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Biodegradation of Crystal Violet by *Agrobacterium radiobacter*

G. K. Parshetti[1,*], S. G. Parshetti[1], A. A. Telke[2], D. C. Kalyani[3], R. A. Doong[1], S. P. Govindwar[4]

1. Department of Biomedical Engineering and Environmental Sciences, National Tsinghua University, 101, Sec. 2, Kuang-Fu Rd. Hsinchu, 30013, Taiwan. E-mail: ganeshgp2000in@gmail.com
2. Division of Applied Life Sciences, Gyeongsang National University, 900 Gajwa-dong, Jinju, Gyeongnam 660-701, Korea
3. Division of Chemical Engineering, Konkuk University, 1 Hwayang-Dong, Gwangjin-Gu, Seoul 143-701, Korea
4. Department of Biochemistry, Shivaji University, Kolhapur, Maharashtra 416004, India

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Abstract

Agrobacterium radiobacter MTCC 8161 completely decolorized the Crystal Violet with 8 hr (10 mg/L) at static anoxic conditions. The decreased decolorization capability by *A. radiobacter* was observed, when the Crystal Violet concentration was increased from 10 to 100 mg/L. Semi-synthetic medium containing 1% yeast extract and 0.1% NH_4Cl has shown 100% decolorization of Crystal Violet within 5 hr. A complete degradation of Crystal Violet by *A. radiobacter* was observed up to 7 cycles of repeated addition (10 mg/L). When the effect of increasing inoculum concentration on decolorization of Crystal Violet (100 mg/L) was studied, maximum decolorization was observed with 15% inoculum concentration. A significant increase in the activities of laccase (184%) and aminopyrine *N*-demethylase (300%) in cells obtained after decolorization indicated the involvement of these enzymes in decolorization process. The intermediates formed during the degradation of Crystal Violet were analyzed by gas chromatography and mass spectroscopy (GC/MS). It was detected the presence of *N,N,N′,N″*-tetramethylpararosaniline, [*N*, *N*-dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone, *N*, *N*-dimethylaminobenzaldehyde, 4-methyl amino phenol and phenol. We proposed the hypothetical metabolic pathway of Crystal Violet biodegradation by *A. radiobacter*. Phytotoxicity and microbial toxicity study showed that Crystal Violet biodegradation metabolites were less toxic to bacteria (*A. radiobacter*, *P. aurugenosa* and *A. vinelandii*) contributing to soil fertility and for four kinds of plants (*Sorghum bicolor, Vigna radiata, Lens culinaris* and *Triticum aestivum*) which are most sensitive, fast growing and commonly used in Indian agriculture.

Key words: *Agrobacterium radiobacter*; Crystal Violet; biodegradation; gas chromatography and mass spectroscopy; toxicity study

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Introduction

Large amounts of chemically different group of dyes such as azo, disperse, acidic, basic, triphenylmethane were used for textile dyeing. Significant proportions of these dyes enter in the environment as wastewater. Discharge of colored textile effluents into rivers and lakes results in reduced dissolved oxygen concentration and creates toxic conditions to aquatic flora and fauna (Gill et al., 2002; Liu et al., 2004). Among many classes of synthetic dyes used in the textile and dyeing industries, triphenylmethane dyes are the largest and most versatile, and play a predominant role in various industrial applications (Azmi et al., 1998). The triphenyl methane dye, Crystal Violet has been extensively used in human and veterinary medicine as a biological stain and as a textile dye in textile processing industry (Au et al., 1978; Azmi et al., 1998). Crystal Violet has been classified as recalcitrant dye and remains in the environment for longer period. It is toxic to aquatic and terrestrial life (Au et al., 1978; Azmi et al., 1998). Investigations on Crystal Violet *in vitro* concluded that this dye was a mitotic poisoning agent. In addition *in vivo* studies proved that, Crystal Violet should be regarded as a biohazard substance. Crystal Violet is a potent clastogenes, which is responsible for promoting tumor growth in some species of fish and also known as potent carcinogenic (Au et al., 1978; Fan et al., 2009).

Currently, various chemical and physical treatment methods including adsorption, chemical precipitation and flocculation, oxidation by chlorine, hydrogen peroxide and ozone, electrolysis, reduction, electrochemical treatment, and ion-pair extraction were used to remove the dye (Azmi et al., 1998; Cheng et al., 2008; Minero et al., 2008; Fan et al., 2009). Because of the high cost, disposal problems and generation of toxic products most of the chemical and physical methods for treating dye waste are not widely

* Corresponding author. E-mail: ganeshgp2000in@gmail.com

applied in the textile industries. Physio-chemical methods of dye removal are effective only if the effluent volume is small and sometimes the degradation products are toxic (Robinson et al., 2001).

Bacterial decolorization is associated with involvement of various enzymes such as lignin peroxidase, laccase, azoreductase and biotransformation enzymes (Telke et al., 2008; Parshetti et al., 2010). Decolorization of Crystal Violet by water borne pathogenic mycobacteria is mainly due to membrane bound fraction (Jones and Falkinham, 2003). El-Naggar et al. (2004) reported the biodegradation of Crystal Violet using air bubble bioreactor packed with *Pseudomonas aeruginosa*. However, most of the studies were carried out with Crystal Violet and a few researches have reported the degradation mechanisms or pathways underlying the decolorization of this triphenylmethane dye, with fewer reporting on enzyme actions (McDonald and Cerniglia, 1984; Bumpus and Brock, 1988; Sani and Banerjee, 1999; Hayase et al., 2000; Chen et al., 2008; Moturi and Singaracharya, 2009).

Genus *Agrobacterium* is a recognized group of phytopathogenic bacteria, particularly with *A. radiobacter* and *A. tumefaciens*. *A. radiobacter* lacks the tumorigenic Ti plasmid that is present in *A. tumefaciens* and is thus non-phytopathogenic. It has been reported that some bacteria in genus *Agrobacterium* could degrade xenobiotics such as atrazine (Struthers et al., 1998), quinoline-4-carboxylic acid (Schmidt et al., 1991), 4-aminobenzenesulfonate (Singh et al., 2006), phenanthrene (Aitken et al., 1998) and phenol (Baek et al., 2003). There have been reports on the isolation of *Agrobacterium* sp. from activated sludge treating domestic and industrial wastewaters (Lauff et al., 1993; Dangmann et al., 1996; White et al., 1996), and strains of *A. radiobacter* appear to be the most frequently reported ones (Drysdale et al., 1999; Singh et al., 2004).

Various organic and inorganic compounds enhanced the decolorization rate of bacteria by acting as electron donor or stabilizing the enzymes involved in decolorization of textile dyes (Telke et al., 2009). Therefore, it would be a meaningful and helpful work to study the decolorization of triphenyl methane dyes in the presence of various organic and inorganic compounds. Thus, the objectives of the present study were to investigate the ability of *A. radiobacter* to degrade Crystal Violet and to elucidate the possible degradation mechanism of Crystal Violet with enzyme system involved. Various operational parameters were optimized for maximal decolorization of Crystal Violet. The intermediates formed during the degradation of Crystal Violet were analyzed by gas chromatography and mass spectroscopy (GC/MS).

1 Experimental

1.1 Microorganisms and culture conditions

The pure culture of bacterium *Agrobacterium radiobacter* MTCC 8161 was obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. Pure culture was maintained on nutrient agar and stored at 4°C. The nutrient broth containing (g/L) beef extract, 1; yeast extract, 2; peptone, 5; NaCl, 5; was used for decolorization experiment.

1.2 Dyes, chemicals and microbiological media

ABTS (2,2-Azinobis (3-ethylbenzothiazolin-6-sulfonic acid) was purchased from Sigma-Aldrich (Mumbai, India). Yeast extract and glucose were obtained from Hi Media Laboratory (Mumbai, India). Catechol, *n*-propanol, and other fine chemicals were purchased from Sisco Research Laboratories (India). Crystal Violet was obtained from S.D. Fine Chemicals Limited (Biosar, India). All chemicals were of the highest purity and of an analytical grade.

1.3 Decolorization experiments

A loopful of microbial culture (105 CFU/mL, determined from Direct Microscopic Counts) was inoculated in 250 mL Erlenmeyer flask containing 100 mL nutrient broth and incubated at 30°C for 24 hr. Crystal Violet was added at a concentration of 10 mg/L after 24 hr, and 3 mL of the culture media was withdrawn at different time intervals. The aliquot was centrifuged at 5000 r/min for 20 min to separate the bacterial cell mass. The clear supernatant was used to determine decolorization by measuring the change in absorbance of culture supernatants at the maximum absorption wavelength (λ_{max}) of Crystal Violet (580 nm). Decolorization performance of Crystal Violet was measured under static conditions. Decolorization at different initial concentrations of Crystal Violet (10–100 mg/L) and effect of increasing inoculums percentage on decolorization of Crystal Violet were tested at 30°C in the nutrient broth. Studies on the effect of different culture conditions were carried out in synthetic medium (dye concentration 10 mg/L) at 30°C using 10% inoculum of optical density 1.0 (620 nm). To study the effect of carbon and nitrogen sources on degradation of Crystal Violet, semi-synthetic medium having following composition was used (g/L): Crystal Violet 0.010; $(NH_4)_2SO_4$ 0.28; NH_4Cl 0.23; KH_2PO_4 0.067; $MgSO_4{\cdot}7H_2O$ 0.04; $CaCl_2{\cdot}2H_2O$ 0.022; $FeCl_3{\cdot}6H_2O$ 0.005; yeast extract 0.2; NaCl 0.15; $NaHCO_3$ 1.0; and 1 mL/L of a trace element solution containing (g/L) $ZnSO_4{\cdot}7H_2O$ 0.01; $MnCl_2{\cdot}4H_2O$ 0.1; $CuSO_4{\cdot}5H_2O$ 0.392; $CoCl_2{\cdot}6H_2O$ 0.248; $NaB_4O_7{\cdot}10H_2O$ 0.177; $NiCl_2{\cdot}6H_2O$ 0.02 with different carbon (glucose, lactose, molasses, sucrose) and nitrogen (peptone, yeast extract, malt extract, urea, NH_4Cl) sources. Further decolorization of repeated addition of dye (10 mg/L) aliquots to culture media was also studied in nutrient broth at static conditions.

The percentage of decolorization was measured at different time intervals. All decolorization experiments were performed in triplicates. Abiotic controls (without microorganisms) were always included. Percentage of decolorization (D, %) was calculated by using a procedure reported earlier (Parshetti et al., 2010).

$$D = \frac{A_i - A_o}{A_i} \times 100\% \quad (1)$$

where, A_i (cm^{-1}) is the initial absorbance, A_o (cm^{-1}) is the observed absorbance.

1.4 Enzyme assays

1.4.1 Crude enzyme preparation

A. radiobacter cells were grown in the nutrient broth at 30°C for 24 hr consider being control, centrifuged at 10,000 r/min for 20 min. These cells (75 mg/mL) were suspended in a potassium phosphate buffer (50 mmol/L) at pH 7.4 and sonicated (Sonics-vibracell ultrasonic processor, USA), keeping sonifier output at 40 (amps) and giving 7 strokes each of 30 sec, with 1 min interval at 4°C. The homogenate was centrifuged at 10,000 r/min for 20 min and supernatant was used as a source of crude enzyme. A similar procedure was followed with the cells obtained after complete decolorization (8 hr).

1.4.2 Enzyme activities

Activities of lignin peroxidase, laccase, and tyrosinase were assayed spectrophotometrically in cell free extract. Laccase activity was determined in a reaction mixture of 2 mL containing ABTS (10%) in 0.1 mol/L acetate buffer (pH 4.9) and the increase in optical density at 420 nm was measured (Hatvani and Mecs, 2001). Tyrosinase activity was determined in a reaction mixture of 2 mL, containing catechol (0.01%) in 0.1 mol/L phosphate buffer (pH 7.4) at 410 nm (Parshetti et al., 2007). Lignin peroxidase activity was determined by monitoring the formed propanaldehyde at 300 nm in a reaction mixture of 2.5 mL containing 100 mmol/L *n*-propanol, 250 mmol/L tartaric acid, and 10 mmol/L H_2O_2 (Kalyani et al., 2008). All enzyme assays were carried out at 30°C where reference blanks contained all components except the assayed enzyme. One unit of enzyme activity was defined as amount of enzyme required to increase 1.0 unit of absorbance per min.

The NADH-DCIP reductase activity was determined by using a procedure reported earlier (Salokhe and Govindwar, 1999). The assay mixture contained 50 μmol/L DCIP, 28.57 mmol/L NADH in 50 mmol/L potassium phosphate buffer (pH 7.4) and 0.1 mL of enzyme solution (cell free extract) in a total volume of 5.0 mL. The DCIP reduction was calculated using the extinction coefficient of 19 L/(mol·cm). The activity of aminopyrine *N*-demethylase was determined using the procedure reported earlier (Jadhav et al., 2007). The assay of aminopyrine *N*-demethylase activity included incubations in 50 mmol/L N-2-hydroxymethyl piperazine-N-2 ethane sulfonic acid buffer (pH 7.8), containing NADPH-generating system (NADPH 2.6 mmol/L, glucose 6-phosphate 12.5 mmol/L, glucose 6-phosphate dehydrogenase 4 units) and 0.5 mL of enzyme solution (cell free extract) of incubation medium. After the addition of 0.4 mL of aminopyrine (80 mmol/L) to the incubation medium, the reaction mixture was incubated at 37°C for 10 min and 1 mL of ice cold 20% trichloroacetic acid solution was added to terminate the reaction. The amount of formaldehyde liberated was determined colorimetrically using Nash reagent. All enzyme assays were run in triplicates and average rates were calculated to represent the enzyme activity.

1.5 Microbial toxicity studies

For the purpose of assessing the impact of Crystal Violet (500 and 1000 mg/L) and its degradation product (extracted and dried, 500 and 1000 mg/L), microbial toxicity tests were performed on the microorganism *A. radiobacter*, phosphate-solubilizing bacterium *Pseudomonas aeruginosa* and nitrogen-fixing bacterium *Azatobacter vinelandii*. The microbial toxicity assessment was evaluated based agar well diffusion technique. Test microorganisms cultures were grown overnight (24 hr) in nutrient broth and standardized to $OD_{600\,nm}$ = 0.1. Test organisms were then spread-plated onto nutrient agar plates containing 1.5% agar, using sterile cotton swabs. A flame sterilized cork borer with a diameter of 6 mm was used to bore 4 wells into the agar and 100 μL of the control dye (500 and 1000 mg/L) and its metabolites extract (500 and 1000 mg/L) was loaded into the wells. The dye and extract was allowed to diffuse into the agar before the plates were incubated under aerobic conditions at 37°C for 24 hr. At the end of the incubation period, the plates were observed for zones of inhibition around the wells. Inhibition zone is defined as the area free of growth in a bacterial lawn which results from the toxic effect of compounds that has diffused into the medium from its applied source.

1.6 Phytotoxicity study

Phytotoxicity tests were conducted to assess the impact of Crystal Violet and its degradation metabolites on vegetation and to explore the possible reuse of the treated solution in the irrigation of agriculture fields. Tests were carried out on four kinds of plants which are most sensitive, fast growing and commonly used in Indian agriculture: *Sorghum bicolor*, *Vigna radiata*, *Lens culinaris* and *Triticum aestivum* (Kalyani et al., 2008). Fifteen seeds of each plant were sowed into a plastic sand pot. The sand pot was prepared by adding 20 g washed sand into the plastic pot. Crystal Violet and ethyl acetate extracted metabolites (dry) were dissolved separately in distilled water and made the final concentration of 500 mg/L. A toxicity study was done by watering (5 mL) the seeds of each plant with Crystal Violet and extracted metabolites sample. The control was run by watering the seeds with distilled water. The watering was done twice a day. Germination and length of shoot and root were recorded after 13 days. The experiment was carried out at room temperature.

1.7 Analysis of metabolites formed after decolorization of Crystal Violet

The culture medium was centrifuged after complete decolorization of Crystal Violet at 7000 r/min for 20 min. The metabolites present in the culture supernatant were extracted using equal volume of ethyl acetate, dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator. The crystals obtained were dissolved in small volume of HPLC grade methanol and used for further analysis. The GC-MS analysis of metabolites was carried out

using a Shimadzu 5050-QP-MS Engine (Japan), equipped with integrated gas chromatograph with a DB-5 column (60 m long, 0.25 mm i.d.). Helium was used as carrier gas at a flow rate of 1.3 mL/min. The injector temperature was maintained at 300°C with oven conditions. The initial column temperature was 40°C for 1 min, then increased linearly at 10°C/min to 325°C, and held for 2 min. The ionization was carried out in the electron impact mode (70 eV). The electron multiplier voltage and automatic gain control target were set automatically. The transfer line and ion trap mainfold were set at 280 and 230°C, respectively. Degradation products were identified by comparison of retention time and fragmentation pattern, as well as with mass spectra in the NIST spectral library stored in the computer software (version 1.10 beta, Shimadzu) of the GC-MS.

1.8 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparison test.

2 Results and discussion

2.1 Decolorization studies

A. radiobacter cells showed complete decolorization of Crystal Violet (10 mg/L) within 8 hr at static anoxic conditions. When autoclaved cells were tested for their ability to decolorize Crystal Violet, no decolorization was observed. It indicates that the decolorization was due to biodegradation. To confirm whether this decolorization was due to microbial action or change in pH, the change in pH range 6.6–6.8 was recorded. UV-Visible spectra of Crystal Violet did not show any change at this pH range. Chen et al. (2008) also reported the 100% decolorization of Crystal Violet under static anoxic conditions. The percentage of decolorization was decreased with increasing dye concentration. The time required for 100% decolorization of 10, 30, 50, 70 and 100 mg/L Crystal Violet concentration by *A. radiobacter* was 8, 24, 36, 48 and 86 hr respectively. The rate of decolorization was constant at different concentration, but with higher loads of dye substrate demand more time to be degraded. Ren et al. (2006) reported that decreased decolorization ability of bacterial isolate *Aeromonas hydrophila* to Crystal Violet was observed, when its concentration was increased.

2.2 Effect of different carbon and nitrogen source on decolorization

Remediation is usually limited by different culture conditions (Bogan et al., 2001; Parshetti et al., 2009). The efficacy of *A. radiobacter* to decolorize 10 mg/L of Crystal Violet in the presence of different culture conditions was tested to obtain efficient and faster decolorization. In the synthetic medium 70% decolorization was observed within 5 hr and no further decolorization was observed (Fig. 1). Crystal Violet decolorization of 100% was observed with 1% yeast extract and 0.1% NH_4Cl within 5 hr, whereas less than 100% decolorization was observed with other

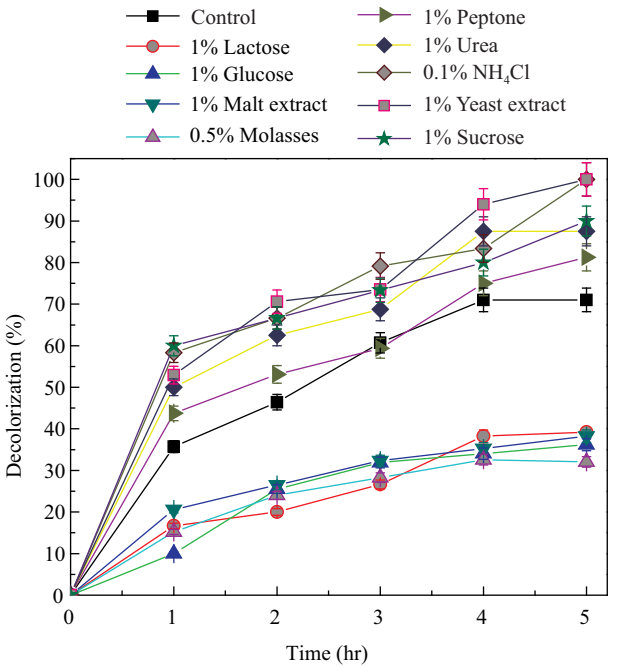

Fig. 1 Effect of different carbon and nitrogen sources decolorization of Crystal Violet (10 mg/L) by *A. radiobacter*.

supplements within 5 hr. In the presence of urea, peptone and malt extract the culture exhibited 87.5%, 81.25% and 38.23% decolorization respectively. The presence of 1% each of sucrose, lactose and glucose showed 90%, 39.23% and 36.17% decolorization respectively. The decolorization observed with 0.5% molasses was 32.06%. Presence of different culture conditions might be having stimulatory or inhibitory effect on enzymes involved in the decolorization of dyes, which results in the variation in time required for decolorization as well as decolorization percentage (Parshetti et al., 2006; Jadhav et al., 2007). Struthers et al. (1998) reported that the herbicide atrazine is degraded by *A. radiobacter* in soil by injecting soluble nutrients, like nitrogen sources, a few centimeters under the surface of soil.

2.3 Effect of inoculum concentration and fed batch addition

With the increase in inoculum concentration of *A. radiobacter*, the decolorization of Crystal Violet (100 mg/L) was increased. With 2%, 5%, 7%, 10% and 15% inoculum the time required for decolorization of Crystal Violet was 40, 30, 25, 18, 15 hr respectively (Fig. 2).

A. radiobacter showed 100% decolorization of Crystal Violet within 8 hr up to seven cycles. Further addition of dye to the same broth showed reduction in decolorization to 80% and 70% for 8th and 9th cycle respectively (Fig. 3). The mixed bacterial culture isolated from domestic waste treatment plant showed the decolorization of azo dye Methyl Red up to only three cycles (Vijaya and Sandhya, 2003).

2.4 Enzyme analysis

The demand for removal of synthetic dyes from the textile industry waste using fungal/bacterial enzymes is

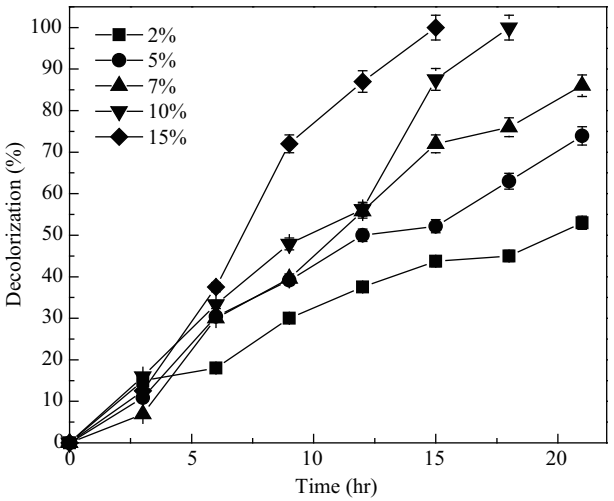

Fig. 2 Effect of increasing inoculum percentage on decolorization of Crystal Violet (100 mg/L) by *A. radiobacter*.

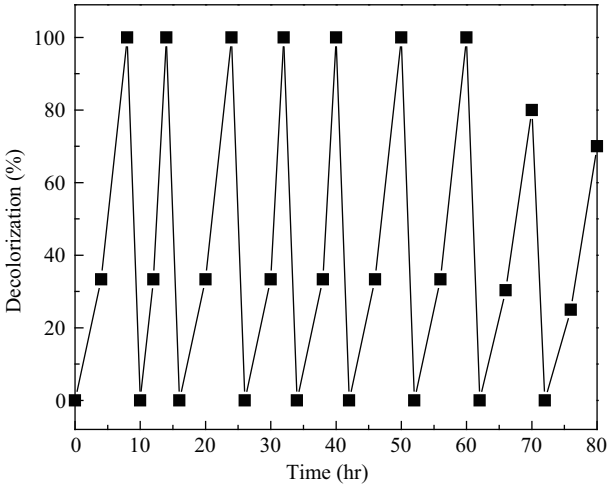

Fig. 3 Stability and longitivity of *A. radiobacter* cells for decolorization of Crystal Violet (100 mg/L).

being increased tremendously. Several microorganisms are known to decolorize triphenylmethane dyes and many reports showed that biodegradation mechanisms of dyes depend mainly on the enzyme secreted by them (Yatome et al., 1991, 1993; Cha et al., 2001; Parshetti et al., 2006, 2009). In order to get additional insight into the decolorization mechanism of Crystal Violet by *A. radiobacter*, enzyme activities of lignin peroxidase, laccase, tyrosinase, aminopyrine *N*-demethylase and NADH-DCIP reductase were monitored before and after decolorization.

In the present study, significant increase in the enzyme activities of laccase (184%) and aminopyrine *N*-demethylase (300%) were observed over the period of Crystal Violet decolorization compared to tyrosinase, lignin peroxidase and DCIP reductase (Table 1). This suggests that laccase and aminopyrine *N*-demethylase were presumably involved in the decolorization of Crystal Violet. Recently, Yan et al. (2009) reported the biodegradation mechanism of Crystal Violet by laccase with low molecular mass fraction (LMMF) from the white rot fungus *Pleurotus ostreatus* BP. Only 2.8% Crystal Violet was decolorized by the purified laccase. However, Crystal Violet was significantly degraded when it was treated by

Table 1 Enzyme activities profile in cells before (control) and after decolorization

Enzyme assay	Control cells at 0 hr	Cells obtained after decolorization for 8 hr
Laccase (min/mL)	0.076 ± 0.009	0.140 ± 0.012*
Lignin peroxidase (min/mL)	0.180 ± 0.022	0.210 ± 0.005**
Tyrosinase (min/mL)	0.136 ± 0.010	0.055 ± 0.003**
NADH-DCIP reductase (g DCIP reduced/(min·mg protein))	30.002 ± 1.200	40.000 ± 0.660
Aminopyrine *N*-demethylase (nmol formaldehyde librated/(min·mg protein))	0.073 ± 0.008	0.219 ± 0.019**

Values are mean of three experiments SEM (± standard error of mean). Significantly different from control cells at $*P < 0.05$, $**P < 0.001$ by one-way ANOVA with Tukey-Kramer multiple comparisons test.

crude enzyme, LMMF or LMMF and laccase after 24 hr incubation. It shows that along with laccase, some other enzymes could also be involved in decolorization process.

Bumpus and Brock (1988) found that substantial degradation of Crystal Violet also occurred in nonligninolytic cultures of *Phanerochaete chrysosporium,* suggesting another mechanism exists in this fungus which is also able to degrade Crystal Violet. Cha et al. (2001) reported that Crystal Violet could be transformed by the filamentous fungus *Cunninghamella elegans* ATCC 36112 through sequential *N*-demethylation to *N*,*N*′,*N*″-trimethylpararosaniline. Thus, it is hypothesized that the biological degradation of Crystal Violet was based on the reduction reaction, reductive splitting reaction (Henderson et al., 1997; Chen et al., 2008; Yan et al., 2009) and demethylation reaction (Sarnaik and Kanekar, 1999; Parshetti et al., 2009).

2.5 Toxicity studies

In spite of the effectiveness of the biological treatments, in some cases micro-organisms can transform dyes in to compounds more toxic than the original compound. Consequently, there is need to assess the toxicity of end product after the biological treatments. For this purpose some toxicological assays such as microbial toxicity and phytotoxicity were used to determine the toxicity of degradation products (Colarieti et al., 2006; Kalyani et al., 2008).

2.5.1 Microbial toxicity studies

A number of dyes have been tested for mutagenicity using Ame's bioassay. Several of them have found to be carcinogenic and mutagenic (Mathur et al., 2005). All the tested strains are microflora contributing to soil fertility as they take part in the biotransformation of organic materials and nutrients in the soil. Microbial toxicity studies revealed that zone of inhibition was observed with Crystal Violet by all bacterial strains studied, whereas, its metabolites showed comparatively less zone of inhibition (Table 2).

These findings suggest that the degradation products were less toxic compared with the Crystal Violet to an exploited microorganism's *A. radiobacter*, a phosphate-solubilizing bacterium *P. aeruginosa* and nitrogen-fixing

Table 2 Microbial toxicity studies of Crystal Violet and metabolites obtained after its decolorization

Bacteria	Diameter of inhibition zone (cm)			
	Crystal Violet at 500 mg/L	Degradation product at 500 mg/L	Crystal Violet at 1000 mg/L	Degradation product at 1000 mg/L
A. radiobacter	0.5	N.I.	1.1	N.I.
P. aurugenosa	0.6	0.2	0.7	0.2
A. vinelandii	0.6	0.1	1.1	0.2

N.I.: no inhibition.

bacterium *A. vinelandii*.

2.5.2 Phytotoxicity study

Untreated dyeing effluents may cause serious environmental problems and health hazards. They are being discharged in water bodies and this water could be used for agriculture. Thus, it is of immediate concern to assess the phytotoxicity of the effluent before and after degradation. For this purpose, one of the most common phytotoxic assays used in the literature was performed (Kalyani et al., 2008). This assay was applied to evaluate the phytotoxicity of plant growing media based on the germination percentage of seeds. The germination percentage combines measurements of relative root and shoot elongation as both are sensitive to the presence of phytotoxic compounds. Although several species have been traditionally used for evaluating phytotoxicity, there are no standardized seed species in use worldwide (Osma et al., 2010).

Phytotoxicity study revealed the toxicity of Crystal Violet to the *Sorghum bicolor*, *Vigna radiata*, *Lens culinaris*, and *Triticum aestivum* plants. Germination percentage of all four plants was less with Crystal Violet treatment as compared to metabolites obtained after its decolorization and distilled water treatment (Table 3).

Crystal Violet was significantly reducing the length of shoot and root than its metabolites. Phytotoxicity study revealed the toxic nature of Crystal Violet and less toxic nature of the metabolites obtained after decolorization of Crystal Violet. Kalyani et al. (2008) reported that, the phytotoxicity testing with seeds of *Sorghum vulgare* and *Phaseolus mungo* showed more sensitivity towards the dye, while the products obtained after dye decolorization have less inhibitory effects. Moawad et al. (2003) reported the phytotoxicity of different soluble textile dyes estimated by measuring the relative changes in seed germination of four plants: clover, wheat, tomato and lettuce. Thus, the process of biodegradation of Crystal Violet by *A. radiobacter* would be useful for environmental point of view.

2.6 Biodegradation analysis

To disclose the possible mechanism of the dye decolorization, we also analyzed the products of biotransformation of Crystal Violet by GC-MS analysis. Five intermediates, *N,N,N′,N″*-tetramethylpararosaniline, [*N*, *N*-dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone, 4-methyl amino phenol, *N*, *N*-dimethylaminobenzaldehyde, and phenol with a molecular mass of 345, 253, 124, 150 and 95, were deduced with the corresponding mass spectrum at GC retention times of 25.49, 22.34, 19.10, 11.67 and 7.32 min respectively (Fig. 4).

We have proposed a pathway for degradation of Crystal Violet by *A. radiobacter* on the basis of enzyme activities and GC-MS data (Fig. 5).

From the five intermediate compounds detected by GC-MS in extracted samples collected during color removal, we cogitated that Crystal Violet was first biotransformed into *N,N,N′,N″*-tetramethylpararosaniline. It was not clear whether Crystal Violet was reduced to its leuco derivative before reductively splitting into *N,N,N′,N″*-tetramethylpararosaniline, although the production and transformation of leuco-Crystal Violet might have occurred shortly after incubation (Henderson et al., 1997). Few reports are available on the intermediates or the products of biodegradation of triphenylmethane dyes. Several studies had shown that Crystal Violet is converted to its colorless leuco derivatives by intestinal microflora and several anaerobic bacteria (McDonald and Cerniglia, 1984; Henderson et al., 1997). In addition we observed the formation of four more compounds, [*N*, *N*-dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone, *N*, *N*-dimethylaminobenzaldehyde, 4-methyl amino phenol and phenol. Yatome et al. (1991)

Table 3 Phytotoxicity of Crystal Violet and metabolites formed after its decolorization

Observation	*Sorghum bicolor*			*Vigna radiata*			*Lens culinaris*			*Triticum aestivum*		
	I	II	III	I	II	III	I	II	III	I	II	III
Germination (%)	70	20	70	90	20	70	70	0	40	80	20	50
Shoot length (cm)	4.50 ±0.43	0.80 ±0.58	3.30 ±0.40$^{\$\$\$}$	8.31 ±0.38	1.20 ±0.53***	5.20 ±0.53$^{\$\$}$	5.80 ±0.64	0 ±0	3.00 ±0.40*	6.20 ±0.38	1.40 ±0.10***	3.53$^{\$\$}$ ±0.84
Root length (cm)	5.50 ±0.96	0.9 ±0.06	3.20 ±0.25*	4.40 ±0.44	1.11 ±0.46***	2.10 ±0.49**	3.70 ±0.63	0 ±0	1.97 ±0.10**	3.62 ±0.20	0.35 ±0.05***	2.37 ±0.2$^{\$\$\$}$

I: seeds treated with distilled water; II: seeds treated with Crystal Violet (500 mg/L); III: seeds treated with metabolites obtained after decolorization of Crystal Violet (500 mg/L).

Values are mean of germinated seeds of three experiments, SEM, significantly different from the control (seeds germinated in water) at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ and significantly different from dye treated sample at $^{\$\$}P<0.01$ and $^{\$\$\$}P < 0.001$ by one way analysis of variance (ANOVA) with Tukey-Kramer multiple comparison test.

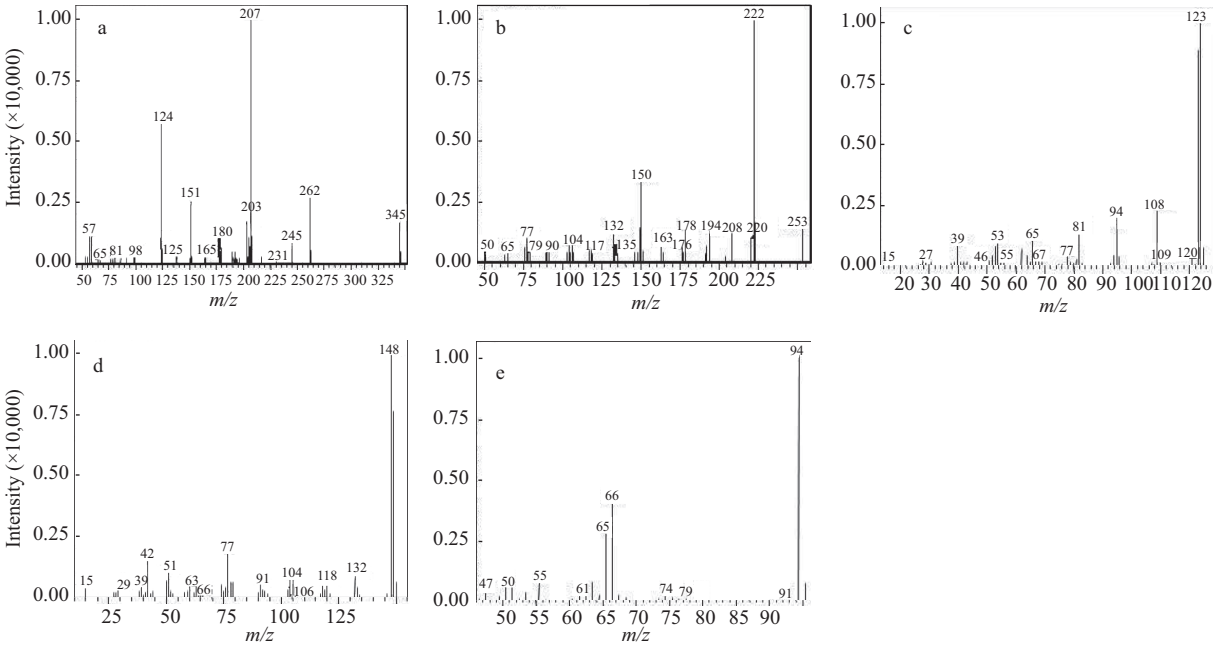

Fig. 4 Identification of metabolites of Crystal Voilet by GC-MS. (a) *N*, *N*,*N*′,*N*′′-tetramethylpararosaniline (Ret. time 25.49 min, *m*/*z* 345), (b) *N*, *N*-dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone (Ret. time 22.34 min, *m*/*z* 253), (c) 4-methyl amino phenol (Ret. time 11.67 min, *m*/*z* 124), (d) *N*,*N*-dimethyl aminobenzaldehyde (Ret. time 19.10 min, *m*/*z* 150), (e) phenol (Ret. time 7.32 min, *m*/*z* 95).

reported that the major degradation product of Crystal Violet by growing cells of *Bacillus subtilis* IFO 13719 was Michler's Ketone. The same degradation product was also detected by Yatome et al. (1993) and Chen et al. (2008) with the growing cell of *Nocardia coralline* and *Shewanella decolorationis* NTOU1 respectively. On the other hand, compounds identified as *N,N,N′,N′,N′′*-penta-, *N,N,N′,N′′*-tetra-, and *N,N′,N′*-trimethylpararosaniline were detected during the degradation of Crystal Violet by white-rot fungi under ligninolytic conditions (Bumpus and Brock, 1988). We speculated that *N,N,N′,N′′*-tetramethylpararosaniline was first reductively split into [*N*, *N*-dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone and 4-methylaminophenol, then [*N*, *N*−dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone was further degraded into *N*,*N*-dimethylaminobenzaldehyde and 4-methylaminophenol. Furthermore, phenol was identified as a degradation product of Crystal Violet which leads us to speculate that *N*, *N*-dimethylaminobenzaldehyde might be further sequentially degraded into phenol. Further more formation of phenol also speculated that 4-methylaminophenol might be degraded into phenol. A novel biodegradation mechanism of Crystal Violet by *A. radiobacter* was deduced. The Crystal Violet decolorization mainly attributed to the existence of laccase and aminopyrine *N*-demethylase and proposed degradation pathway was elucidated. The special biodegradation mechanisms of Crystal Violet by *A. radiobacter* might exist.

3 Conclusions

The present study indicates potential of *A. radiobacter* to 100% decolorize and degrade Crystal Violet. The culture has ability to decolorize Crystal Violet in repeated additions for 7 cycles. As decolorization progresses the inducible nature of laccase and aminopyrine *N*-demethylase suggest their involvement in the dye degradation. Based on the degradation products detected by GC-MS, it was proposed that Crystal Violet was first bio-transformed into *N,N,N′, N′′*-tetramethylpararosaniline. Further *N,N,N′,N′′*-tetramethylpararosaniline was first reductively split into [*N*, *N*-dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone and 4-methylaminophenol, then [*N,N*-dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone was further degraded into *N,N*-dimethylaminobenzaldehyde and 4-methylaminophenol. Furthermore, phenol was identified as a degradation product of Crystal Violet. Degradation products were less toxic for microorganisms (*A. radiobacter*, *P. aeruginosa*, and *A. vinelandii*) and plants (*Sorghum bicolor*, *Lens culinaris*, *Vigna radiata* and *Triticum aestivum*) compared with the original dye. Overall findings suggested the ability of *A. radiobacter* for the decolorization of triphenylmethene dye and ensured the ecofriendly degradation of Crystal Violet.

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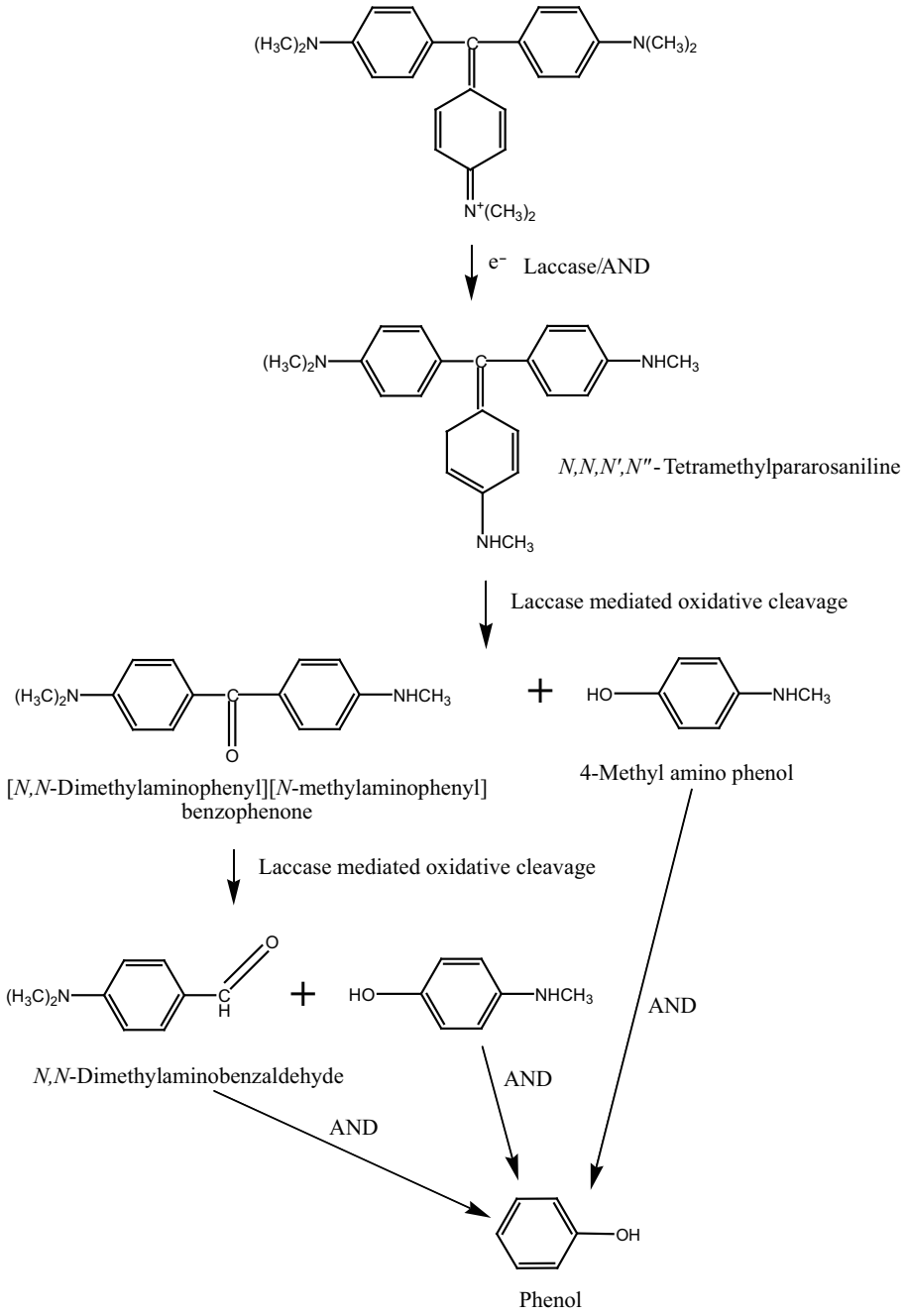

Fig. 5 Proposed pathway for degradation of Crystal Violet using *A. radiobacter*. AND: Aminopyrine *N*-demethylase.

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