African Journal of Biotechnology Vol. 10 (4), pp. 624-631, 24 January, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1616
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Characterization and identification of *Pseudomonas fluorescens* NCIM 2100 degraded metabolic products of Crystal Violet

R. R. Pandey[1]*, B. V. Pandey[2] and R. S. Upadhyay[3]

[1]Department of Life Sciences, Manipur University, Canchipur, Imphal-795 003, India.
[2]Department of Botany, Sri Agrasen Kanya Autonomous P.G. College, Varanasi-221 001, India.
[3]Laboratory of Plant Pathology and Microbial Technology, Department of Botany, Banaras Hindu University, Varanasi-221 005, India.

Accepted 23 December, 2010

Crystal violet (CV), a recalcitrant molecule, used commonly as biological stain and commercial dye, was subjected to biodegradation in this study by *Pseudomonas fluorescens* NCIM 2100 and its biodegraded metabolic products were identified by UV, ^{1}HNMR and IR Spectrophotometry. During degradation process CV dye was first broken down into an intermediate compound which was further converted into either 4-benzhydrylidene-cyclohexa-2,5-dienylamine hydrochloride or 4-benzhydrylidene-cyclohexa-2,5-dienol or 4-benzhydryl-cyclohexa-2,5- diennol in broth medium and were non-toxic in nature. Hence, *P. fluorescens* can be used for bioremediation of textile effluents containing CV dye in sequential cycles.

Key words: Crystal violet, bioremediation, *Pseudomonas fluorescens,* metabolic products, spectrophotometry.

INTRODUCTION

Crystal violet (N, N, N', N', N'', N''- hexamethylpararosalinine), a triphenylmethane dye, has been used extensively in textile dyeing and dye-stuff manufacturing industries, in human and veterinary medicines as a biological strain and in paper, leather, food and cosmetic processing industries (Bangert et al., 1977; Bumpus and Brock, 1988; Gregory, 1993). This dye has been categorized as a recalcitrant molecule indicating that, it is poorly metabolized by microbes and therefore, is persistent in environment for longer period when discharged (Chen et al., 2007). Increasing concerns about the color and toxic ingredients in the dyes effluents are leading towards the worldwide efforts for developing their effective removal processes. Physical and chemical facilities available for wastewater treatments are often unable to remove completely the commercial dyes from contaminated environments, thus, contributing to the pollution in aquatic habitat (Reife, 1993). Some triphenylmethane dyes were also proved to be mutagenic and carcinogenic to biota (Black et al., 1980). However, the processes used for the chemical treatment of dyes effluents are much costly, less efficient and produce large amount of sludge, hence, biological degradation processes are getting more attention since it is cost effective, eco-friendly and produce less quantity of sludge as compared to physical-chemical treatments (Azmi et al., 1998; Ayed et al., 2009).

Various microbes such as bacteria, fungi, actinomycetes and algae have been reported to degrade different pollutants (Bumpus and Brock, 1988; Leahy and Colwell, 1990; Spain, 1995; Azmi et al., 1998). Interest is now focused on the bacteria which can perform high rate of discoloration under anoxic conditions and provide detoxification of aromatic products under aerobic environment (Stolz, 2001; Upadhyay, 2002; Chen et al., 2007). Several isolates of *Pseudomonas*, *Alcaligenes, Acinetobacter* and *Bacillus* are a few important bacteria useful in bioremediation of halogenated organic compounds (Chaudhary and Chapalamadugu, 1991; Yatome et al.,

*Corresponding author. E-mail: pandey.rr@rediffmail.com. Tel: +919436025986. Fax: +91-385-2435327.

1991). However, few reports are available on the biodegradation products or intermediates of different dyes. Therefore, the objective of the present study was to characterize and identify spetrophotometrically, the biodegradation metabolic products of crystal violet by a CV-degrading bacterium, *Pseudomonas fluorescens* NCIM 2100.

MATERIALS AND METHODS

Microbial strain and culture medium

The bacterial culture of *P. fluorescens* NCIM 2100, used in the present study for biodegradation of crystal violet, was obtained from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India, that was well adopted to grow on this dye. The test bacterium was grown on King's B medium. The pH of broth medium was adjusted to 7.2 with phosphate buffer before sterilization.

CV-degradation by *P. fluorescens* NCIM 2100

The experiments were performed as described by Kar et al. (1997) into 250 ml conical flasks (in triplicate) containing 50 ml King's B broth (pH 7.2) supplemented with CV dye (ca. 200 mg/l in H_2O). Cell suspension of the test bacterium containing 10^6 cfu/ml was used as inoculum. Cultures were established by inoculating the CV supplemented broth with 0.1 ml bacterial cells and the flasks were transferred into a temperature controlled incubator shaker. Ambient temperature of the cultures was maintained at 37°C. The cultures were then flushed with oxygen, resealed and further incubated for 4 days on a rotary shaker (200 rev/min).

Preparation of cell extract

The flasks were removed after 4 days of incubation period and were centrifuged at 4°C temperature for 5 min by high speed cooling centrifuge (Remi C-24) at 10000 rev/min. The test bacterial cells were harvested after centrifugation, washed with phosphate buffer (10 mM, pH 7.2) and then cells were disrupted by adding 1 mm glass beads into the suspension. The resulting suspension was again centrifuged and the supernatant was stored on ice until use. The bioremediation experiments with cell-free extract before and after degradation of crystal violet dye by *P. fluorescens* NCIM 2100 were performed spectrophotometrically.

UV spectrophotometry

The UV and visible spectra of the samples were measured in ethanol with a Perkin Elmer double beam UV-Vis Spectrophotometer 117. Quartz cells (1 cm sq) having 1.0 cm path length was used for the determination. Hydrogen discharge tungsten filament lamp was applied as a source of light and maximum absorbance was recorded.

^{1}HNMR spectroscopy

The samples were subjected to ^{1}HNMR spectroscopy. Proton nuclear magnetic resonance spectra of the test samples were run at Brukar AC 200 M Hz in D_2O using TMS as an interval standard and 0.5 ml aliquot of the samples along with 0.5 ml of solvent were taken in 5 ml O.D glass tube for the determination.

IR spectroscopy

The infra red (IR) spectra of the test samples were obtained by placing solid compound in Nujol between salt plates (NaCl, 0.1 mm cells), without a spacer, in one of the beams of a Perkin Elmer-783 spectrophotometer Nernst glower, molded rod containing a mixture of zirconium oxide, yttraium oxide and erbium oxide and heated to 1500°C by electrical means, which was used as a light sources of IR irradiation. Grating was employed to obtain monochromatic light. The stretching and bending position of bands were observed and compared with reference compounds. Band position was presented in wave number unit (cm^{-1} reciprocal) and band intensities were expressed as transmittance (T). The functional groups present were counted according to band positions (Dyer, 1991).

RESULTS AND DISCUSSION

Ultraviolet (UV) spectrum of crystal violet before degradation by *P. fluorescens* NCIM 2100 showed maximum absorbance in methanol at 578 nm (Figure 1). After degradation, maximum absorbances were obtained at 238 and 276.7 nm. The peaks were completely different which reveal that, Crystal Violet changed into another compound (Figure 2). Before degradation by the test bacterium, ^{1}HNMR spectrum of crystal violet (CV) showed three singlets at 4.25, 6.00 and 7.25 ppm (Figure 3). After degradation, it also showed three singlets in aromatic region at 7.25, 6.1 and 4.32 ppm. It can be concluded that, there is the formation of a new compound, which possessed benzene rings attached by benzylic carbon that contained one proton (Figure 4). IR spectrum before degradation, revealed a medium peak at 1350 cm^{-1}, a strong peak at 1450 cm^{-1}, a medium peak at 1550 cm^{-1} and a broad peak at 3350 cm^{-1} (Figure 5). After degradation, IR spectrum showed a medium peak at 1350 cm^{-1}, a strong peak at 1450 cm^{-1}, two medium peaks at 1530 and 1620 cm^{-1} and two broad peaks at 3200 cm^{-1} and 3450 cm^{-1}. This indicate the presence of C-O, C=O, C-H, OH or NH group in the compounds (Figure 6).

Our findings on UV, ^{1}HNMR and IR spectrum after biotreatment of crystal violet by *P. fluorescens* NCIM 2100 revealed that, CV was degraded into an intermediate compound which further converted into either 4-benzhydrylidene-cyclohexa-2,5- dienylamine hydrochloride or 4-Benzhydrylidene-cyclohexa-2,5- dienol or 4-Benzhydryl-cyclohexa-2,5- diennol in broth medium that were non-toxic in nature (Figure 7). Buckingham and Macdonald (1996) have also reported that, the end products obtained as a result of textile dyes biodegradation by *P. fluorescens* were non-toxic. A similar result has been observed by Pandey and Upadhayay (2006) in case of Acid Yellow-9 dye which after degradation by *P. fluorescens* changed into three non-toxic intermediate compounds such as 2,4 dihydroxy-benzene sulphonic acid sodium salt, 2 amino-4 dihydroxy-benzene

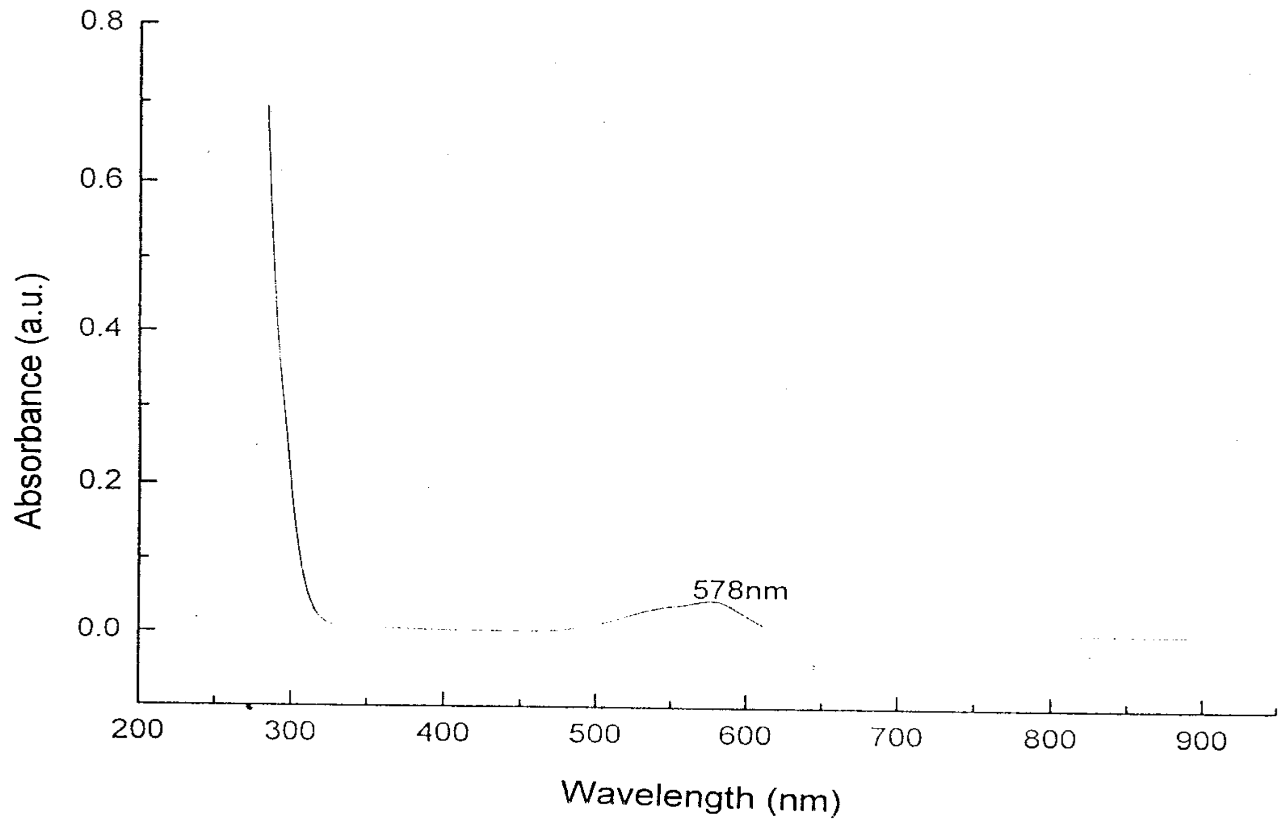

Figure 1. UV spectrum of crystal violet before degradation by *P. fluorescens* NCIM 2100.

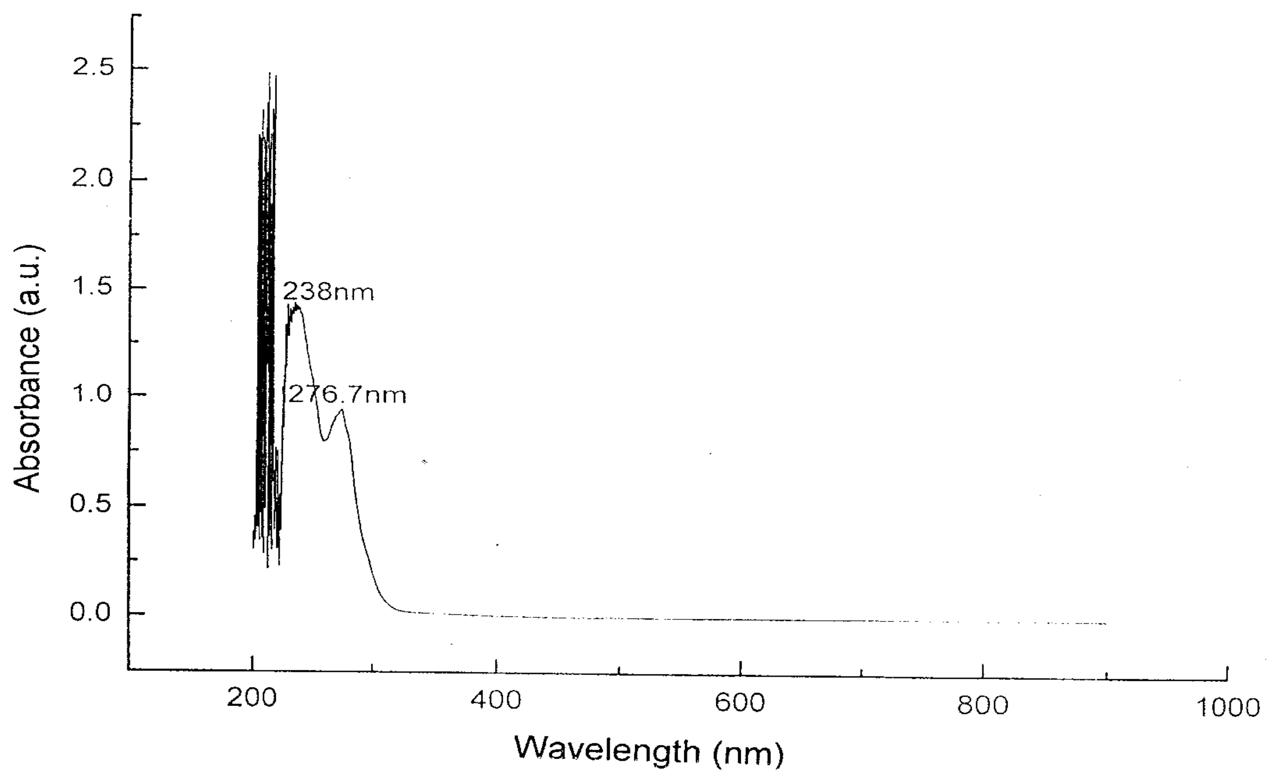

Figure 2. UV spectrum of crystal violet after degradation by *P. fluorescens* NCIM 2100.

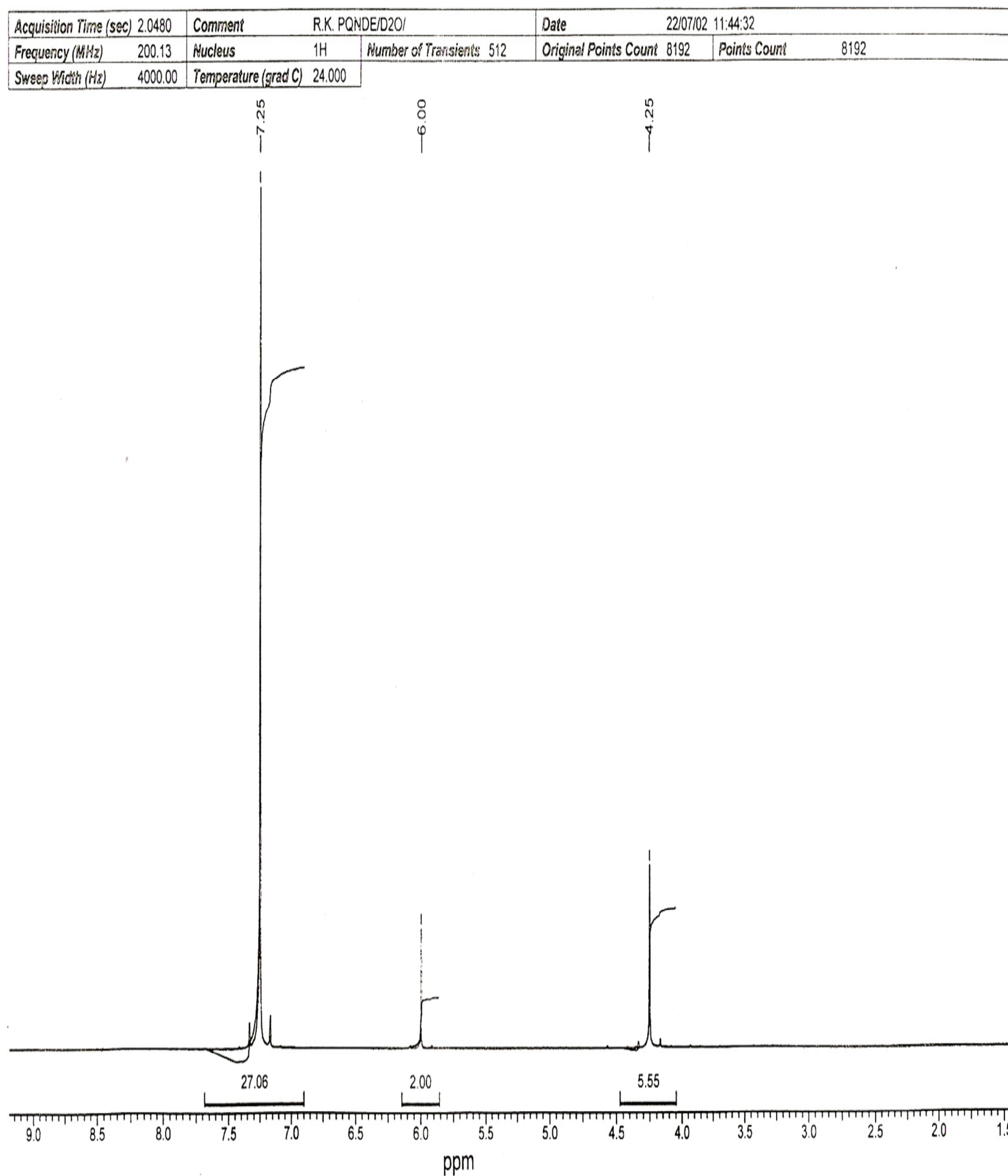

Figure 3. ^{1}HNMR spectrum of crystal violet before degradation by *P. fluorescens* NCIM 2100.

sulphonic acid sodium salt or 4-amino- 2 hydroxybenzene sulphonic acid sodium salt. Bacterial strains have the initial degradative dehalogenation steps yielding hydroxy benzoic acid, which is subsequently metabolized through meta-ring cleavage (Mark et al., 1984; Kobayashi et al., 1997). The degradation mechanism is mainly associated with the presence of NAD (P) which acts as electron donor. Bumpus and Brock (1988) reported the biodegradation and mineralization of CV by a white rot fungus, *Phanerochaete chrysosporium* and observed

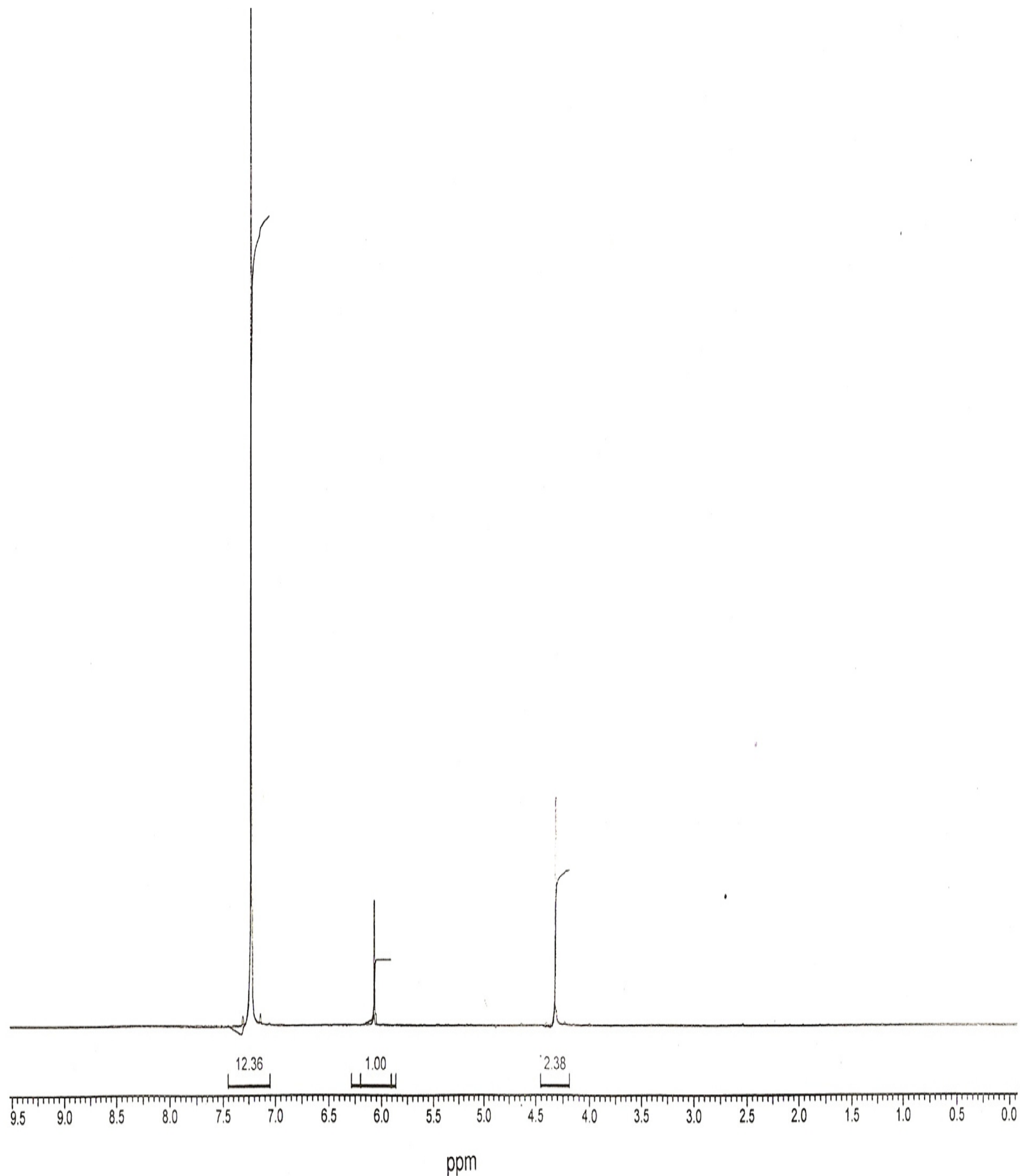

Figure 4. ^{1}HNMR spectrum of Crystal violet after degradation by *P. fluorescens* NCIM 2100.

that, the initial degradation starts with sequential demethylation of the tested dye which resulted into appearance of metabolic products that were identified as N, N, N', N', N''-penta-, N, N, N', N''-tetra- and N, N', N''-trimethylpararosalinine. Chen et al. (2007) found that *Pseudomonas putida* is also capable of degrading the industrial CV dye after determining their demethylation degradation pathway in which a sequential identification of primary and secondary metabolites that is, a series of N-demethylates intermediates, were observed under visible spectral region. Several studies on degradation of crystal violet by the microorganisms have reported that, the dye is relatively resistant to decolorization in the environment (Michaels and Lewis, 1986; Yatome et al., 1981) and

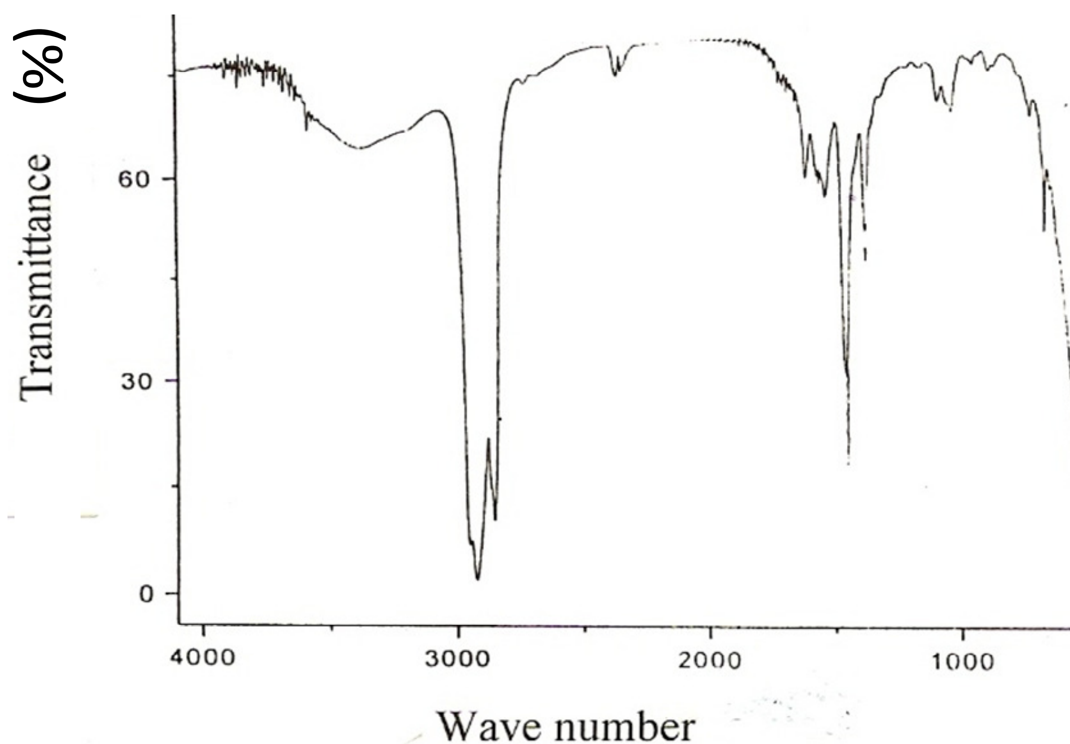

Figure 5. Infra red spectrum of crystal violet before degradation by *P. fluorescens* NCIM 2100.

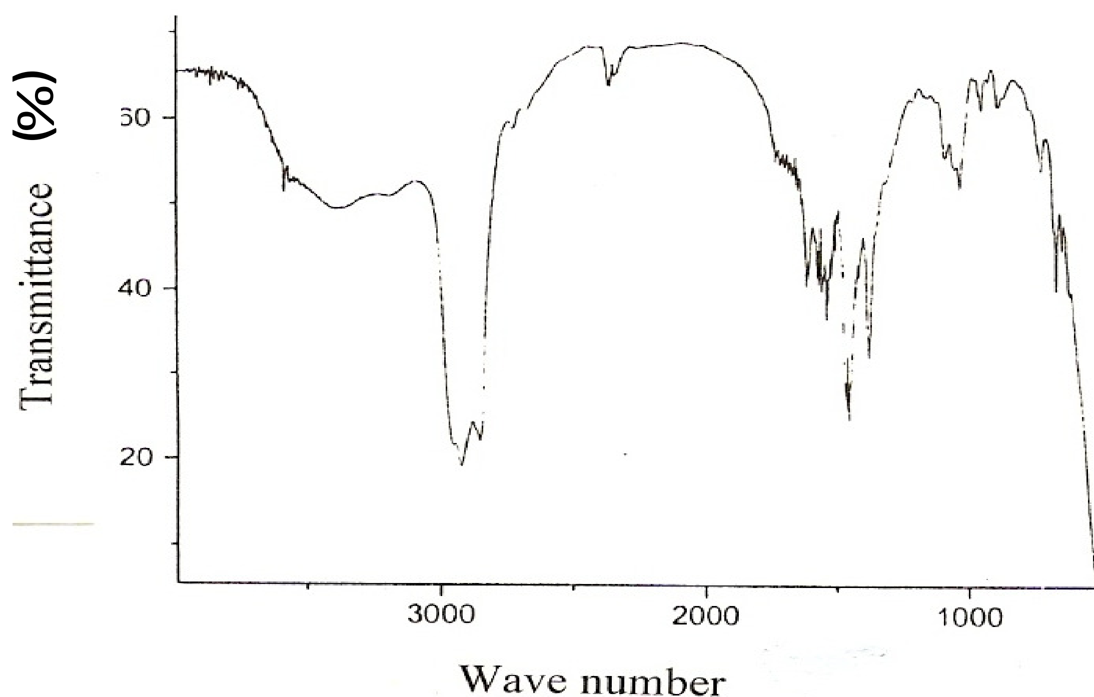

Figure 6. Infra red spectrum of crystal violet after degradation by *P. fluorescens* NCIM 2100.

attributed to the fact that CV is toxic to several bacterial strains and inhibits their growth and activity during treatment process.

Conclusion

Our findings suggest that, crystal violet is non-toxic to the

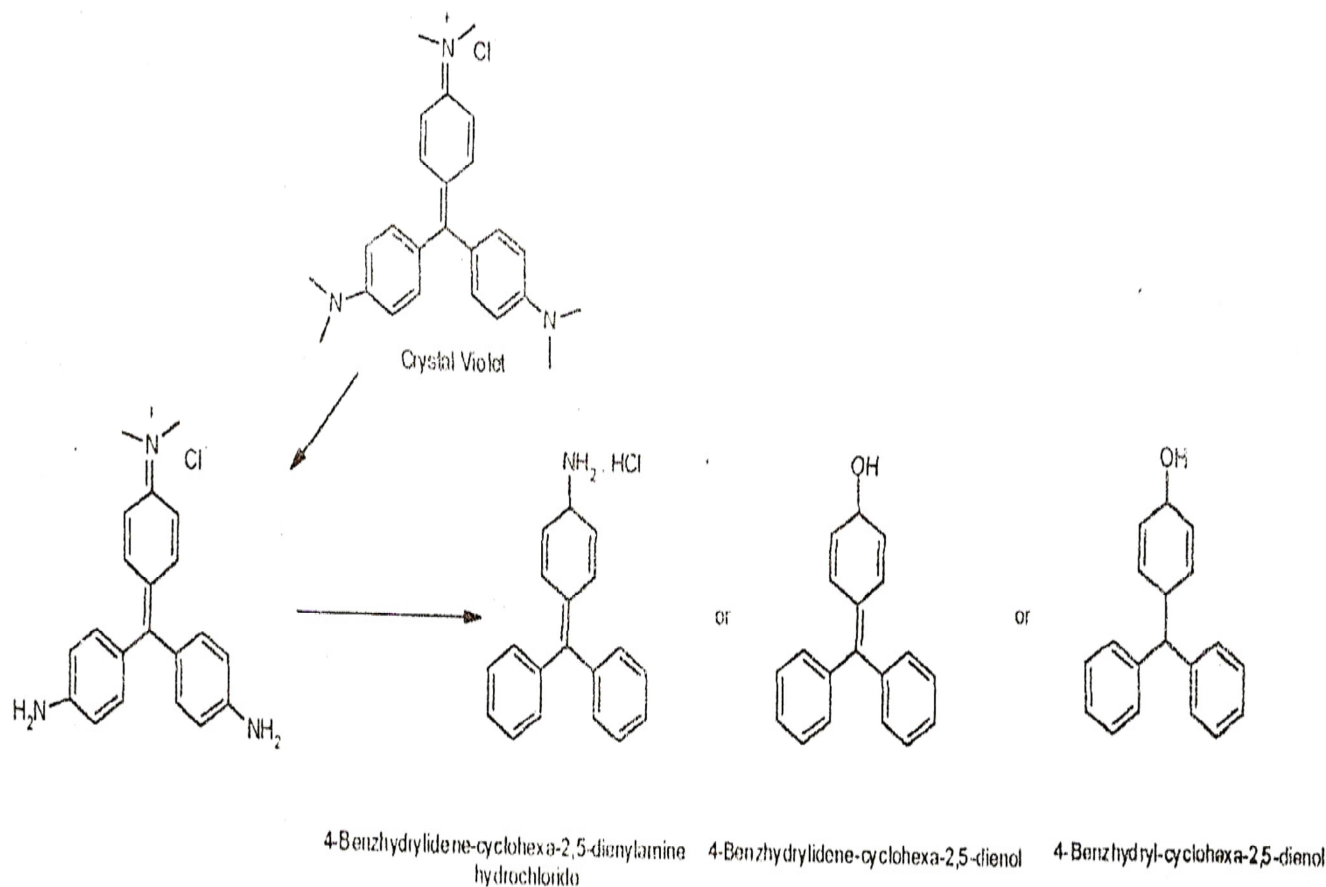

Figure 7. Possible pathway of degradation of crystal violet.

test bacterium strain, *P. fluorescens* NCIM 2100, which has the capability to successfully degrade crystal violet in sequential cycles. Further studies on enzymatic system responsible for CV degradation would enhance our understanding in determining the potential of this bacterial strain for effective bioremediation of the test dye effluents.

REFERENCES

Ayed L, Cheriaa J, Laadhari N, Cheree A, Bakhrouf A (2009). Biodegradation of crystal violet by an isolated *Bacillus* sp. Ann. Microbiol. 59: 267-272.

Azmi W, Sani RK, Banerjee UC (1998). Biodegradation of triphenylmethane dyes. Enzyme Microb. Technol. 22: 185-191.

Bangert R, Aichele W, Schollmeyer E, Weimann B, Herlinger H (1977). Photo-oxidation von Malachitgrum and Kristall-violett. Melliand Textilber, 5: 399-404.

Black JJ, Holmes M, Dymerski PP, Zapisek WF (1980). Fish tumor pathology and aromatic hydrocarbon pollution in a Great lakes estuary. In: Afghan BK, Mackoy D (eds). Hydrocarbon and Halogenated Hydrocarbons in the Aquatic Environment. Plenum Press, New York, pp. 559-565.

Buckingham J, Macdonald F (1996). Dictionary of Organic Compounds. 6th Edn. Electronic Publishing Division, New York.

Bumpus JA, Brock B J (1988). Biodegradation of Crystal Violet by the white rot fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 54: 1143-1150.

Chaudhary GK, Chapalamadugu S (1991). Biodegradation of halogenated organic compounds. Microbiol. Rev. 55: 59-79.

Chen CC, Liao HJ, Cheng CY, Yen CY, Chung YC (2007). Biodegradation of Crystal violet by *Pseudomonas putida*. Biotechnol. Lett. 29: 391-396.

Dyer JR (1991). Application of Absorption Spectroscopy on Organic Compounds. Prentice Hall, Englewood Cliffs, New Jersey.

Gregory P (1993). Dyes and dyes intermediates. In: Kroschwitz JI (ed). Encyclopedia of Chemical Technology. Vol. 8, John Wiley & Sons, New York, pp. 544-545.

Kar S, Swaminathan T, Baradarajan A (1997). Biodegradation of phenol and cresol isomer mixture by *Arthrobacter.* World J. Microbiol. Biotechnol. 13: 659-663.

Kobayashi K, Katayama-Hirayama K, Tobita S (1997). Hydrolytic dehalogenation of 4-chlorobenzoic acid by *Acinetobacter* sp. J. Gen. Appl. Microbiol. 43: 105-108.

Leahy JG, Colwell RR (1990). Microbiol degradation of hydrocarbons in the environment. Microbiol. Rev. 54: 305-315.

Mark TS, Smith ARW, Qurik AV (1984). Degradation of 4-chlorobenzoic acid by *Arthrobacter* sp. Appl. Environ. Microbiol. 48: 1020-1025.

Michaels GB, Lewis DL (1986). Microbial transformation rates of azo and triphenylmethane dyes. Environ. Toxicol. Chem. 5: 161-166.

Pandey BV, Upadhyay RS (2006). Spectroscopic characterization and identification of *Pseudomonas fluorescens* mediated metabolic product of Acid Yellow-9. Microbiol. Res. 161: 311-315.

Reife A (1993). Dyes: Environmental Chemistry. In: Kroschwitz JI (ed). Encyclopedia of Chemical Technology. Vol. 8, John Wiley & Sons, New York, p. 754.

Spain JC (1995). Biodegradation of nitroaromatic compounds. Ann. Rev. Microbiol. 49: 523-555.

Stolz A (2001). Basic and applied aspects in the microbial degradation

of azo dyes. Appl. Microbiol. Biotechnol. 56: 69-80.
Upadhyay RS (2002). Microbial Bioremediation of textile effluents. In: Singh VP, Stapleton Jr. RD (eds). Biotransformation: Bioremediation Technology for Health and Environmental Protection. St. Apleton, Elsevier Science BV, Amsterdam, The Netherlands, pp. 331-348.
Yatome C, Ogawa T, Koga D, Idaka E (1981). Biodegradability of azo and triphenylmethane dyes by *Pseudomonas pseudomallei* 13NA. J. Soc. Dyers Colour, 97: 166-169.
Yatome C, Ogawa T, Matsui M (1991). Degradation of Crystal Violet by *Bacillus subtilis*. J. Environ. Sci. Health, A26: 75-87.