-(1′-phenyl)-methyl]-2,4-hexenoic acid	Gomare et al. (2009)
Shewanella sp. NTOU1	LMG, *N,N,N′*-trimethyl-4,4′-benzylidenedianiline, [*N,N*-dimethylaminophenyl] [phenyl]benzophenone, *N,N*-dimethylaminophenol	Chen et al. (2010)
Pseudomonas sp. strain DY1	Malachite green carbinol, (dimethyl amino-phenyl)-phenyl-methanone, *N,N*-dimethylaniline, (methyl amino-phenyl)-phenyl-methanone, (amino phenyl)-phenyl methanone, di-benzyl methane	Du et al. (2011)
Pseudomonas aeruginosa NCIM 2074	LMG, (4-(4-aminophenyl) (phenyl) methyl) *N–N*-dimethylaniline), 4-dimethylamino, aniline, benzophenone	In this study

Fig. 6 A proposed pathway for the biodegradation MG with by *P. aeruginosa* NCIM 2074 in shaking condition (pH 7.0). The *square brackets* signify that no quantitation was done
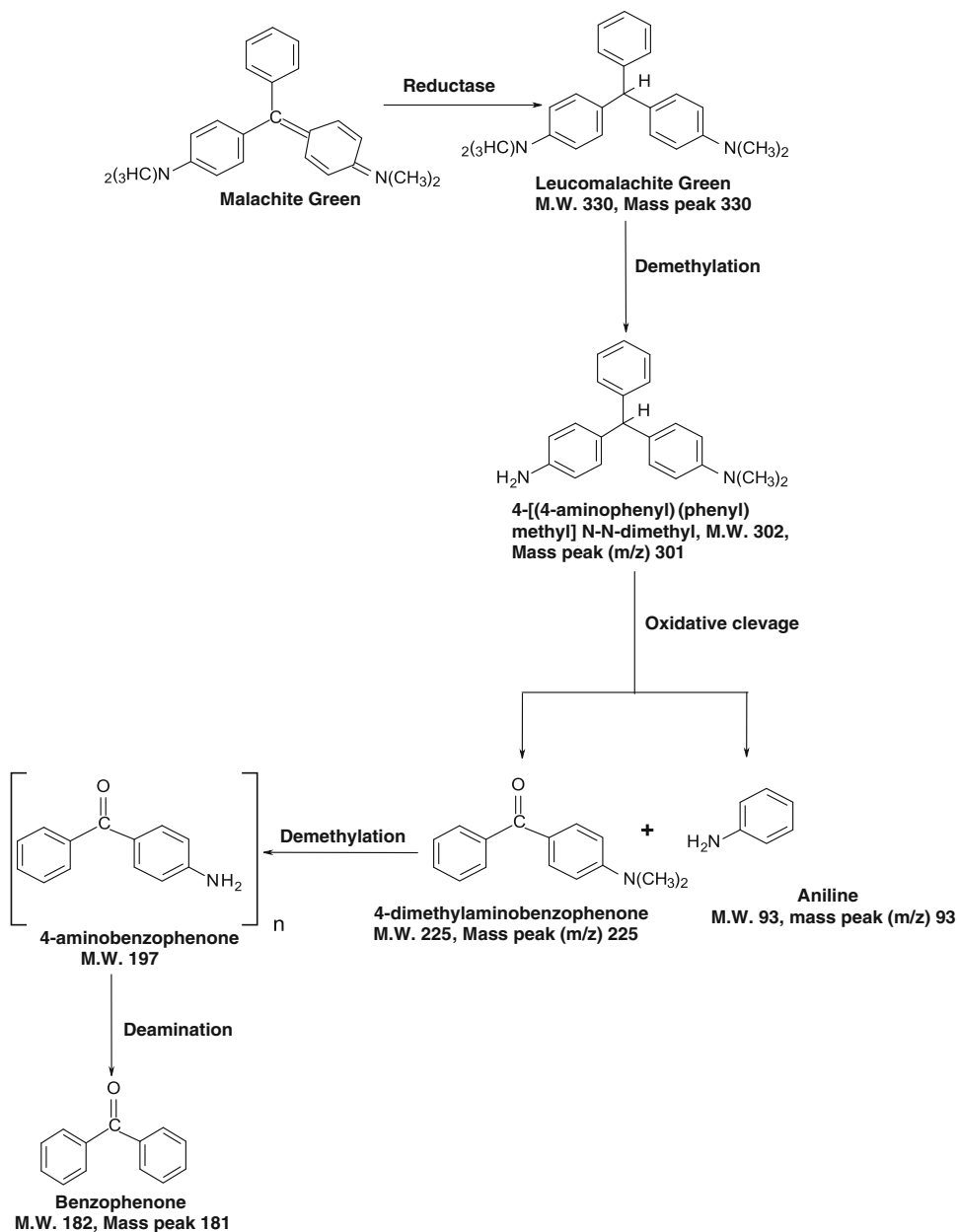
Table 5 Effect on root length, MI, obtained for the *A. cepa* tests

Analysis	Concentration (ppm)						
	Control	Sample[a]			Sample[b]		
		100	300	500	100	300	500
RL (cm)							
Mean ± SD	4.40 ± 0.30	3.10 ± 0.30	2.40 ± 0.65	1.10 ± 0.40	4.20 ± 0.30	4.10 ± 0.30	4.00 ± 0.15
MI							
Mean ± SD	9.32 ± 1.51	12.37 ± 1.01*	5.68 ± 2.01**	4.87 ± 0.84**	9.37 ± 2.16	9.30 ± 1.28	9.79 ± 2.45
TCA	2,145	2,186	2,068	2,201	2,161	2,009	2,113

Values are mean of three experiments, SD (±), significantly different from the control (roots meristem germinated in water) at * $P < 0.01$; ** $P < 0.001$, by ANOVA with Tukey–Kramer comparison test

Sample[a], before MG degradation; Sample[b], after MG degradation; *RL* root length; *MI* mitotic index; *TCA* total number of cells analyzed

and biodegraded MG. The cytotoxicity was analyzed on the basis of MI and frequency of chromosome aberrations using the *A. cepa* plant model. *A. cepa* roots exposed to various concentrations (100, 300, and 500 ppm) of MG had root lengths reduced by 28, 45, and 74% as compare to control (Table 5). On the other hand, the roots exposed to the same concentrations of biotreated MG sample had same average length to control. The inhibition of root growth and the appearance of stunted roots indicate the retardation of growth and cytotoxicity. To measure the MI, approximately 2,000 cells were observed for each concentration. Table 4 shows the MIs for the examined cells from MG and biodegraded MG samples. The control exhibited the MI (9.32%), while cells exposed to MG sample at a concentration of 500 ppm had the lowest MI (4.87%). With increasing concentration of MG, concentration-dependent decrease in the MI was noticed (Table 5). The effect of MG concentration on MI was significantly different ($P < 0.01$) for 100 ppm, and ($P < 0.001$) for 300 and 500 ppm, as compared with the control. On the other hand, biotreated samples of all concentrations (100, 300, and 500 ppm) did not show any significant difference compared with control (Table 5). The cytotoxicity level of a test compound can be determined based on the increase or decrease in the MI, which can be used as a parameter of cytotoxicity in the study of environmental biomonitoring (Carita and Marin-Morales 2008). The MIs being significantly lower than that of the control indicates that the alterations in the growth and development of exposed organism may be derived from the chemical action dyes. On the other hand, MIs being higher than the control is the result of an increase in cell division, which can be harmful to the cells, leading to a disordered cell proliferation and even to the formation of tumor tissues (Carita and Marin-Morales 2008). The present investigation and earlier observations indicate that the dyes and textile effluents exert effects on chromosome and cell division (Jadhav et al. 2010).

The second bioassay was assayed to evaluate chromosomal abnormalities in dividing cells and to estimate the toxicity potential of MG and biotreated MG samples. In this study, different kinds of chromosomal aberrations were observed: the most common chromosomal abnormalities in all treatments were c-mitosis, laggards, chromosome breaks, anaphase bridges, stickiness, and micronuclei (Table 6). Statistical analysis of the cytotoxicity tests showed that the percentage of aberrant mitotic cells caused by all concentrations of the MG sample was significantly different from that of the control, as shown in Table 4. Among the tests carried out with *A. cepa*, chromosome aberrations provide important information and may be considered an efficient test to investigate the cytotoxic

potential of the various textile dyes and effluent. The chromosomal stickiness might be due to degradation or depolymerization of chromosomal DNA. Stickiness is a common sign of toxic influence on the chromosomes and is probably an irreversible effect (Kumari et al. 2009). The disturbed metaphase observed might be due to disturbance in the spindle apparatus. The previous study suggests that chromosome breaks are associated with the formation of chromosome fragments and micronucleated cells (Carita and Marin-Morales 2008). These results indicate a mutagenic effect of the MG dye, when tested at a concentration of 100 ppm ($P < 0.01$). Recently, a few authors had reported MI inhibition and induction of several mitotic abnormalities in roots exposed to the tested effluent, showing that the dyeing industry effluents presented a mutagenic potential (Carita and Marin-Morales 2008; Jadhav et al. 2010).

The higher concentrations (300 and 500 ppm) had significant indices of mutagenicity. The biotreated concentrations of the MG (100, 300, and 500 ppm) did not produce mutagenic effects in *A. cepa*; this signifies the nontoxic nature of metabolites formed after degradation of MG by *P. aeruginosa* NCIM 2074.

Phytotoxicity

The previous article shows the toxicity effect of the MG (Culp and Beland 1996). Despite the possibility that untreated dyeing effluents may cause serious environmental problems and health hazards, they are being discharged into water bodies, and this water is being used for agricultural purpose (Kalyani et al. 2008). Thus, it was a matter of concern to assess the toxicity of the dye before and after decolorization. For this purpose, one of the most common phytotoxic assays used in the literature was performed (Parshetti et al. 2006). This assay is applied to evaluate the phytotoxicity of plant-growing media based on the germination (%) and length of plumule and radical (cm). Tests were carried out on two kinds of plants (*Vigna sinesis*, and *Vigna aconitifolia*), which are among the most sensitive, fast growing, and commonly used plants in Indian agriculture.

Results shown in Table 7 indicated that the germination (%) of the two plants was less with MG treatment as compared to metabolites obtained after its decolorization and distilled water treatments (Table 7). The MG was significantly reducing the length of shoot and root than the metabolites obtained after its decolorization. This study reveals that the metabolites generated after the biodegradation of MG are less toxic as compared with original dye. Thus, the process for biodegradation of MG by *P. aeruginosa* NCIM 2074 would be useful from environmental point of view.

Table 6 The number and frequency of chromosome aberrations obtained for the *A. cepa* tests

Analysis	Concentration (ppm)						
	Control	Sample[a]			Sample[b]		
		100	300	500	100	300	500
AB	1	7	10	16	2	3	5
DA	2	3	5	7	1	1	2
BC	1	1	3	4	0	2	2
LC	0	2	3	7	2	1	1
MC	0	1	0	2	0	0	0
ML	1	0	2	0	1	1	1
AL	1	0	2	2	1	0	1
TCA	6	14	25	38	7	8	12
TMO	200	274	122	107	203	186	207
Frequency of TCA	3.43 ± 0.60	5.12 ± 0.54	20.15 ± 3.58*	35.50 ± 2.63*	3.46 ± 0.63	4.42 ± 0.57	5.78 ± 0.70

Values are mean of three experiments, SD (±), significantly different from the control (roots meristem germinated in water) at * $P < 0.001$, by ANOVA with Tukey–Kramer comparison test

Sample[a], before MG degradation; Sample[b], after MG degradation; *AB* anaphase with bridge; *DA* delayed anaphase; *BC* binucleated cell; *LC* laggard chromosome; *MC* multipolar chromosome; *ML* metaphase with loss; *AL* anaphase with loss; *TCA* total number of cells with alterations; *TMO* total number of mitotic cells observed

Table 7 Phytotoxicity study of MG and its degradation product

Parameters	*Vigna sinesis*			*Vigna aconitifolia*		
	Water	MG[a]	Extracted metabolites[a]	Water	MG[a]	Extracted metabolites[a]
Germination (%)	100	30	80	100	0	100
Plumule (cm)	8.32 ± 0.22	3.32 ± 0.15***	6.35 ± 0.88	6.56 ± 0.39	0.0***	4.45 ± 0.10*
Radical (cm)	6.01 ± 1.59	1.65 ± 0.02***	3.50 ± 0.52**	4.67 ± 0.82	0.0***	3.05 ± 0.33

Values are mean of three experiments, SD (±), significantly different from the control (seeds germinated in water) at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by ANOVA with Tukey–Kramer comparison test

[a] 500 ppm concentration

Conclusions

The present study revealed the ability of the *P. aeruginosa* NCIM 2074 to decolorize and detoxify MG. Results obtained from this study showed complete decolorization of MG with significant reduction in COD. During the biodegradation, the induction of reductase, laccase, and aminopyrine *N*-demethylase suggests its involvement in the degradation process. The results obtained from the studies of mass spectrum, proposed degradation pathway, and toxicity revealed that this strain has the ability to convert MG into nontoxic compounds.

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