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Biodegradation of crystal violet by an isolated *Bacillus* sp.

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Abstract - Synthetic dyes are widely used in the textile, cosmetic, printing, drug, and food processing industries. Triphenylmethane dyes belong to the most important group of synthetic dyes. They are generally considered as the xenobiotic compounds, which are very recalcitrant to biodegradation. *Bacillus* sp., was isolated from the treatment plant effluent of a textile and dyeing industry (SITEX) located in Ksar Hellal, Tunisia, decolorizes crystal violet (500 ppm) within 2.5 h under shaking condition at pH 7 and temperature 30 °C. The effect of dye concentration, temperature and initial pH of the solution were studied. The results obtained from the batch experiments revealed the ability of bacteria in removing dye. UV-Vis spectroscopy and FTIR analysis of samples before and after dye decolorization in culture medium confirmed decolorization of crystal violet. The phytotoxicity and microbial toxicity studies of extracted metabolites suggest the less toxic nature of them.

Key words: decolorization, *Bacillus* sp., trihenylmethane dyes, crystal violet, phytotoxicity.

INTRODUCTION

Large numbers of chemically different dyes are used for various industrial applications and significant proportion appears in the form of wastewater and is spilled into the environment. Improper chemical disposal of dyes leads to the reduction in sunlight penetration in receiving water bodies that causes decrease in photosynthetic activity and dissolved oxygen. The physical and chemical treatments available have limited use and are having high operational cost (Alexander, 1994) Synthetic dyes used are recalcitrant to be removed by conventional wastewater treatments such as adsorption, photo-oxidation, coagulation, flocculation, photodegradation and chemical degradation and biological process. The biological degradation is highly promising, relatively inexpensive, ecofriendly and presents a reduced sludge production in comparison with chemical-physical treatment. Currently an extensive research is focused to find optimal microbial biomass, which is as cheap as possible for removal of contaminating dyes from large volumes of polluted water (Jadhav and Govindwar, 2006). There are a few reports on biodegradation of triphenylmethane dyes. Some authors have reported degradation by aerobic bacteria or consortium, by yeast, by microalgae and by anaerobic bacteria (Casas *et al*., 2009). The triphenylmethane groups of dyes are used extensively in textile dyeing and dye-stuff manufacturing industries, as a biological stain and in paper printing. Some of the triphenylmethane dyes are used as dermatological agents, the best among them is gentian violet, which has also been used for oral consumption for pinworms and tropical application in human and in domestic animals and has been shown to be effective in controlling fungal growth under varying conditions(Au *et al*., 1978).

Crystal violet is a triphenylmethane dye used as a biological stain or as dermatological agent. For many years it was used as oral medication for treatment of pinworms and other tropical diseases because of its great effect in controlling fungal growth under varying conditions (Casas *et al*., 2009). 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 (Au *et al*., 1978). Unfortunately, wastewater treatment facilities are often unable to remove commercial dyestuffs including crystal violet dye and this effluent contaminates aqueous habitats (Micheals and Lewis, 1985). Crystal violet was also found to cause reduced RNA and protein synthesis and decreased oxygen consumption in rabbit granulation tissue (Diachenko, 1979). Diachenko, 1979 reported the deposition of crystal violet and malachite green and aniline in the sediments and water of Buffalo River, New York, USA thus posing a threat to aquatic environment and human population. It was shown that theses dyes have mutagenic and carcinogenic properties. These chemicals have been suggested to be responsible for the promotion of tumor growth in several bottom feeding species of fish (Diachenko, 1979).

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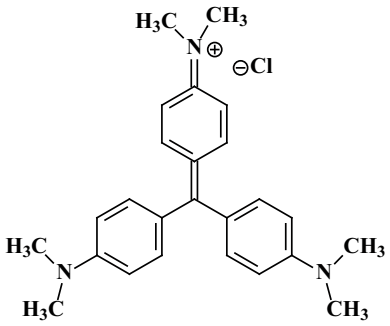
TABLE 1 - Chemical structure of crystal violet

Dye	Structure	λ_{max} (nm)	Chemical class
Crystal violet		592	Triphenylmethane

The textile and dyestuff industrial wastes are generally treated by physicochemical methods (including adsorption, chemical precipitation and flocculation, oxidation by chlorine, H_2O_2 and ozone electrolysis, electrochemical treatment and ion pair extraction), which are significantly different in color removal, volume capability, operating speeds and capital costs. It was found that 15% of triphenylmethane dyes, which are currently manufactured, are lost to waste water as a result of inefficiency in the chemical and physical treatment processes (Chen *et al*., 2008). Yatome *et al*. (1993) reported that four triphenylmethane dyes (basic fuchsin, methyl violet, crystal violet and Victoria blue) were biodegraded using *Pseudomonas pseudomollei* 13NA. The half-decolorization time was 54 h for both methyl violet and crystal violet when 4.08 mg/l dye was applied. In addition Yatome *et al*. (1991) noticed that the decolorization of several triphenylmethane dyes could be obtained using *Bacillus subtilis*, *Escherichia coli* and some species of *Pseudomonas* (*P. cepacia, P. cruciviae* and *P. stutzeri*). They reported *B. subtilis* remarkably decolorized 2.85 mg/l of crystal violet after 24 h.

This study aims to investigate the potential of a *Bacillus* sp. isolated from textile wastewater for decolorizing a solution containing a triphenylmethane dye, crystal violet. Correlation of the kinetic properties with dye concentration and other rate-dependent environmental parameters (dye concentration, pH and temperature) were characterized. The phytotoxicity and microbial toxicity of the products formed after decolorization were studied.

MATERIAL AND METHODS

Dyes and chemicals. The triphenylmethane dye Crystal Violet (CV) was obtained from the Sigma Chemical Company, MO, and USA. The dyes concentration was measured with a spectrophotometer UV-Visible (UV-2401 PC, Shimadzu, Japan) at maximum absorption wavelengths λ_{max} = 592 nm (Table 1).

Growth medium. Mineral salts medium (MSM) were prepared by adding the following components (in g): $MgSO_4$ (0.1), $(NH_4)_2SO_4$ (0.6), NaCl (0.5), K_2HPO_4 (1.36), $CaCl_2$ (0.02), $MnSO_4$ (1.1 mg/l), $ZnSO_4$ (0.2 mg/l), $CuSO_4$ (0.2 mg/l), $FeSO_4$ (0.14 mg/l) in 1000 ml of distilled water.

The MSM pH was adjusted to 7.0 (Moutaouakkil *et al*., 2003; Ayed *et al*., 2009) and was supplemented with 1.25 g/l glucose and 3 g/l yeast extract at different concentration unless mentioned. The yeast extract, glucose and dyes were added to sterilized MSM from their respective filter sterilized stock solutions.

Isolation and screening of dye degrading microorganism. The Agar-plate screening method used for dyes decolorization was described by Khehra *et al*., (2005). Briefly, activated sludge samples were collected from a textile dyeing industry and screened for dye decolorization organisms. Samples of activated sludge (10 g) were added to 100 ml of mineral basal medium (6 g/l Na_2HPO_4 , 0.5 g/l NaCl, 3 g/l K_2HPO_4, 1 ml/l $MgSO_4$, 0.14 g/l NH_4Cl) and kept on an orbital shaker (SI-600, Lab Companion, Jeio-tech, Korea) at 150 rpm at 37 °C for 12 h. After 48 h of incubation, 1 ml of the culture broth was appropriately diluted and plated on MSM-agar containing crystal violet (500 ppm). The colonies were selected on their ability to form clear zones on the MSM-agar plates. Such colonies were subsequently transferred to MSM medium containing different concentrations of dyes. Crystal violet was decolorized on solid media by *Bacillus* sp. which was used for decolorization assay.

Decolorization assay and COD removal. Decolorizing activity was expressed in terms of decolorization percentage as indicated by Khehra *et al*. (2005) and determined by monitoring the decrease of the absorbance at λ_{max} = 592 nm CV. Chemical oxygen demand (COD) was determined spectrometrically by 5B-1 Quick COD analyzer (LianHua Environmental Instrument Institute, Langzhou, PR China). Experiments were performed in flasks containing 100 ml MSM medium and 500 ppm of dye. The flasks were inoculated with 5 ml *Bacillus* sp. (isolated from industrial textile wastewater). The inoculum's size were adjusted at 1 OD at λ = 600 nm (7 x 10^8 cells/ml) and incubated under shaking condition (150 rpm) at 37 °C (El-Naggar *et al*., 2004). The samples were withdrawn at different time intervals and analysed for decolorization assay. The flask containing dyes and MSM without the bacteria served as control. From time to time a 5 ml samples was taken and centrifuged at 10000 rpm for 15 min. The absorbance of supernatant was spectrophotometrically determined using spectrophotometer (UV-2401 PC, Shimadzu). All assays were performed in triplicate and compared with the control. Decolorization activity was calculated as described by Parshetti *et al*. (2006), Daneshvar *et al*. (2007) and Ayed *et al*. (2009).

$$\text{Decolorization \%} = \frac{(I - F)}{I} \times 100$$

were I = initial absorbance and F = absorbance of decolourized medium.

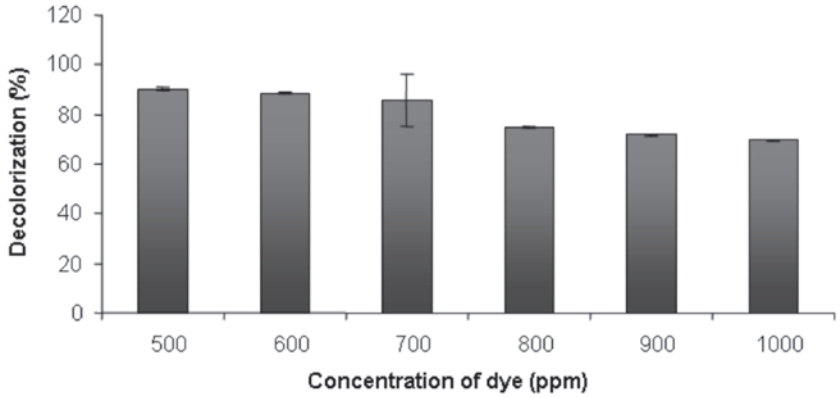


FIG. 1 - Effect of initial concentration of crystal violet on decolorization by *Bacillus* sp. in MSM. Temperature = 30 °C, pH = 7.0, bacterium concentration = 7 x 10^8 cells/ml.

Decolorization at different concentration of dye. The decolorization of crystal violet was tested at different concentrations of the mentioned dyes (500, 600, 700, 800, 900 and 1000 ppm) as described by El-Naggar *et al*. (2004). The respective dyes were added separately to 250 ml Erlenmeyer flasks containing 100 ml of MSM medium. The flask containing dyes was inoculated with 5 ml of *Bacillus* sp. suspension which was adjusted at 1 OD at λ=600 nm (7 x 10^8 CFU/ml). The inoculum size was incubated under shaking condition (150 rpm) at 30 °C.

Decolorization at different pH and temperatures. The decolorization studies of crystal violet (500 ppm) were carried out at pH 3, 5, 7, 9 and 11 by adjusting pH of the medium using 0.1 N HCl and 0.1 N NaOH. *Bacillus* sp. was grown at respective pH at 30 °C for 96 h under shaking condition and used for decolorization studies. Similarly the decolorization of crystal violet (500 ppm) was carried out at different temperatures 5, 25, 30, 40 and 50 °C by using 96 h grown bacterium at pH 7. Flasks were incubated at respective temperatures for 30 min before addition of the dye and decolorization was observed.

UV-Vis spectral analysis and Fourier Transform Infrared Spectroscopy (FTIR). Decolorization was monitored by UV-Vis spectroscopic analysis (Hitachi U-2800), whereas biodegradation was monitored by FTIR. The same sample was used for UV–Vis spectral analysis, and FTIR analysis. Spectral analysis was carried out using UV-Vis spectrophotometer (2401 PC, Shimadzu), and changes in its absorption spectrum (400-800) were recorded. FTIR analysis was carried out using Perkin Elmer 783 Spectrophotometer and changes in % transmission at different wavelengths were observed. The FTIR analysis of extracted metabolites was done on Perkin Elmer, Spectrum one instrument and compared with control dye in the mid IR region of 400-4000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio 2:200, pellets were fixed in sample and the analysis was carried out.

Phytotoxicity and microbial toxicity studies. The phytotoxicity studies were carried out using 500 ppm concentration of crystal Violet and its extracted metabolites using seeds of *Triticum aestivum* and *Sorghum bicolor*, with distilled water as a control. The microbial toxicity of control CV and its degradation products (500 ppm) was carried out using *Bacillus* sp. on Mueller and Hinton agar plate having composition (g/l): beef infusion solids, 4.0; starch, 1.5; casein hydrolysate, 17.5; agar, 15.0).The pH of the medium was adjusted to 7. The plates are incubated at 30 °C and the zones of inhibition are measured.

RESULTS AND DISCUSSION

Decolorization performance and removal of COD by *Bacillus* sp. under shaking conditions

The effect of initial concentration of CV on decolorization of CV was studied at different initial concentrations of CV dye 500, 600, 700, 800, 900 and 1000 ppm. The percent decolorization decreased with increase in CV concentration at 24 h of incubation (Fig. 1). Results show that with 500 ppm dye concentration in MSM, 100% of the colour was removed after 2.5 h (Fig. 2). COD removal was 85%. Dafale *et al*. (2008) showed that 70% COD was removed during shaking condition for Remazol Black B (RBB). *Bacillus subtilis* was able to decolorize crystal violet dye up to 40 mg/l and the maximum decolorization rate (1.25 mg/l/h) was obtained when 15 mg/l crystal violet was applied. Other investigators reported that, crystal violet can be degraded by *Nocardia corralina* and a complete removal of 0.49 ppm crystal violet was observed after 24 h of incubation with a decolorization rate of 0.039 mg/l/h (Yatome *et al*., 1993). Moreover, Bumpus and Brock (1988) reported that, a complete degradation of 5 mg/l crystal violet by *Phanerocheate chrysosporium* was carried

FIG. 2 - Decolorization of crystal violet (500 ppm) within 2.5 h of incubation by *Bacillus* sp. Temperature = 30 °C, pH = 7.0, bacterium concentration = 7 x 10^8 cells/ml. C_1: crystal violet before degradation; D_1: crystal violet after degradation.

TABLE 2 - Effect of pH and temperature on the decolorization of crystal violet

Parameters	pH					Temperature (°C)				
	3	5	7	9	11	5	25	30	40	50
Decolorization (%)	80	82	90	84	75	76	90	92	80	77
Decolorization time (h)	6.5	5.5	2.5	3.5	4.8	9.0	4.0	3	8	10

out within 72 h of incubation. (Khehra *et al*., 2005) suggested that the decrease in decolorization efficiency might be due to the toxic effect of dyes.

The various physicochemical parameters such as pH, temperature were found to affect the rate of decolorization as well as percent decolorization data shown in Table 2. The pH optimum for decolorization was 7 and temperature optimum was 30 °C.

The results showed essentially no thermal inactivation of decolorization activity under operational temperatures, indicating that *Bacillus* sp. could acclimatize them to a broad range of temperature of practical dyeing wastewater. Previously, several researches had proved that biosorption processes using algae were highly pH dependent and the most important parameters to be considered (Aksu and Tezer, 2005; Kumar *et al*., 2006).

These results indicate that *Bacillus* sp. could treat practical basic dyeing wastewater at normal operational temperature and largely decreases the economic cost for acidification. The trend of pH dependence of decolorization is similar to that observed in *Pseudomonas luteola* (Kumar *et al*., 2006). For *Bacillus* sp., the isoelectric point would be at a pH of 7.0 as. In addition we noted that, at lower pH value, the H^+ ions compete effectively with dye cations, causing a decrease in colour removal efficiency. Furthermore, at higher pH above this point charge, the surface of biomass gets negatively charged, which enhances the positively charged dye cations through electrostatic force of attraction. This can be observed from the considerable increase in colour removal efficiency from 10 to 25% for an increase in pH from 3.0 to 4.0 (Daneshvar *et al*., 2007).

Degradation product analysis

UV-Vis analysis (400-800) of supernatants of different time intervals showed decolorization and decrease in dye concentration from batch culture. Crystal violet displays three absorption peaks of CV at λ_{max} 592, 411, 330 nm in the UV-vis spectra. The intensity of these peaks decreased remarkably after aerobic treatment due to decolorization. Further, a new peak was observed at λ_{max} = 265 nm consistently in all the spectra of final samples. This suggests the formation of an intermediate during aerobic decolorization of CV. Peak observed at 592 nm (0 h) was decreased without any shift in λ_{max} up to complete decolorization of medium (2.5 h) (Fig. 3). 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 (Fig. 3).

Remarkable variations in the fingerprint region (1500 to 500 cm^{-1}) of the FTIR (Fig. 4A) spectroscopy of control CV and 2.5 h extracted metabolites indicate biodegradation of the dye by *Bacillus* sp. FTIR spectra of control CV showed the specific peaks in fingerprint region (1500 to 500 cm^{-1}) for the mono-substituted and para disubstituted benzene rings which is supporting to the peak at 1587 cm^{-1} for the C=C stretching of the benzene ring. Also the peak at 1171 cm^{-1} for aromatic C-N stretching vibrations. At 2923 cm^{-1} showed C-H asymmetric stretching and free $-NH_2$ group showed amide antisymmetric stretching vibration at 3413 cm^{-1}. The peak at 758 cm^{-1} indicates symmetric out of

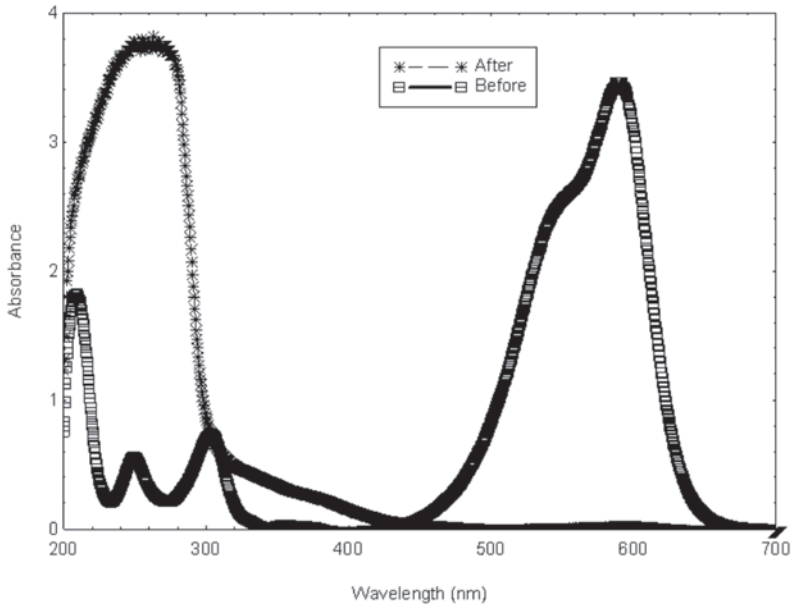


FIG. 3 - UV-Vis spectra of crystal violet (500 ppm) biodegraded by *Bacillus* sp. before and after optimised condition. T = 30 °C, pH = 7.0, bacterium concentration = 7 x 10^8 cells/ml.

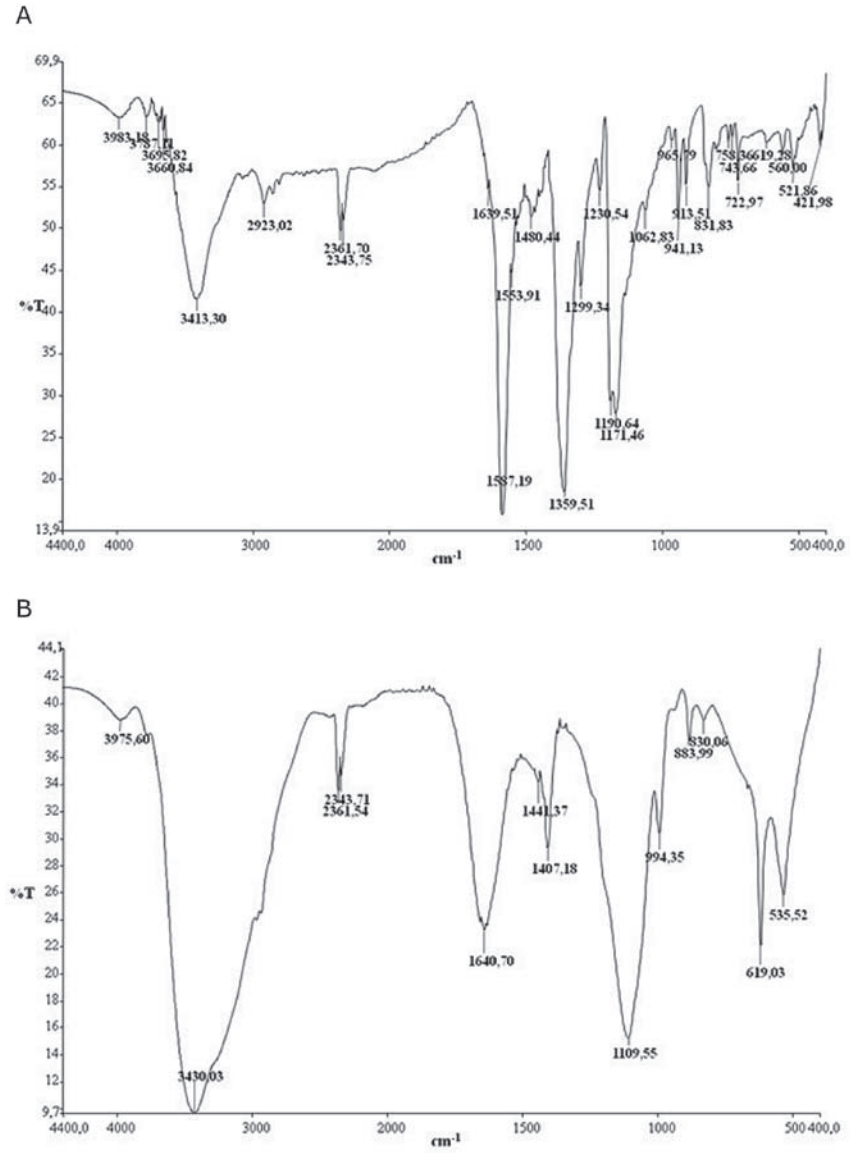


FIG. 4 - FTIR analysis of control crystal violet (A) and its degradation products (B).

plane bending of the ring hydrogen's. The peak at 1480 cm^{-1} indicate OH bend, at 1639 cm^{-1} showed C=C stretching bend. Peaks corresponding to C-O stretch at 1190 and 1171. The peaks at 743 and 722 correspond to symmetric out of plane bending of the ring hydrogen's.

FTIR spectra of degradation product (Fig. 4B) displays peak at 3430 cm^{-1} shown in the spectrum of CV bead is attributed to stretching vibration of hydroxyl group. The bands observed at 1640 cm^{-1} correspond to N–H bending vibrations. The peaks at 1444, 994 and 1407 indicate a sideways ring stretch absorbs $=CH_2$ out of plane twist and $-CH_2$ scissoring bend. The absorption bands below 900 cm^{-1} indicate aromatic nature of the compound. Absence of peaks at 688 cm^{-1}, 726 cm^{-1}, 765 cm^{-1} and 827 cm^{-1} indicates loss of aromaticity or benzene ring.

Phytotoxicity and microbial toxicity studies

Disposal of the untreated dyeing effluent, without any treatment, in water bodies cause serious environmental and health hazards. Besides the use of dye contaminated water is harmful to agriculture. Thus it was of prime interest to assess the phytotoxicity of the dye and its extracted metabolites after degradation. The relative sensitivities towards the crystal violet and its degradation products in relation to *S. bicolor* and *T. aestivum* seeds are presented in Table 3. Phytotoxicity study shows well germination rate as well as significant growth in shoot and root for both the plants, grownin metabolites extracted after decolorization, as compared to the dye (500 ppm). Table 3 displays the comparative growth of plumule and radical in *S. bicolor* and *T. aestivum* seeds. Hence phytotoxicity studies revealed that biodegradation of the dye by a microbial culture, resulted in its detoxification. Thus treated effluent can be used for ferti-irrigation.

Microbial toxicity on *Bacillus* sp. showed growth inhibitory zone (1.0 cm) surrounding the well containing dye, while product did not show inhibitory zone also confirmed the nontoxic nature of the extracted metabolite.

CONCLUSION

The present study revealed the ability of *Bacillus* sp. to decolorize crystal violet. The results showed that the decolorization depend on the dye concentration, pH and temperature. Over the range

TABLE 3 - Phytotoxicity study of crystal violet and its extracted metabolites formed after biodegradation

Parameters studied	*Triticum aestivum*			*Sorghum bicolor*		
	Distilled water	Crystal violet (500 ppm)	Extracted metabolites (500 ppm)	Distilled water	Crystal violet (500 ppm)	Extracted metabolites (500 ppm)
Germination (%)	100	73	100	100	53	90
Plumule (cm)	14.64 ± 0.42	0.2 ± 0.1	4.5 ± 0.39	3.68 ± 0.15	0.12 ± 0.021	2.48 ± 0.35
Radical (cm)	9.13 ± 0.60	0.23 ± 0.06	3.83 ± 0.378	8.33 ± 0.72	0.34 ± 0.051	3.68 ± 0.22

Values of germinated seeds are mean of three experiments ± Standard Deviation.

of 5-50 °C the decolorization rate increased with the temperature rise. The optimal decolorization pH and temperature is attributed at pH 7 temperature 30 °C. In this study we demonstrated a typical time-dependent UV-Vis spectrum of CV solution during biodegradation (Fig. 3). The absorbance peaks, corresponding to dye, diminished which indicated that the dye had been removed. The spectrum of CV in visible region exhibited a main peak with a maximum at 592 nm. The decrease of absorbance peak of CV at λ_{max} = 592 nm in this figure indicated a rapid degradation of the dye. According to the pervious literature (Chang and Kuo, 2000) decolorization of dyes can be due to adsorption to biomass or biodegradation. If the dye removal is attributed to biodegradation, either the major visible light absorbance peak will disappear or a new peak will appear. In addition, extra absorbance peaks appeared in decolorized solution, probably resulting from the absorbance of metabolites or degraded fragments of dye molecules. These results indicate that the color removal by *Bacillus* sp. may be largely attributed to biodegradation. CV was degraded into non-toxic compound by *Bacillus* sp. As previous reports showed crystal violet degradation into leucocrystal violet this was found to be equally toxic to malachite green. This strain has also the ability to decolorize other dyes including textile dyes and the use of cheap sources such as molasses for decolorization.

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