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Biodegradation of Crystal Violet by *Agrobacterium radiobacter*

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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

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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.