Decolorization of Malachite Green by Newly Isolated *Bacillus Strain* MTCC -3330

Nand Lal*, Alok Kumar Srivastava
Chemistry Department, V.S.S.D.College, Kanpur-208002, India
**To whom correspondences should be addressed*
E-mail: drnandlal71@rediffmail.com

Received February 14, 2011, Revised manuscript received September 03, 2011, Accepted October 21, 2011

Abstract

A isolated bacterium species was studied for its degradation /biodegradation potential using Malachite Green (MG) as a model dye pollutant. MG (0.02 mM) was completely decolorized within 30 h by isolated bacterium sp. in the culture medium. The decolorization was confirmed by UV-VIS spectrophotometer. The initial dye solution showed high peak at the wavelength of 617 nm (λmax of the dye). The decolorized dye showed disappearance of peak, which indicated that the decolorization is due to dye degradation. The result of morphological, physiological biochemical test and list of carbohydrates supporting the growth of the isolated bacterium have been mentioned.

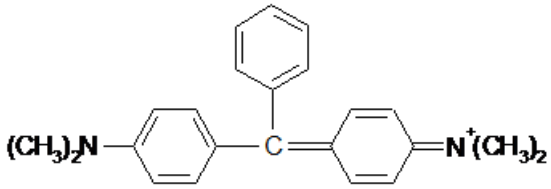
Keywords: Malachite Green (MG), Decolorization, Toxicity, Biosorption, *Bacillus Strain* MTCC -3330

1. Introduction

A major class of synthetic dyes is widely used in textile, paper, printing, colour photography, rubber product, pharmaceuticals, food colouring, cosmetic and many other industries [1]. In textile industries, during dyeing process, upto 15% of the total textile dye remains unreacted and are directly lost in the effluents [2,3]. Some of the dyes and dye degradation products are reported to be carcinogenic and mutagenic in nature [4-7]. Thus, the effluents must be treated before releasing into the natural environment. MG is a tri-phenylmethane dye which has been used extensively for dyeing silk, wool, jute, leather, ceramic and cotton [8]. It is highly soluble in water and has also been used in aquaculture industry as a fungicide, parasiticide and disinfectant [9]. MG has been banned in the United States since 1983 in food-related applications. It is banned in the UK also [10]. Malachite green, a fungicide, was banned internationally in the 1990s, but still illegally used in some salmon hatcheries and for juvenile fish [11]. It is highly toxic to mammalian cells [12-14] at concentrations as low as 0.1 μg/ml, and has been reported to enhance tumor formation in rats [15]. MG was nominated by the Food and Drug Administration as a priority chemical for carcinogenicity testing by the National Toxicology Programe [8]. Reduction of MG to leucomalachite green has been reported but leucomalachite green is also not environmentally friendly [16-18]. However, biodegradation of MG into non-toxic compounds [19] and into simpler metabolic intermediates [20] have been reported.

In addition, there is a need to isolate such type of micro-organisms which could remove MG completely. This short communication reports a strain of Bacillus sp., which completely removes malachite green from its culture medium.

Figure 1. Structure of Malachite Green



2. Methodology

Beef extract (bacteriological) and peptone (bacteriological) were obtained from BDH and Qualigens Chemical India Pvt. Ltd. MG dye was obtained form Ranbaxy laboratories India Pvt. Ltd. All other chemicals and reagents used in this research were obtained either from Central Drug House (P) Ltd., Mumbai or from Loba Chemie Pvt. Ltd., Mumbai.

The bacterial strain has been isolated from a soil sample collected from the site where waste waters of Sanjai Paper and Chemical Industries, Khalilabad, Sant Kabir Nagar (U.P.) India is discharged. 1g of soil sample was extracted with 10 ml of quartz double distilled sterilized water. The soil extract was diluted serially 1:10 using

sterilized double distilled water and 1 ml of 7^{th} dilution was plated on sterilized nutrient agar [13] contained in petridishes. The culture medium composition was 0.5 g peptone, 0.3 g beef extract, 0.5 g NaCl, 1.5 g agar and 1g saw dust in 100 ml double distilled water. There visibly distinct types of bacteria appeared in the petridishes were purified by four transfers of the micro organisms on sterilized agar plates using streaking technique [14]. The purified bacterial strain were tested for their capability to degrade different dyes, only one bacteria was found having the capability to degrade some dyes, was got identified by the MTCC centre at the Institute of Microbial Technology, Chandigarh, India . The strain has been deposited at same centers as MTCC-3330.

The decolorization of malachite green by the isolated bacterial strain was studies in sterilized liquid culture medium containing 0.5 g peptone, 0.3 g beef extract and 0.5 g NaCl in 100 ml water in 250 ml culture flasks in which varied concentrations of malachite green (0.01 mM to 1.0 mM) were added. 1 ml of bacterial suspensions O.D. 1.55, λ = 540 nm were aseptically inoculated in each culture flask. The controlled culture flasks were not inoculated with bacterial suspension. Triplicates of culture flasks were set for each concentration of MG and the culture flasks were incubated in an orbital shaker maintained at 30℃ and 130 rpm. The bacterial suspension was prepared by transferring bacterium from agar slants to the liquid culture medium mentioned above and growing the culture under similar conditions till O.D., reaches a value of 1.55, λ = 540 nm.

In order to study the removal of MG from the culture medium, an aliquot of 2 ml was withdrawn at different time intervals, bacterium was removed by centrifugation using Sigma (Germany) refrigerated centrifuge model K-30 for 20 minutes at 4℃ & 4000 g (~16000 rpm) and absorbance of the supernatant solution was determined at λ = 617 nm using UV/VIS spectrophotometer Hitachi (Japan) model U-2000. In cases where initial O.D. was very high, the supernatant solution was appropriately diluted in order to bring concentration in the range which obeys Lambert Beer's Law. MG obeys Lambert-Beer's Law upto 0.06 mM concentration. The absorbance verses time curves were drawn to show the removal of MG from the culture medium. Decolorization activity was calculated as follows:

$$\text{Decolorization}(\%) = \frac{\text{Intial absorbance - Observed absorbance}}{\text{Initial absorbance}}$$

In order to confirm that MG has been completely removed from the culture medium, the UV/VIS spectra of the supernatant solutions prepared by withdrawing culture media at the start and at the end of decolorization of MG were scanned in the range of 190-800 nm. Supernatant solution of culture medium with the bacterium alone was analyzed UV/VIS spectrophotometer. The different morphological, physiological and biochemical tests on the isolated bacterium were done at MTCC, Institute of Microbiology Technology, Chandigarh, India, utilizing their services.

Table 1. Results of morphological tests on the isoloated bacterium

Tests		Results
Colony Morphology:	Configuration	Round
	Margin	Wavy
	Elevations	Convex
	Surface	
	Density	
	Pigments	
Gram's reaction:		+ve
	Shape	Rods
	Size	Long
	Arrangement	Mostly single
Spore:	Endospore	+ve
	Position	Central
	Shape	Round
	Sporangia bulging	+
Capsule		
Motility		+
PHB accumulation		
Fluorescence (UV)		-

3. Results and Discussions

Under morphological examination the bacterial colony has been found to be of round configuration, wavy margin and convex surface. The bacterium is Gram-Positive, rod shapped and gives positive endo-spore test. (Table 1) Gram Staining is an empirical method of

differentiating bacterial species into two large groups i.e. Gram-positive and Gram-negative which involves the application of a series of dyes that leaves some bacteria purple and other's pink, bacteria that stain purple are termed Gram-positive, and those that stain pink, Gram-negative. The method is named after its inventor, the Danish scientist Hans Christion Gram (1853-1938), who developed the technique in 1884 [21]. Gram-positive bacteria have a thick mesh like cell wall, gram-negative have a thin cell wall and an outer phospholipids bilayer membrane. The specific strain reaction of bacterium results from the structure of its cell wall. Positive endo-spore tests indicates under unfavourable conditions (i.e. under conditions of extreme temperature, dryness, or lack of food), the bacterium develops a waterproof cell wall that protects it from being dried out or damaged. Bacteria have been known to remain unreactive but alive in the form of endospores for long periods of time, even thousands of years, also called endosporium.

Table 2. Results of physiological tests on the isoloated bacterium

Growth at temperature	Results
4°C	-
10°C	-
15°C	-
22°C	+
26°C	+
30°C	+
37°C	+
42°C	+
55°C	+
65°C	-
Growth pH	
pH 5.0	+
pH 5.7	+
pH 6.8	+
pH 8.0	+
pH 9.0	+
pH 11.0	-
Growth of NaCl (%)	
2.5	+
5.0	+
7.0	-
8.5	-
Growth under anaerobic condition	+

The bacterial strain grows between 22℃ to 55℃ in the pH range 5.0-9.0 (Table 2). This strain can tolerate NaCl upto 5.0% and grow under anaerobic conditions. The results of biochemical tests on the bacterium are shown in (Table 3) and carbohydrates supporting the growth of the bacterium are listed in (Table 4). All the features described in the Tables 1-4 are on the basis of report given by experts at Microbial Type Culture Collection Centre, (MTCC), Institute of Microbial Technology, Chandigarh, India.

Table 3. Results of bio-chemical tests on the isoloated bacterium

Tests	Results
Growth on MaCconkey agar:	
(a) Lac-fermenter	
(b) non-lacfermenter	-
Indole test	-
Methyl red test	-
Voges proskauer test	-
Citrate utilization	
Casein hydrolysis	-
Starch hydrolysis	+
Urea hydrolysis	-
ONPG hydrolysis	+
Nitrate reduction	+
Nitrite reduction	-
H_2S production	-
Cytochrome oxidase test	+
Catalase test	+
Oxidation/fermentation (O/F)	0
Gelatin liquefaction	+
Lysine decarboxylase	-
Ornithine decarboxylase	-

Figure 2 illustrates the decolorization of MG in liquid culture medium at varying concentration (a) 0.01 mM, (b) 0.02 mM, (c) 0.04 mM and (d) 0.02 mM (controlled). As can be seen, 100% decolorization of MG was observed at 0.01 mM and 0.02 mM concentrations within 24 h and 30 h respectively whereas 95% decolorization of MG was observed at 0.04 mM concentration within 50 h. The above data reveals that rate of decolorization was decreased beyond the 0.02 mM concentration, indicating reduction in decolorization with increase in dye concentration. It has been shown that 1 mM concentration of MG completely inhibits the growth of bacterium, which indicates toxicity of MG at higher dye concentration (data not shown). Similar observation was also reported

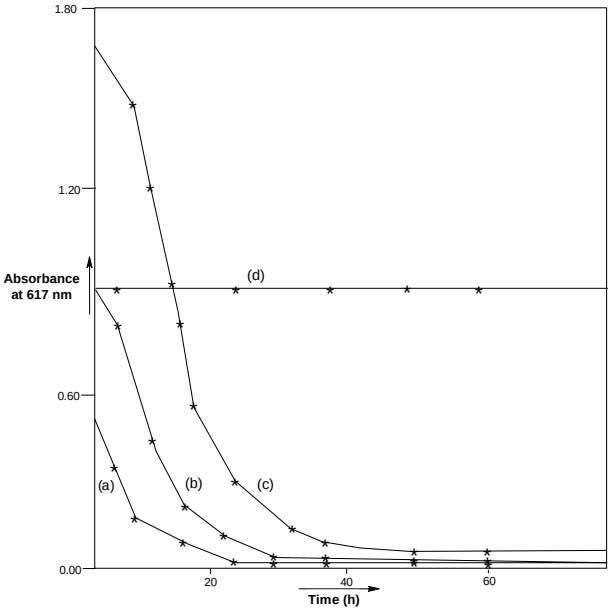
by Cha et al. for inhibition of fungal growth at higher concentration of MG [22].

Table 4. Carbohydrates supporting the growth of the reported bacterium

Carbohydrates	Results
Adonitol	+
Arabinose	+
Cellobiose	+
Dextrose	+
Dulcitol	+
Fructose	+
Galactose	+
Inositol	+
Inulin	-
Lactose	+
Maltose	+
Mannitol	+
Mannose	+
Melibiose	-
Raffinose	-
Rhamnose	+
Salicin	-
Sorbitol	+
Sucrose	+
Trehalose	+
Xylose	+

The UV/VIS spectra of supernatant, corresponding to just after inoculation of the bacterium in culture medium containing 0.02 mM of MG [Fig. 3(a)], after 30 h growth of bacterium [Fig. 3(b)] and controlled experiment in which no bacterium has been inoculated [Fig. 3(c)] where as [Fig. 3(d)] corresponds the UV/VIS spectra of supernatant of the culture medium in which no MG has been added but the bacterium was grown for 30 h. The absorbance was analyzed from 200 nm to 800 nm. The initial dye (just after inoculation) showed high peak at wavelength of 617 nm (λmax of MG) whereas after decolorization, there is no peak observed at 617 nm [Fig. 3(b)]. It is also observed that there is no peak found at wavelength of ~260 nm (λmax of Lucomalachite green). Lucomalachite green is a biodegradation product of MG and equally toxic to malachite green [23] [Fig. 3(b)]. The comparison study of spectra [Fig. 3(b)] and [Fig. 3(d)] also indicates that completely removal of MG from culture medium. These results supporting that the decolorization is most probably due to biosorption [24] of dye in which aromatic ring of dye can take as sole carbon and energy source [25].

Figure 2. Variation of absorbance at 617 nm (λmax of malachite green) of supernatant solutions prepared by withdrawing aliquots at different time intervals from the growth medium containing malachite green of different concentrations. (a) 0.01 mM; (b) 0.02 mM; (c) 0.04 mM; (d) 0.02 mM (control)



4. Conclusions

Newly isolated *Bacillus strain-3330* is a gram positive bacteria gives positive endo-spore test and can work in anaerobic condition. This bacterial culture can also work even in hot condition (growth temperature range 22℃ to 55℃) with either in acidic or in basic medium because of its growth pH range 5.0-9.0. The present study confirms the ability of reported bacterial culture *Bacillus-3330* to decolorize 0.02 mM of triphenyl methane dye MG with decolorization efficiency of 100%. As previous reports showed MG degradation into Leucomalachite green, that is equally toxic to MG [23]. The UV/Visible spectra confirm that there are no peak observed at ~260 nm (λmax of the Leucomalachite green) and at 617 nm (λmax of the MG), which reveals that completely removal of MG from culture medium is due to biosorption of dye.

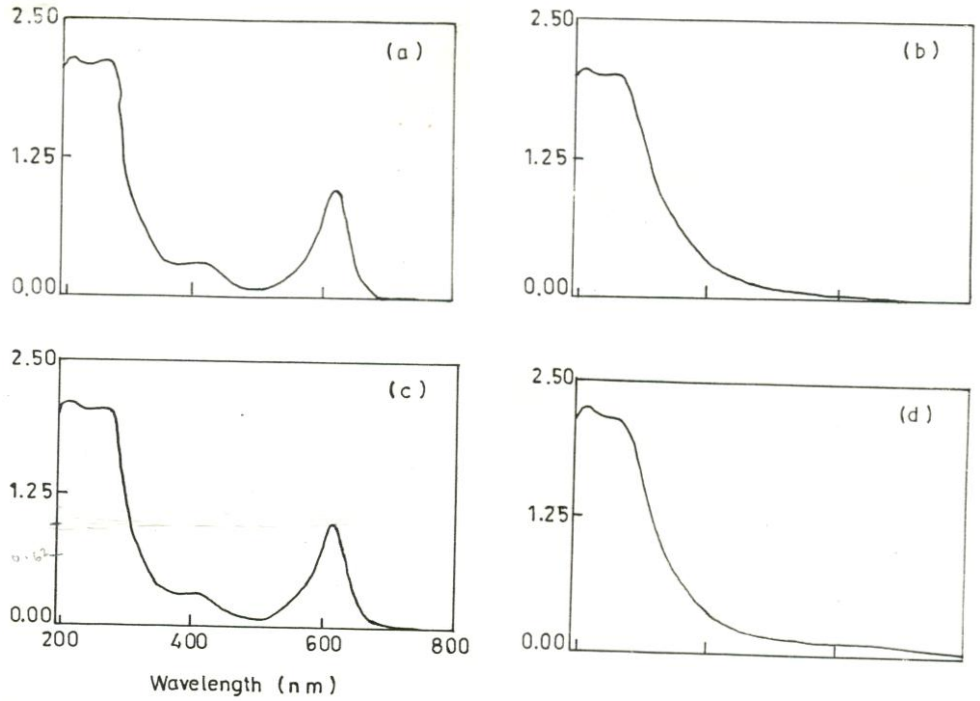
Figure 3. UV/VIS spectrum of supernatant solutions prepared by withdrawing aliquots from the culture medium containing malachite green at different time interval.

(a) Just after inoculation of the bacterium

(b) After 30 hrs of inoculation when MG was completely decolorized.

(c) Control experiment.

(d) After 30 hrs of the growth of the bacterium containing no MG.



Acknowledgement

The author are thankful to Dr. K. D. S. Yadav, Emeritus Professor, Chemistry Department, D.D.U. Gorakhpur University, Gorakhpur, U.P. (India) for valuable guidance and providing necessary facilities and also the Department of Environment and forest, Govt. of India, New Delhi for the financial support.

References

[1] Raffi F, Hall JD & Cernigila CE (1997). Mutagenecity of Azodyes Used in Foods, Drugs and Cosmetics before and after Reduction of *Clostridium Species* from the Human Intestinal Tract. *Food and Chemical Toxicology*, 35: 897-901.

[2] O' Neill C, Hawkes FR, Hawkes DL, Lourenco ND, Pinherio HM and Delee W (1999). Colour in Textile Effluents Sources, Measurements, Discharge Consents and Simulation. *A Review Journal of Chemical Technology & Biotechnology,* 74:1009-1018.

[3] Jadhave JP, Govindwar SP (2006). Biotransformation of Malachite Green by Saccharomyces cerevisiae. *Yeast*, 23:315-323.

[4] Michaels GB & Lewis DL (2006). Sorption and Toxicity of Azo and Triphyenylmethane Dyes to Aquatic Microbial Populations. *Environmental Toxicology and Chemistry*, 4:45-50.

[5] Littlefield NA, Blackwell BN, Hewitt D and Gaylor D (1985). Chronic Toxicity and Carcinogenicty Studies of Gention Violet in Mice. *Fundamental of Applied Toxicology*, 5:902-912.

[6] Henderson AL, Schmitt TC, Heinze TM and Cerniglia CE (1997). Reduction of Malachite Green to Leucomalachite Green by *Intestinal Bacteria*. *Applied and Environmental Microbiology*, 63:4099-4101.

[7] Verma P Madamwar D (2003). Decolorization of Synthetic Dyes by a Newly isolated strain of *Serratia Marcescers*. *World Journal of Microbiology & Biotechnology*,19:615-618.

[8] Culp SJ and Beland FA (1996). Malachite Green: A Toxicological Review. *J. Am. Coll. Toxicology*,15:219-238.

[9] Alderman DJ (1984). Malachite Green: *A Review. J. Fish Dis.*, 8:289-298.

[10] Malachite green- Wikipedia, the free encyclopedia; http://en.wikipedia.org/wiki/Malachite_green

[11] Farmed Salmon and Human Health; http://www.puresalmon.org/human_health.html#ftn1

[12] Clemmensen S, Jensen JC, Meyer O, Olsen P & Wurtzen. G (1984). Toxicological Studies on Malachite Green: A Triphenylmethane Dye. *Arc. Toxicology*, 56:43-45.

[13] Pandiker A, Fernandes C and Rao KVK (1992). The Cytotoxic Properties of Malachite Green are associated with the Increased Demethylase, Arylhydrocarbon Hydroxylase and Lipid Peroxidation in Primary Cultures of *Syrian Hamster Embryo Cells. Cancer Letter*, 67:93-101.

[14] Rao KVK (1990). Comparative Cytotoxic Effects of the Non-Permitted Food Colouring Agents Metanil Yellow, Orange II, Rhodamine B and Melachite Green on Hamster Dermal and C3H/10 T1/2 Fibroblasts. *Bombay Hosp. J.*, 32:61-65.

[15] Rao KVK (1995). Inhibition of DNA Synthesis in Primary Rat Hepatocyte Cultures by Malachite Green: A New Liver Tumor Promoter. *Toxicology Letter*, 81:107-113.

[16] Allison L, Henderson Thomas C, Schmitt Thomas M, Henze and Carle Cerniglia (1997). Reduction of Malchite Green to Leucomalachite Green by Intestinal Bacteria. *Applied and Environmental Microbiology*, 63(10):4099-4101.

[17] Culp SJ, Blankenship LR, Kusewitt DF, Doerge DR, Mulligan LT and Beland FA (1999). Toxicity and Metabolism of Malachite Green and Leucomalachite Green During Short-Term Feeding to Fisher 344 Rates and BCC 3F Mice. *Chem. Biol. Interact.*, 122(3):153-170.

[18] Fessand V, Godard T, Huet S Mourot A. Paul JM, (1999). Mutagenicity of Malachite Green and leucomalachite Green in Invitro Tests. *J. Appl. Toxicology*, 19(6):421-430.

[19] Ganesh Parshetti, Satish Kalme, Ganesh Saratale, Sanjay Govindwar (2006). Biodegradation of Malachite Green by *Kocuria Rosea MTCC 1532*. *Acta Chim Slov.*, 53:492-498.

[20] Deepak Kumar Sharma, Harvinder Singh Saini, Manjinder Singh. (2004). Bio-treatment of Simulated Textile Dye Effluent Containing Malachite Green by an Up-Flow Immobilized Cell Bioreactor. *World Journal of Microbiology & Biotechnology*, 20:431-434.

[21] Gram C (1884). The Differential Staining of *Schizomycetes* in Tissue Sections and in Dried Preparations. *Fortschitte Der Medicin.*, 5(2):123-127.

[22] Chang-Jun Cha, Daniel R Doerge and Carl E Cerniglia**,** (2001). Biotransformation of Malachite Green by the Fungus Cunninghamella elegans. *Appl. Environ. Microbiol*ogy, 67:4358-4360.

[23] Burchmore S, Wilkinson M (1993). United Kingdom department of the Environment, Water Research Center, marlow, Buckinghamshire, United Kingdom. Report no. 316712 (November 1993).

[24] Jadhav JP, Govindwar SP (2006). Biotrnsformation of Malachite Green by *Saccharomyces Cerevisiac MTCC-463*. *Copyright John Wiley & Sons Ltd.*

[25] Suzanne Parrott, Simon Jones and Ronald A Cooper (1987). 2-Phenylethylamine Catabolism by *Escherichia coli K1*. *Journal of General Microbiology*, 133:347-351.